BIOLOGY AND DEVELOPMENT OF TWO WILT FUNGI OF POTATO:

VERTICILLIUM DAHLIAE AND *COLLETOTRICHUM COCCODES*

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Julie Sherman Pasche

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Julie Sherman Pasche

The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

SUPERVISORY COMMITTEE:

Neil C. Gudmestad
Chair

Gary A. Secor

Luis del Rio

Asunta L. Thompson

Approved:

April 25, 2012

Jack Rasmussen
Department Chair

ABSTRACT

The demand for high quality tubers for the frozen processing industry has exerted increased pressure on producers to control diseases that can compromise tuber quality, including, *C. coccodes* and *V. dahliae*. Infection of potato plants by *C. coccodes*, the causal agent of black dot, can result in foliar necrosis, plant wilting, yield loss, tuber vascular discoloration and skin blemishes. Black dot of potato can originate from foliar-, seed- of soil-borne infections and, all plant tissues can be affected. The development of colonization in above- and below-ground plant tissues, from each inoculation/infestation source, was evaluated at two sites across two growing seasons. Colonization of potato tissue was detected at the first sampling date, even prior to plant emergence. While disease resulted from natural inoculum only, as inoculation/infestation sources increased, so did host colonization. Disease development also was greater in treatments with more than one site of inoculation/infestation. Overall, it was determined that stem tissue was colonized at greater frequency than root or stolon tissue. Infection of potato plants with *V. dahliae,* the causal agent of Verticillium wilt, occurs mainly through root contact with infested soil and can result in premature senescence as well as losses from decreased tuber yield and quality. Control of this pathogen is difficult and expensive and, therefore, efforts recently have increased towards the development of resistance. Resistance to *V. dahliae* in potato is thought to be multigenic, and therefore, quantification of the host:pathogen interaction is required to accurately define the level of resistance in a particular cultivar. Unfortunately, current methods used for quantification of *V. dahliae* in potato stems are time and labor intensive. A real-time duplex quantitative PCR assay was developed to simplify pathogen quantification to help breeding programs and researchers identify resistance in germplasm and cultivars. QPCR assays were validated using plant material from greenhouse and field trials, demonstrating specificity

for *V. dahliae*, as well as sensitivity and accuracy when compared to traditional plating assays. Results from greenhouse and field evaluations also indicated that resistance is present among the eight russet-skinned cultivars evaluated in these studies.

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LITERATURE REVIEW

The soil-borne disease complex

The Potato Early Dying Complex (PED) was named for the early death of a crop infected with any combination of many pathogens including *Colletotrichum coccodes* (Wallr.) Hughes, *Verticillium dahliae* Kleb, *V. albo-atrum* Reinke & Berthold, *Fusarium* spp. and the root lesion nematode *Pratylenchus penetrans* (Cobb) Sher and Allen (Powelson and Rowe, 1993; Rowe et al., 1987; Tsror (Lakhim) et al., 2001). While these pathogens can be devastating alone, in combination have been regarded as the most costly problem in potato production due to yield and quality losses and as well as the cost of control (Powelson and Rowe, 1993). Among these pathogens, *C. coccodes* and *V. dahliae* are unique in that they overwinter as sclerotia in plant debris and soil and these sclerotia can serve as infective propagules for subsequent crops. While yield losses have been attributed to both *C. coccodes* and *V. dahliae* alone, increased losses have been documented when both pathogens are present (Davis and Howard, 1976; Tsror (Lahkim) and Hazanovsky, 2001). Many similarities exist between these two pathogens, not limited to comparable resting structures and wide host ranges, including potato. However, each has many unique characteristics that allow them to be successful pathogens.

Colletotrichum coccodes

<u>Taxonomy</u>. *Colletotrichum* first was identified as the genus *Vermicularia* (Tode) Fr., although it has been difficult to discern if these original species would be classified as *Colletotrichum* today (Sutton, 1992). Tode's description of *Vermicularia* is absent of the mention of setae, and although setae are characteristic of *Colletotrichum,* this does not completely eliminate the possibility of a synonymous relationship. There are known examples of species classified in the genus which do not produce setae, including *C. coffeanum* Noack, *C.*

crassipes (Spreg.) von Arx, *C. falcatum* Went and some species where setae are observed as being sparse to absent (Barron, 1968; Sutton, 1992).

Colletotrichum is classified as a coelomycete, producing conidia in acervuli (Alexopoulos, 1996). The classification of *Colletotrichum* into species is similar to that of other deuteromycetes, such as *Penicillium* Link and *Aspergillus* (Micheli) Link, but has met with several challenges, including the practice of creating novel species for isolates found on a new host (Cannon et al., 2000; Sutton, 1992). In the 1950's the number of accepted species of *Colletotrichum* was reduced from 750 to 11 (Cannon et al., 2000; Sutton, 1992). Since that time the number of species has grown, in part due to better identification of morphological characteristics which aid in classification (Cannon et al., 2000; Sutton, 1992). There are approximately 40 accepted species of *Colletotrichum* currently, some of which infect a single host, and some with an extensive host ranges (Cannon et al., 2000; Sutton, 1992).

Colletotrichum coccodes (Wallr.) Hughes first was described in the literature in 1833 as *Chaetomium coccodes* Wallr. (Mordue and Elizabeth, 1967). Shortly following that description, it was re-classified as *Vermicularia atramentarium* Berk. And Br. In 1913 it was moved back to the genus *Colletotrichum*, but the species name *atramentarium* was preserved. Finally, in 1958, Hughes restored the original species name of *coccodes* (Mordue and Elizabeth, 1967; Sutton, 1980). *C. coccodes* also has been identified as a synonym of *C. phomiodes* (Sacc.) Chester (Dillard, 1992; Sutton, 1980).

Glomerella is the only known teleomorph to be associated with *Colletotrichum* sp. (Dennis, 1981; Sutton, 1992). The genus *Glomerella* is classified in the Kingdom, *Fungi*, Phylum *Ascomycota*, Family *Pyrenomycetes*, and Order *Phyllachorales* (Agrios, 1997; Alexopoulus, 1996; Bryson, et al., 1992). There are approximately 80 species in the genus

Glomerella, several of which have anamorphic stages classified into the genus *Colletotrichum* including *G. glycines* Lehman & Wolf, *G. fusariodes* Edgert., and *G. lindemuthiana* Shear (Bryson, et al., 1992). Among *Colletotrichum* species known to have identified teleomorphs, some have very strong relationships in which a single spore culture has produced structures of the opposite morph, and some have more informal relationships (Sutton, 1992). The only reported teleomorph which is produced in nature is that of *G. cingulata* (Bryson, 1992; Edgerton, 1909).

Pathogen distribution and host range. In the United States it generally is believed that plant diseases caused by *Colletotrichum* Corda species and the teleomorph *Glomerella* von Schrenk & Spaulding are very important, however, this pathogen group is much more devastating in tropical and sub-tropical regions of the world (Agrios, 1997; Bailey et al., 1992). Diseases caused by *Colletotrichum* species are important worldwide as both a pre- and post-harvest pathogen, but especially are devastating after harvest because of an extended latent period (Sutton, 1992). The *Colletotrichum* spp. have a very wide host range causing anthracnose symptoms on pepper, squash, onion, coconut, strawberry, cereals and grasses, pepper, watermelon, cantaloupe, cucumber and eggplant. *C. coccodes* is damaging particularly to *Solanaceous* hosts, causing tomato anthracnose and black dot of potato, and has been isolated from symptomless hosts including cabbage, lettuce and some grasses (Dillard, 1992; Powelson and Rowe, 2008; Tsror (Lahkim) and Johnson, 2000). Crops planted in rotation with potatoes including canola, soybeans, and alfalfa also are susceptible to *C. coccodes* (Powelson and Rowe, 2008). *C. coccodes* has been associated with potato in various parts of the world including Europe, South America, the Middle East, Australia, South Africa and North America (Dickson, 1926; Sutton, 1980; Tsror (Lahkim) and Johnson, 2000).

Biology. *C. coccodes* is similar to other species of *Colletotrichum* in that it is capable of expressing a wide range of disease symptoms, as well as cultural characteristics. In culture, *C. coccodes* is characterized by abundant black sclerotia, 100 µm to 5 mm in diameter (Dickson, 1926; Tsror (Lahkim) and Johnson, 2000). Sclerotia typically are found, either singly or in groups, but can be absent. They have three layers, superficial hyphae make up the epidermis, agglutinated hyphae make up the cortex and a central medulla is made up of free or partially agglutinated hyphae (Dillard and Cobb, 1998). Acervuli also are produced, but only are found sub-epidermally on stems, roots and fruit tissue, disrupting the host epidermis (Dillard, 1992; Tsror (Lahkim) and Johnson, 2000). Acervuli are 200-300 µm in diameter (Dillard, 1992; Sutton, 1992; Thirumalachar, 1967). Dark pigmented, short, septate setae commonly are present in acervuli and sclerotia on host tissue, but frequently may be absent from sclerotia in culture (Barron, 1968; Dickson, 1926). Aerial mycelium is white to grey, but typically sparse (Barron, 1968; Tsror (Lahkim) and Johnson, 2000). Conidiophores are simple hyaline structures produced in the acervulus (Barron, 1968). Phialospores do not stay in chains, but slime into a mass and are 16-24 µm in length by 2.5-4.5 µm wide (Barron, 1968; Dickson, 1926; Tsror (Lahkim) and Johnson, 2000). Significant differences were observed in the length (10.5 to 17.0 µm) and width (2.8 to 3.4 µm) of conidia of *C. coccodes*, depending on vegetative compatibility group and type of in vitro culture media (Aqeel et al., 2008). Unlike other species of *Colletotrichum*, conidia of *C. coccodes* are straight or may be medianly constricted, have obtuse ends and are aseptate (Barron, 1968; Sutton, 1980). In mass, the conidia have been described as pink, orange to salmon or honey colored in appearance (Barron, 1968; Dickson, 1926; Sutton, 1980). Appressoria are common in *C. coccodes* and are brown, obclavate, elliptical or irregular in shape (Sutton, 1980; Sutton, 1992). While it has been demonstrated that *C. coccodes* will grow in a

wide range of temperatures, it is interesting to note that the optimum germination temperature for conidia is 22°C, 6°C lower than that of sclerotia, possibly indicating some sort of evolution by the fungus in response to environmental conditions (Dillard, 1992). This also may provide an explanation for how the fungus can become established in areas of widely varying climates.

Life cycle. Infected seed-tubers often are responsible for the introduction of *C. coccodes* into non-infested soil, although wind-blown debris and soil moved by equipment or animal traffic also can harbor the pathogen (Barkdoll and Davis, 1992; Komm and Stevenson, 1978; Read and Hide, 1995a, 1995b; Tsror (Lahkim) et al., 1999b; Tsror (Lahkim) and Johnson, 2000). Early season infections originate from infected seed-tubers and the pathogen subsequently accumulates in the soil on infected crop debris (Komm and Stevenson, 1978; Read and Hide, 1995b). *C. coccodes* overwinters in the soil and in plant debris as sclerotia. Sclerotia of *C. coccodes* have been documented to survive up to 13 years in infested soil, depending on soil depth, moisture and association with plant debris, making this an important source of inoculum for subsequent crops (Dillard and Cobb, 1998). These sclerotia germinate and infect susceptible roots, stolons, tubers and stems even before the plant has emerged from the soil (Dillard, 1992). Additionally, sclerotia and conidia on the surface of plant tissue may be wind-blown, splashed by rain or irrigation or disseminated by people, animals or equipment to infect healthy foliage (Barkdoll and Davis, 1992; Dillard, 1992; Johnson, 1994; Johnson and Miliczky, 1993; Mohan et al., 1992). While penetration into host tissue occurs within 24h, the latent period of this pathogen may last from weeks to months and, therefore, the effects of these early-season infections may not be observed until premature plant death occurs near the end of the growing season (Dillard, 1992; Powelson and Rowe, 2008). The infection progress in roots is similar to the sigmoidal curve of root growth (Huisman, 1982). Plant roots make increasingly more contact with the

pathogen in the soil as they grow. After these initial infections, colonization of roots tissue increases as plants reach the flowering stage (Hornby, 1968). Infected plant tissue is returned to the soil after harvest and the cycle is repeated.

Vegetative compatibility groups. In many host-pathogen systems, the high variation in virulence and host range present at the sub-specific level may impact disease severity and management (Joaquim and Rowe, 1991). Vegetative compatibility is the genetically determined ability of fungal isolates within a species to form viable heterokaryons via hyphal anastomosis. In fungal species with no known teleomorph, or ability to reproduce sexually, hyphal anastomosis is a major means of genetic exchange. Isolates that have the ability to anastomose are classified as belonging to the same vegetative compatibility group (VCG), and tend to have similar pathogenic and physiologic traits (Joaquim and Rowe, 1990; Joaquim and Rowe, 1991; Puhalla, 1985). Fungal isolates belonging to the same VCG are known to be from a common lineage and, therefore, are considered clones (Leslie, 1993; Puhalla, 1985). Vegetative compatibility has been described in many host-pathogen systems including *Verticillium albo-atrum* (Correll et al., 1988) and *V. dahliae* (Puhalla, 1979; Puhalla and Hummel, 1983), *Fusarium oxysporum* f. sp. *apii* (Puhalla, 1984a; 1984b) and *Fusarium oxysporum* f. sp. *asparagi* (Elmer and Stephens, 1989).

Vegetative compatibility also has been described in several *Colletotrichum* spp. (Brooker et el., 1991) including *C. orbiculare* (Wasilwa et al., 1993), *C. acutatum* (Freeman et al., 2000), *C. kahawe* (Beynon et al., 1995), *C. dematium* f. sp. *spinaciae* (Correll et al., 1993), and *C. coccodes* (Nitzan et al., 2002). Initially, four VCGs were identified among 110 isolates of *C. coccodes* from Israel, France and the Netherlands. Of these, EU/I-VCG3 was the most aggressive as measured by the density of sclerotia on the roots and crown (Nitzan et al., 2002). EU/I-VCG4

was least aggressive by the same measurements. Thirty isolates could not be assigned to a EU/I-VCG, indicating that there are most likely more VCGs within the population that may not be identified without a larger sample population (Nitzan et al., 2002). Later, North American isolates of *C. coccodes* were classified into six NA-VCGs using the same technique (Nitzan et al., 2006c). None of these isolates from North America formed heterokaryons with the European isolates evaluated and, therefore, represent a genetically distinct population. In this study, 18 of the total 123 isolates could not be assigned to any NA-VCG (Niztan, 2006c). As was demonstrated with the European population, differences in aggressiveness existed among North American VCGs. Inoculation with *C. coccodes* isolates belonging to NA-VCGs 1, 2, and 5 resulted in yield reductions (Nitzan et al., 2006c). Over all parameters evaluated including sclerotial production on host tissue, isolates belonging to NA-VCGs 2 and 5 were most aggressive. Further research performed to investigate aggressiveness among NA-VCGs corresponded somewhat with original findings (Aqeel et al., 2008; Shcolnick et al., 2007). Similar tendencies where observed in sclerotial density, but there were no significant differences among isolates of EU/I-VCGs (Shcolnick et al., 2007). *C. coccodes* isolates belonging to EU/I-VCG3 again were the most aggressive when compared directly with the four EU/I-VCGs originally evaluated. However, isolates belonging to newly described EU/IVCGs 5, 6, and 8 displayed higher sclerotial density than all four original EU/I-VCGs (Shcolnick et al., 2007). Differences in tuber weight could be detected among NA-VCGs when inoculum was applied to the foliage, but not when applied to the roots (Aqeel et al., 2008). The greatest tuber weight reductions were observed when plants were inoculated with isolates of NA-VCGs 1, 2, and 6; however, inoculation with isolates of NA-VCG2 resulted in the least foliar disease. While these differences in aggressiveness are not always apparent, they may have important implications for

applications such as screening breeding stocks for resistance to *C. coccodes*. More research needs to be directed in the area to further clarify any relationship. A strong relationship was observed between the molecular technique of AFLP and traditional NA-VCGs, as both techniques resulted in the placement of North American *C. coccodes* isolates into the same groups a majority of the time (Heilmann et al., 2005). This technique allows for more rapid evaluation of VCGs among *C. coccodes* isolates, therefore, facilitating further research.

Symptoms of black dot of potato. Black dot affects all plant tissues including the foliage, roots, stolons, above- and below-ground stems as well as tubers (Andrivon et al., 1998; Davis and Johnson, 2001; Dickson, 1926; Mohan et al., 1992). The most diagnostic sign of black dot is the characteristic sclerotia which may be produced on any infected host tissue, and are the source of the common name for the disease (Jellis and Taylor, 1974; Powelson and Rowe, 2008). However, they do not appear until the plant is senescent, or nearly so (Andrivon et al., 1998; Dickson, 1926; Powelson and Rowe, 2008). Belowground portions of the plant can become infected very early in the growing season, but these early stages of disease development are not as easy to identify (Andrivon et al., 1998). Initially, stems may begin to split, leaflet margins yellow, roll and eventually overall plant wilting occurs (Dickson, 1926). Foliar symptoms resembling that of early infection by *A. alternata* or *A. solani* start as small black irregularly shaped necrotic spots, however, acervuli occasionally may be observed within the necrotic tissue of a black dot lesion (Powelson and Rowe, 2008). Symptoms of black dot increase as plants begin to senesce and the pathogen begins the necrotrophic phase of plant colonization (Dillard and Cobb, 1997; Hornby, 1968; Nitzan et al., 2006b). As the disease progresses and the plant begins to die, the cortical tissue on the stem, roots and stolons may start to slough off, revealing an amethyst color on underlying tissue (Davis and Johnson, 2001; Dickson 1926; Dillard and

Cobb, 1997; Pavlista and Kerr, 1992; Read and Hide, 1988). Infected stolons may stay attached to tubers during and after harvest operations giving a characteristic 'pig-tail' appearance (Davis and Johnson, 2001; Dickson 1926; Pavlista and Kerr, 1992). Tubers develop a gray to silver diseased regions which resemble that of silver scurf caused by *Helminthosporium solani* (Davis and Johnson, 2001; Jellis and Taylor, 1974). As opposed to the small lesions with discrete borders produced by silver scurf, black dot lesions tend to grow larger and have a diffuse border (Jellis and Taylor, 1974; Powelson and Rowe, 2008). Tuber lesions caused by *C. coccodes* may be sparse at harvest, but become larger and more discolored during storage (Jellis and Taylor, 1974). In some instances, black dot lesions can be sunken and result in tuber shrinkage from water loss (Glais and Andrivon, 2004; Hunger and McIntyre, 1979; Read and Hide, 1988; 1995a), or are raised and become dark to give a 'bumpy tuber' appearance (Nitzan et al., 2005b). In both lesion types, sclerotia can be observed within the black dot tuber lesion (Jellis and Taylor, 1974; Rich 1983).

<u>The importance of black dot of potato</u>. The debate over whether *C. coccodes* is a true pathogen, or merely an opportunistic invader which thrives only when the plant has been compromised by another pathogen or abiotic stress, has endured since the first descriptions of black dot of potato (Davis and Everson, 1986; Dickson, 1926; Kotcon et al., 1985; Otazu et al., 1978; Rowe et al., 1987; Scholte et al., 1985; Thirumalachar, 1967). For many years, *C. coccodes* was considered a saprophyte, or at best, a weak pathogen. Early research indicates symptom development in the absence of other pathogens, providing evidence that although it is described as a weak parasite, *C. coccodes* may be important as a pathogen (Dickson 1926). In a survey of 37 commercial potato fields in Idaho, significant correlations were observed between the level of *C. coccodes* in the soil, and wilt, as well as the amount of the pathogen recovered

from stem tissue, but no relationship of either of these parameters to yield was reported (Davis and Everson, 1986). *C. coccodes* infection did not reduce yield unless combined with other factors including infection by *V. dahliae* (Scholte et al., 1985).

Many recent examples exist to support *C. coccodes* as a true pathogen, causing losses in both tuber yield and quality (Barkdoll and Davis, 1992; Johnson 1994; Mohan et al., 1992; Nitzan et al., 2005a; 2005b; Stevenson et al., 1976; Tsror (Lahkim) et al., 1999b). Generally, effects on yield and tuber quality are variable and may depend on site of infection, or agronomic factors. Research has indicated that measurable yield losses up to 30% are possible under field conditions following *C. coccodes* infection, although these results are not always repeatable (Barkdoll and Davis, 1992; Denner et al., 1998; Johnson, 1994; Mohan et al., 1992; Tsror (Lahkim) et al., 1999b). Yield was reduced by more than 50% in a single greenhouse study performed with field soil collected in Indiana and amended with sclerotia of *C. coccodes* when compared to non-amended soil (Stevenson et al., 1976). Severe seed-tuber infection resulted in significant yield losses in only one of two field trials, while soil infestation did not significantly affect yield in either year (Read and Hide, 1995a). Field trials in which foliage was inoculated with conidia of *C. coccodes* conducted in Idaho over two growing seasons resulted in no yield effect at the first site-year, even though a significant difference in disease was observed (Mohan et al, 1992). However, a significant increase in disease incidence, and reductions in yield, were observed at both sites the second year the trials were conducted. In greenhouse assays, foliar inoculation of nine *C. coccodes* isolates resulted in reductions in total tuber weight (Barkdoll and Davis, 1992). In field trials, foliar inoculation resulted in significant yield reduction with only one of three isolates. While yield was reduced significantly in two greenhouse trials where foliage was inoculated, a significant yield reduction was observed in only two of three field trials

also comparing inoculated foliage to non-inoculated plants (Johnson, 1994). Greenhouse trials performed to compare foliar to root inoculations indicated that while both inoculation methods significantly reduced total tuber weight when compared to non-inoculated plants, root inoculations did more-so than foliar inoculations (Aqeel et al., 2008). Much difficulty may be involved in interpreting results from experiments which rely upon the use of seed-tubers and/or field soil harboring the pathogen at low, possible undetectable, levels (Johnson. 1994). Therefore, this debate may continue until a better understanding exists concerning the possible presence of undetected inoculum, as well as disease epidemiology and etiology.

Common agronomic practices such as tillage may have a major effect on the survival of soil pathogens (Rothrock, 1992) and specifically *C. coccodes* sclerotia (Denner et al., 2000). Changes in tillage and other practices have been attributed to the increase in occurrence of black dot as a result of the increase in sclerotia in the soil (Bockus and Shroyer, 1998). Black dot of potato also is gaining importance due to the increased demand for blemish-free potatoes by the potato processing industry, as well as fresh-pack consumers (Andrivon et al., 1997; Denner et al., 1997; Lees and Hilton, 2003; Nitzan et al., 2002; Tsror (Lahkim) et al., 1999b). Quality losses from infection by *C. coccodes* can come in several forms including, decreased specific gravity, vascular discoloration, skin blemishes, and tuber shrinkage from water loss (Barkdoll and Davis, 1992; Hunger and McIntyre, 1979; Read and Hide, 1988). These quality losses affect both fresh-pack and processing potatoes. As a consequence of increased quality standards, fungicide use to control silver scurf has increased (Andrivon et al., 1997; Hide et al., 1994a). Because of the competitive nature of black dot and silver scurf, the decrease in the later via the use of fungicides allowed more tuber tissue to be affected by the former (Andrivon et al., 1997; Hide et al., 1994a; Jellis and Taylor, 1974). Additionally, tuber symptoms of black dot can be confused easily with

that of silver scurf, compounding management problems (Jellis and Taylor, 1974; Powelson and Rowe, 2008).

C. coccodes etiology. Black dot of potato is a complicated disease affecting all plant tissues. Additionally, inoculum originates from the seed, soil, and also may be airborne. In order to develop more effective control measures, much research has been dedicated to elucidating which source of inoculum is most important and accordingly, which host tissue is colonized first and to the greatest extent. Seed inoculum is important for mainly two reasons. Planting infected seed is a major means of introducing *C. coccodes* into non-infested soil (Barkdoll and Davis, 1992; Komm and Stevenson, 1978; Tsror (Lahkim) et al., 1999a) and, several reports have indicated that seed-borne inoculum increased early season stem infections (Komm and Stevenson, 1978; Johnson et al., 1997). In Indiana, infection incidence of seed-tubers was reported to be as high as nearly 88%; resulting in up to 93 and 72% root and stem infection, respectively (Komm and Stevenson, 1978). *C. coccodes* was recovered from the periderm of 95% of seed-tubers evaluated of cv. Maris Piper in 1986 (Dashwood et al., 1992). Pathogen levels on roots was higher in plants generated from seed-tubers, with or without visual tuber-blemish symptoms, when compared to soil inoculated, disease-free, micropropagated plants at three points in the growing season. Therefore, seed-borne inoculum contributed to early season infections and increases in overall disease levels (Dashwood et al., 1992). In Idaho, the incidence of seed-lot infection with *C. coccodes* ranged from 0 to 90%, and mean infection frequencies were 25 and 41% in 1979 and 1980, respectively (Barkdoll and Davis, 1992). Also, 100% of soil sampled from potato production fields were infested with *C. coccodes,* while no soil infestation could be detected from areas with native vegetation and no history of crop production. Similarly, from zero to 90% of tubers tested from seed-lots from nine Midwestern United States and two

Canadian provinces were infected with *C. coccodes* (Johnson et al., 1997). The frequency with which *C. coccodes* was detected in seed-lots increased with increasing seed generation. No *C. coccodes* was detected in nuclear seed, but 50% of generation 3 seed-lots were infected (Johnson et al., 1997). Additionally, significantly higher frequency and severity of stem infections were observed from plants produced from infected seed-tubers compared to those with no detectable *C. coccodes*. Seed-tubers of cvs. Russet Norkotah and Russet Burbank from Washington State were infected with *C. coccodes* at levels ranging from 0 to 49% (Nitzan et al., 2005a). Infection frequencies increased with increasing seed generation, as was reported in previous studies. Increased seed inoculum also increased colonization on below ground stems for both cultivars (Nitzan et al., 2005a). Increased seed-borne inoculum increased disease incidence on progeny tubers, and decreased yield in cv. Russet Norkotah, but not cv. Russet Burbank, indicating that the role of inoculum source may be cultivar dependent.

Contrary to this, other reports indicate that soil-borne inoculum plays a greater part in progeny tuber infection (Denner et al., 1998; Read and Hide, 1988; 1995a). The effects of seed- and soil-borne inoculum were compared across two growing seasons under irrigated and non-irrigated conditions (Read and Hide, 1988). *C. coccodes* stem infections were significantly higher in seed inoculated treatments in only one year in non-irrigated plots, while they were significantly higher in soil inoculated treatments in all trials, when compared to non-inoculated plants. Soil inoculum also increased root and progeny tuber infections more consistently and to a higher degree than did seed inoculum across years and irrigation treatments (Read and Hide, 1988). Several experiments were conducted in England from 1988 to 1990 to examine the role of seed- and soil-borne inoculum in the development of black dot of potato (Read and Hide, 1995a). Results across all trials were variable, depending on cultivar, trial site, inoculum source, and

parameter evaluated. However, general conclusions indicated that seed infection was a substantial contributor to overall disease only when it was very severe, or when soil infestation levels were low or absent (Read and Hide, 1995a). Overall, soil infestation level was a much larger indicator of eventual levels of plant infections by *C. coccodes*. The importance of seed- and soil-borne inoculum was compared directly using methyl bromide fumigated and non-fumigated soil and non-infected, lightly infected, and severely infected seed (Denner et al., 1998). The level of seed infection had some effect, but this effect was small compared to the effect of soil fumigation. Yield increased and *C. coccodes* infected progeny tubers decreased in fumigated soil, even with severely infected seed (Denner et al., 1998). In areas with low levels of soil-inoculum, limiting seed-borne inoculum or treating seed with fungicides may help reduce black dot symptoms on progeny tubers, but in highly infested soil the effect of seed-borne inoculum was inconsequential (Denner et al., 1998; Read and Hide, 1995a).

In other comparisons of soil- and seed-borne inoculum under greenhouse conditions, soil-borne inoculum, alone or in combination with seed-borne inoculum, significantly reduced yield over non-inoculated seed-tubers in three of four trials, and over seed inoculum alone in two of four trials (Nitzan et al., 2008). No significant difference was observed between soil inoculum alone and the combination of soil and seed inoculum. Seed-borne inoculum alone did not significantly reduced yield in any of the four trials conducted (Nitzan et al., 2008). Similar trends also were observed for sclerotial density on roots and the number of progeny tubers, but these differences were not always significant. Studies were performed in Washington State to compare the influence of low and high internal vascular *C. coccodes* infections in seed-tubers grown in fields with either five year rotation, 30 years since the last potato crop or no recorded potato crop (Dung et al., 2012). Results indicated that no relationship exists between disease development, as

measured by symptom expression, pathogen colonization, or yield reductions, and seed-tuber infection. However, a relationship was observed between crop rotation and these parameters, indicating that soil-borne inoculum plays a more substantial role in disease development than does seed-borne inoculum. Additionally, the authors also concluded that a five year rotation is not adequate for black dot disease management (Dung et al., 2012).

Foliar inoculum may originate as microscelotia carried by, and introduced into, the host via wounds made by windblown sand (Johnson and Miliczky, 1993; Nitzan et al., 2006a; Tsror (Lahkim) and Johnson, 2000; Tsror (Lahkim) et al., 1999b). Additionally, conidia produced in acervuli on crop residue, or current season infections, may be spread by water splashed during rainstorms or irrigation events (Dillard, 1989; Tsror (Lahkim) and Johnson, 2000; Tsror (Lahkim) et al., 1999b). Therefore, research efforts have focused on the role foliar or air-borne inoculum may play in black dot disease progression. Artificial foliar inoculations of *C. coccodes* have been performed under greenhouse and field conditions to evaluate the effect on this source of inoculum on yield and other disease parameters (Aqeel et al., 2008; Barkdoll and Davis, 1992; Johnson 1994; Johnson and Miliczky, 1993; Mohan et al., 1992; Nitzan et al., 2006b; Tsror (Lahkim) et al., 1999b).

Yield reductions resulting from black dot foliar inoculations were observed in cvs. Russet Burbank and Norgold Russet under field conditions in Idaho (Mohan et al., 1992). As has been observed in other *C. coccodes* evaluations, these reductions were not always significant, depending on field location, cultivar, and year. In related research, foliar inoculations were performed by first wounding tissue under both greenhouse and field conditions (Barkdoll and Davis, 1992). Increases in foliar lesions and wilt corresponded with decrease yield in both trials. Infections of potato foliage were significantly higher when tissue was wounded before

inoculation when compared to non-wounded tissue (Johnson and Miliczky, 1993). Additionally, infection frequency decreased significantly with increasing wound age, and increasing wetness duration after inoculation resulted in significantly more disease. Yield was reduced and pathogen colonization increased in wounded and inoculated foliage when compared to non-inoculated, either wounded or non-wounded (Johnson, 1994). Greater than 40% and 12% yield reductions resulted from *C. coccodes* colonization in greenhouse and field experiments conducted in Washington State, respectively. The effect of soil and foliar inoculum sources were examined also in field trials in Israel (Tsror (Lahkim) et al., 1999b). The largest yield reductions were observed in years when the foliage was inoculated, however, these differences were not consistent, and not always significant. Unfortunately, these comparisons were made in different growing seasons, and therefore cannot be compared directly. Therefore, the authors do not attribute this to inoculum source, but rather environmental conditions in a given growing season (Tsror (Lahkim) et al., 1999b). Overall, it has been reported under various environmental conditions that foliar infections by *C. coccodes* have a substantial effect on yield and other disease parameters including wilt. As was reported with seed- and soil-borne inoculum, foliar infections of *C. coccodes* are not always consistent in the effects on potato growth and development and often are dependent upon plant stress from environmental conditions or infections by other pathogens.

Research aimed at discerning frequency and extent of host colonization, or symptom development, has been performed in conjunction with evaluations of inoculum source (Andrivon et al., 1998; Cummings and Johnson, 2008; Komm and Stevenson, 1978; Nitzan et al., 2006b; Read and Hide, 1995a; Tsror (Lahkim) et al., 1999b). Root infection was detected at a greater frequency than was stem infection in plants produced under greenhouse conditions from two

infected certified seed sources (Komm and Stevenson, 1978). Across several cultivars and field trials, roots had higher rates of symptom expression than did stem-bases when plants were grown from infected seed or in infested soil (Read and Hide, 1995a). Also, increasing amounts of inoculum from either source increased the black dot symptoms on root, stem and tuber tissues to varying degrees depending on cultivar. Inoculated seed-tubers of cvs. Bintje and Roseval were used to evaluate the development of black dot symptoms on above and below ground host tissues under greenhouse conditions (Andrivon et al., 1998). Depending on cultivar, symptoms were visible on root tissue from 2 to 5 weeks after emergence, while stem symptoms were not evident until 7 to 10 weeks (Andrivon et al., 1998). Differences between cultivars were attributed to differences in maturity. Colonization of above- and below-ground stem tissue was evaluated 90 days after planting in autumn and spring growing seasons in Israel across four cultivars (Tsror (Lahkim) et al., 1999b). Results of these evaluations were variable, depending on cultivar, growing season and year the trial was performed. This variability may be due to the evaluations being conducted late in the growing season, long after initial infections had occurred (Tsror (Lahkim) et al., 1999b). Several parameters including *C. coccodes* stem colonization and black dot symptom development on stems and tubers were assessed in four field trials using natural inoculum (Cummings and Johnson, 2008). *C. coccodes* was detected more often, and the rate of expansion of the area of stem tissue containing sclerotia was greater, in below- ground stems when compared to above-ground stems. However, these parameters were correlated in most instances.

In other studies, above-ground stem inoculations were performed at a single point on greenhouse reared plants to determine the direction of *C. coccodes* colonization, as well as the relationship between plant colonization and foliar symptom development (Nitzan et al., 2006b).

Results from these experiments indicate that pathogen growth within the plant is minimal until plants begin to senesce, at which time colonization increases. The rate of colonization was significantly higher towards below-ground stems, as compared to above-ground stems (Nitzan et al., 2006b).

Disease management. While some chemical and cultural control measures can aid in limiting disease severity, the complex nature of disease epidemiology, as well as the ubiquitous nature of inoculum makes black dot a very challenging disease to control. Black dot severity is increased when plants are stressed due to a lack of nitrogen, in high temperatures, and high soil moisture, especially under conditions where the soil is saturated for more than 10 continuous hours (Davis and Johnson, 2001). As detailed earlier, the survival structure and primary infective propagule of *C. coccodes* is the sclerotium. Sclerotia can reportedly survive up to 13 years in the soil and are not destroyed by currently registered soil fumigants (Cullen et al., 2002; Dillard and Cobb, 1998; Stevenson et al., 1976). Additionally, due to the wide host range of agricultural crops including soybean and canola, as well as weed species including nightshade and velvetleaf, much of the soil used for crop production is infested with the pathogen (Eberlein et al., 1991; Nitzan et al., 2006b; Raid and Pennypacker, 1987). For example, tomato infections occurred in field soils with no previous history of crops within the family Solanaceae (Dillard and Cobb, 1998). Crop rotation to a non-host, the use of certified disease-free seed-tubers and soil moisture control to limit stress on the plants all can have an effect on reducing yield losses and subsequently limiting the buildup of sclerotia in the field (Lees and Hilton, 2003). Tillage practices aimed at burying crop debris and improving soil drainage, as well as effective reduction in weed hosts, also can aid in lowering infestation levels (Denner et al., 2000; Dillard and Cobb, 1998). Seed treatment fungicides have been utilized, but with varying results (Andrivon et al.,

1997; Denner et al., 1997; 1998; Read and Hyde, 1988; 1995a; 1995b; Read et al., 1995; Tsror (Lahkim) and Johnson, 2000). Limiting the level of seed-tuber infection may reduce early-season infections (Davis and Johnson, 2001). Soil solarization has been used to reduce soil infestation levels with some success, but because of the long duration of treatment and high temperature levels required to kill the heat-tolerant sclerotia, it generally is impractical in most parts of the world (Denner et al., 2000). These disease control measures are aimed mainly at reducing the level of sclerotial soil infestations, but unfortunately, the combination of sclerotial survivability and wide host range render the presently available cultural and chemical disease control means inadequate.

Regardless of the inoculum source, yield losses due to black dot can be substantial. Results from both greenhouse and field trials report significant yield loss due to foliar infections (Aqeel et al., 2008; Barkdoll and Davis, 1992; Johnson 1994; Johnson and Miliczky, 1993; Mohan et al., 1992; Nitzan et al., 2006a). This suggests that targeting foliar infections might prove to be a beneficial strategy for controlling potato black dot. Foliar applications of either mancozeb and chlorothalonil reduced recovery of *C. coccodes* from root segments, but did not reduce the frequency of plants infected overall (Dillard and Cobb, 1997). In recent work with the strobilurin fungicide azoxystrobin, in-furrow and foliar applications resulted in significant decreases in black dot severity on above- and below-ground stems, as well as increased yields (Nitzan et al., 2005a). Related work determined that foliar applications of azoxystrobin made 60-62 days after planting were most effective at reducing black dot disease severity (Cummings and Johnson, 2008). Results were not consistent across years the trial was conducted and varied depending on the parameter assessed; however, overall these results are encouraging and suggest that antifungal compounds may have potential as black dot control agents. Effective chemical

control of the foliar phase of black dot potentially could increase yields and effect the resident population of *C. coccodes* by limiting the amount of inoculum returning to the soil in foliar and stem debris.

Because decreases in tuber quality result from black dot infection, limiting the development of tuber lesions in storage can minimize the effects of the disease on the value of the crop. Excessive water prior to and after harvest has been reported to increase black dot symptoms on tubers; however, quickly drying tubers during storage can decrease black dot symptoms on the periderm (Hide and Boorer, 1991; Hide et al., 1994b; Read and Hide, 1988). This appears to be especially important when harvesting in wet conditions and mud adheres to the tubers, possibly providing increased inoculum from the soil, or the moist environment for infections to take place (Hide et al., 1994b; Read and Hide, 1988). Additionally, similar research has revealed that disease incidence is increased when harvest is delayed, leaving tubers exposed to inoculum and potential wet conditions for extended time periods (Hide et al., 1994a; Read and Hide, 1988).

Resistance to *C. coccodes* in potato has garnered little attention; however, considerable research has been performed to identify the inheritance of resistance to tomato anthracnose caused primarily by *C. coccodes* (Barksdale, 1970; 1971; 1972a; 1972b; Barksdale and Koch, 1969; Miller et al., 1984; Robbins and Angell, 1970; Strommel and Zhang, 2001). Resistance to tomato anthracnose has been difficult to incorporate into elite materials, possibly because there are multiple genes involved, these genes are only partially dominant, or are linked to undesirable traits in non-adapted or wild germplasm (Miller et al., 1984; Strommel and Zhang, 2001). Genetic resistance to *C. coccodes* in tomato has been identified and possibilities exist for these

same genes to be present within wild and cultivated potato species, however, these sources of resistance have not been exploited to this point.

Early research indicates that differences in symptom expression among cultivars exists, although little information is provided about the cultivars and experiments were conducted in only one field trial (Dickson, 1926). Additionally, little literature exists on genetic resistance to black dot in wild or cultivated potato species. It is evident that differences exist among cultivars but the reason for these differences is not clear (Andrivon et al., 1997; 1998; Harrison, 1963; Read, 1991). The susceptibility of several cultivars, as well as hybrids of *S. andigenum* and *S. demmisum* were evaluated under greenhouse conditions in India (Thirumalachar, 1967). All cultivars evaluated were reported to be susceptible to *C. coccodes*, displaying varying levels of diseased stem, stolon, and tuber tissue. Unfortunately, the susceptibility of the hybrids was not reported. Differences in levels of diseased tubers also were observed in black dot susceptibility across 15 cultivars evaluated in the United Kingdom (Read, 1991). These differences were not always related to maturity type. Maincrop cvs. Desiree and Maris Piper were consistently more susceptible than early cvs. Wilja and Marfona. Cv. Pentland Crown was among the most resistant. Additionally, two of three cultivars were intermediately susceptible when compared directly to parental cultivars also included in the study, indicating that black dot susceptibility might be an inherited trait (Read, 1991). In later research by the same group, cv. Desiree was more susceptible to black dot disease development when compared to cvs. Maris Piper, Estima and Pentland Crown (Read and Hide, 1995a). Again, Pentland Crown was the most resistant, but no direct comparisons were made between Maris Piper and Estima. In all cultivars, black dot increased with increasing seed- or soil-borne inoculum, but the disease increase did not always result in decreased yields (Read and Hide, 1995a). Similar research conducted in France

indicated that cv. Roseval exhibited less susceptibility to black dot disease expression than earlier maturing cv. Bintje (Andrivon et al., 1998). *C. coccodes* soil inoculations were performed in the spring and autumn in Israel to determine cultivar susceptibility across one and four cultivars, respectively (Tsror (Lahkim) et al., 1999b). In the spring season, a significant difference was observed between inoculated and non-inoculated plants of cv. Desiree, the only cultivar evaluated. In the fall season, significant differences were observed in cvs. Cara, Nicola and cv. Agria, but not cv. Alpha. Similar trials were performed in two additional years, but using foliar inoculum and four cultivars in each trial (Tsror (Lahkim) et al., 1999b). In the second spring season, differences were observed only with Alpha, and no differences were observed during the third spring season. In the fall season, differences were observed in all four cultivars and only Agria in the second and third years of the study, respectively (Tsror (Lahkim) et al., 1999b). These results indicate that cultivars react differently to infection by *C. coccodes* under varying environments of the two seasons, to inoculation source.

In the United States, the most commonly planted cultivars are susceptible to black dot at varying levels (Barkdoll and Davis, 1992; Komm and Stevenson, 1978; Mohan et al., 1992). When plant foliage was wounded and inoculated with *C. coccodes* under field conditions, yield was not reduced in the first season, while in the second year reduced yield was observed in late maturing cv. Russet Burbank and clone A68113-4, but not early maturing cv. Norgold Russet when compared to non-inoculated plants (Mohan et al., 1992). Similar results were observed in greenhouse soil-inoculation experiments where yield was reduced significantly in cv. Russet Burbank, but not in cvs. Norgold Russet and Superior (Mohan et al., 1992). Some reports of the levels of resistance to infection by *C.coccodes* have indicated that differences may be due to inoculum source (Aqeel et al., 2008; Nitzan et al., 2005a). Yield was reduced and disease

incidence in progeny tubers was increased significantly by seed-borne inoculum in cv. Russet Norkotah, but no effect of inoculum was observed in cv. Russet Burbank (Nitzan et al., 2005a). In greenhouse evaluations, cv. Russet Burbank suffered significantly higher reduction in tuber weight when roots were inoculated, but significantly lower with foliar inoculation methods compared to cvs. Russet Norkotah and Umatilla Russet (Aqeel et al., 2008). Cultivar Russet Norkotah was less affected by either inoculation method than was cv. Umatilla Russet. An expansive evaluation of potato selections was performed recently under field conditions of high soil inoculum (Nitzan et al., 2009). Five of 46 selections were determined to have increased resistance to stem colonization by *C. coccodes,* compared to industry standard cvs. Russet Burbank, Russet Norkotah and Umatilla Russet (Nitzan et al., 2009).

Verticillium dahliae

Taxonomy. Nees von Esenbeck first described the genus *Verticillium* in 1816 (Inderbitzin et al., 2011; Isaac, 1967; Pegg and Brady, 2002). Organisms in this genus are characterized by hyphal branches and erect, septate conidiophores with pointed apices aligned in characteristic whorls or verticils (Inderbitzin et al., 2011; Isaac, 1967; Pegg and Brady, 2002). Three types of resting structures have been described among *Verticillium* species, dark resting mycelium, chlamydospores and microsclerotia (Inderbitzin et al., 2011; Pegg and Brady, 2002). The type of resting structure formed historically has been a major determinant in *Verticillium* taxonomy. *Verticillium* is a genus within the phylum Ascomycota, no teleomorphs have been identified for vascular plant pathogenic species (Barbara and Clewes, 2003).

Up to 190 species have been assigned to the genus *Verticillium*, including insect and fungal pathogens, as well as nematode parasites (Inderbitzin et al., 2011; Zare and Gams, 2008). These organisms since have been placed into other genera based on DNA sequences, leaving

only plant pathogens in the genus *Verticillium* (Inderbitzin et al., 2011; Klosterman, 2009; Zare and Gams, 2008). The number of species within the genus generally is accepted to be between four and seven, depending on the source of the description and the characteristics used to classify the organisms (Inderbitzin et al., 2011; Klosterman, 2009; Zare and Gams, 2008). Expanding the number of *Verticillium* species to ten was proposed recently (Inderbitzin et al., 2011). The creation of new species is based on phylogenic analyses of ribosomal internal transcribed spacer region (ITS) and partial sequences of the protein coding genes. However, some of these newly proposed species do not have any morphological differences from commonly accepted species.

Verticillium albo-atrum was isolated from potato plants with wilt symptoms by Reinke and Berthold in 1879 (Isaac, 1967; Pegg and Brady, 2002). *V. albo-atrum* causes wilt diseases most commonly of hosts within the family Solanaceae in temperate regions and produces dark resting mycelium from hyphae (Isaac, 1967;). *Vertcillium dahliae* was described by Klebahn in 1913 (Isaac, 1967; Pegg and Brady, 2002; Smith, 1965). This microsclerotial-forming relative of *V. albo-atrum* was isolated from dahlia and much controversy followed concerning the validity of establishing a new species (Isaac, 1967; Pegg and Brady, 2002; Smith, 1965). In addition to the formation of microsclerotia by *V. dahliae*, cultures of *V. albo-atrum* are absent of this structure, the two species have different optimal temperature ranges for growth and survival (Isaac, 1967; Pegg and Brady, 2002; Smith, 1965). This characteristic affects geographic distribution of these two otherwise similar pathogens (Isaac, 1967; Pegg and Brady, 2002; Smith, 1965). The two species also differ in optimum pH ranges (Isaac, 1967). *V. dahliae* is better suited to slightly acidic conditions (5.3 to 7.2), while *V. albo-atrum* is better suited to more basic (8.0 to 8.6). Other characteristics including host range, conidial size, conidiophore shape and color and the number of phialides have been proposed as reasons to either combine or separate these two

organisms (Isaac, 1967; Smith, 1965). However, it is well accepted today that *V. dahliae* and *V. albo-atrum* are unique species (Pegg and Brady, 2002). The latest in the specific controversies within the genus *Verticillium* surrounds placing the long-spored variant of *V. dahliae* into a separate species (Karapapa et al., 1997). Variants of *V. dahliae* with "near haploid" nuclear state, elongated microsclerotia and conidia, and a reduction in the number of phialides per node, were found to infect oilseed rape, a non-host for *V. dahliae*. On the basis of these, and other characteristics, isolates of *V. longisporum* (C. Stark) Karapapa, Bainbr. & Heale were elevated into a new species (Karapapa et al., 1997). Additional commonly accepted species within this genus include *V. tricorpus* Isaac, considered more of a soil saprobe and a weak pathogen, produces all three types of resting structures, and *V. nubilum* Pethybridge, which produce only chlamydospores (Barbara and Clewes, 2003; Klosterman, 2009). Sub-specific classifications also have been proposed in some species, but as with the species classifications, the debate over the validity continues (Barbara and Clewes, 2003).

Pathogen distribution and host range. *Verticillium* spp. are primarily wilt pathogens of dicotyledonous hosts worldwide, but have been reported to infect, colonize and in some instances reproduce on the roots of monocotyledons (Ben-Yephet and Szmulewich, 1985; Pegg and Brady, 2002; Smith, 1965). Host ranges include agricultural crops such as lettuce, spinach, potato and tomato, trees and shrubs including maple and olive, and horticultural crops. *Verticillium* spp. are found most commonly in temperate regions, with specific regional importance dependent upon optimal temperatures of species within the genus. *V. dahliae* and *V. albo-atrum* are the causal agents of Verticillium wilt in over 200 herbaceous and woody agricultural and ornamental plants (Agrios, 2005). *V. dahliae* is known to colonize numerous weed species, which have been implicated in disseminating it to areas previously not known to

harbor the pathogen (Evans, 1971). Additionally, it has been reported that isolates recovered from weed hosts caused severe wilt on lettuce (Vallad et al., 2005). Verticillium wilt in United States potato production is caused primarily by *V. dahliae*, however, *V. albo-atrum* Reinke & Berthold may be associated with the disease in areas where temperatures during the growing season typically do not exceed 25°C (Davis and Huisman, 2001; Rowe et al., 1987).

Biology. Mycelium of *V. dahliae* are 2 to 4 µm in diameter, hyaline, and septate with thin walls (Pegg and Brady, 2002; Smith, 1965). Conidiophores are hyaline, arranged in a verticillate or whorled pattern on hyphae, typically are not branched and produce single-celled conidia in a wet mucilage (Pegg and Brady, 2002). Phialides are borne on conidiophores, 3 to 4 per whorl, or directly on hyphae (Smith, 1965). Conidial ontogeny has been difficult to determine, because it appears that the first conidium arising from a conidiophores is formed holoblastically, while subsequent conidia are enteroblastic (Hawksworth et al., 1983). Conidia are uni-cellular, hyaline, ellipsoid in shape, approximately 3-5 × 1.3-2 to 4-6 × 1.8-2 µm (Pegg and Brady, 2002; Smith, 1965). Microsclerotia of *V. dahliae* form from the swelling, budding and melanization of hyphae, and, therefore, are very irregular in size and shape (Isaac, 1967; Pegg and Brady, 2002). The mass of cells within a microsclerotium include older thick-walled cells which may be void of nuclei, as well as younger, less melanized cells (Isaac, 1967). Frequency of germination appears to decrease as cells within a microsclerotium age and become more melanized. The function of the older cells may be to serve as food reserves for the younger cells which germinate (Isaac, 1967). Microsclerotia are the main infective propagule of *V. dahliae* and are reported to survive in the soil for up to 15 years (Agrios, 2005; Pegg and Brady, 2002). Under natural conditions, they germinate to infect via wounds or natural openings in the roots of susceptible host plants and colonize plant vascular tissue (Agrios, 2005; Pegg and Brady, 2002).

The optimal temperature range for the development of Verticillium wilt caused by *V. dahliae* is 21 to 27°C (Powelson and Rowe, 1993). Plant damage can be caused at lower temperatures, however, disease severity and yield losses increase with increasing temperature (Nnodu and Harrison, 1979; Rowe et al., 1985). Temperature increases from 20 to 23°C increased Verticillium wilt, and subsequently decreased yield. These differences were significant depending on soil type (Rowe et al., 1985). Similarly, under greenhouse conditions, increases in disease severity were observed when temperatures were increased from 23.9 to 29.4°C (Nnodu and Harrison, 1979). The effect of temperature was greater with higher inoculum densities. Infection of host plants by *V. dahliae* also is favored by high soil-water content, especially early in the growing season (Cappeart et al., 1992; 1994). Comparisons of excessive to deficit irrigation resulted in significant increases in Verticillium wilt and yield decreases from 14 to 33% depending on growing season (Cappeart et al., 1992). Increases in the level of inoculum increased Verticillium wilt under excess irrigation, but not with deficit irrigation. Additionally, results from microplot trials indicated that Verticillium wilt was affected to a greater extent when excessive irrigation was applied prior to tuber initiation (Cappeart et al., 1994). Under field conditions, excess irrigation significantly increased wilt, and in some instances yield. Reports also indicate that environmental conditions affect the level of microsclerotial production in potato stems (Ben-Yephet and Szmulewich, 1985). Increased populations were observed in stems produced in the cool and moist autumn season in Israel when compared to those produced during the hot and dry summer season. Other factors including soil organic matter also have been found to be associated with severity of Verticillium wilt (Davis et al., 2001). Increasing organic matter led to increases in yield of tubers greater than 280 g, even when root infections were increased (Davis et al., 2001).

Root lesion nematodes of the *Pratylenchus* spp. can exacerbate the effects of *V. dahliae*. At least four *Pratylenchus* spp. have been associated with potato, but *Pratylenchus penetrans* (Cobb) Filipjev & Schuurmans Stekhoven has the most synergistic relationship with *V. dahliae* (Bowers et al., 1996; Riedel et al., 1985). Like *V. dahliae*, *P. penetrans* has a wide host range and can survive in the soil in the absence of a host plant (Rowe and Powelson, 2002). While *Pratylenchus* spp. can damage potatoes by root feeding, more severe losses are observed when found in conjunction with *V. dahliae* (Francl et al., 1987; Kotcon et al., 1985; Martin et al., 1982; Riedel et al., 1985; Rowe et al., 1985).While the exact nature of this relationship is yet unknown, it appears the interaction is not caused by physical damage to the roots (Bowers et al., 1996). Research indicates that feeding by *P. penetrans* has some physiological effect on the root system, possibly increasing root branching, and subsequently the probability of contact with microsclerotia (Bowers et al., 1996; Powelson and Rowe, 1993). The presence of the root lesion nematode *P. penetrans* has been reported to increase losses caused by *V. dahliae* alone by more than 25% (Rowe et al., 1985). Additionally, increased levels of both pathogens resulted in significantly decreased yield over even high levels of a single pathogen (Martin et al., 1982). Reports of decreased yields have been attributed to reduced light use efficiency by the plant (Saeed et al., 2007). Decreases in light use efficiency were reported to be additive when both *V. dahliae* and *P. penetrans* were compared to either pathogen alone. Verticillium wilt also can be exacerbated by other pathogens including *Fusarium* spp., *Pectobacterium carotovora* and *C. coccodes* (Davis et al., 2001; Otazu et al., 1978; Stevenson et al., 1976), but these associations have not been as widely damaging as that observed with *P. penetrans*.

<u>Life cycle</u>. The introduction of *V. dahliae* into non-infested soil occurs through several methods. The fungus can be harbored in or on infected seed-tubers, or carried to the field via soil

or plant debris on equipment, animals, irrigation or other water movement (Rowe, 1985; Powelson et al., 1993). Additionally, *V. dahliae* has been found in association with native vegetation (Rowe, 1985). Following initial introduction into field soil, little disease is observed, but as inoculum builds up over the course of planting several susceptible crops, yield limiting levels of disease are possible (Rowe, 1985). This occurs more quickly when potatoes and other hosts are grown under short rotations or in successive seasons (Rowe, 1985). Microsclerotia of *V. dahliae* survive in the soil and on plant debris and germinate in the presence of root exudates produced by a susceptible host (Rowe, 1985). Hyphae penetrate root tissue, most commonly near the zone of elongation, colonize the root cortex, and spread to xylem tissue (Bowers et al., 1996). The colonization of cortical tissue did not guarantee a successful vascular infection (Gerik and Huisman, 1988). Only 0.02% of cotton root penetration events ended in systemic infections. In potato roots, the formation of lignitubers in root tissues has been reported to halt penetration of some hyphae, but it is thought that cells may not be able to stop the penetration of the multitude of infection points (Perry and Evert, 1983a; 1983b). Penetration and infection by *V. dahliae* causes roots to mature prematurely and stops apical cell growth (Perry and Evert, 1983b). The pathogen then moves within the host as conidia are carried via the transpiration stream to the acropetal portions of the plant, where they cause subsequent infections (Rowe, 1985). Wilting and other symptoms result from toxins produced by the fungus (Rowe, 1985). As plants begin to senesce, microsclerotia are formed and returned to the soil with plant debris after harvest. These resting structures survive in the soil and plant debris until a susceptible host is planted.

While the pathogen can be introduced into fields via infected seed tubers, this does not appear to be major contributor to disease severity in fields with infested soil (Dung and Johnson, 2012; Dung et al., 2012; Hoyman, 1974; Robinson and Ayers, 1961). Incidence of *V. dahliae*

infection in seed lots has been documented to be 64%, 29% and 51% from Israel and North America, respectively (Easton et al., 1972; Omer et al., 2000; Tsror (Lahkim) et al., 1999a). However, the importance of this inoculum has been demonstrated to be very low, especially where inoculum also is carried on the seed surface, or is already present in soil (Dung and Johnson, 2012; Dung et al., 2012; Hoyman, 1974; Robinson and Ayers, 1961). When inoculum carried on the surface of tubers was compared to tubers with vascular infections, the former resulted in a higher frequency of wilted plants in cultivars with some resistance, as well as susceptible cultivars (Robinson and Ayers, 1961). In susceptible cv. Norgold Russet, symptoms of Verticillium wilt were present in plants produced from infected seed-tubers (Hoyman, 1974). Similar results were observed with moderately susceptible cv. Russet Burbank (Dung and Johnson, 2012). Tuber-borne inoculum alone did not significantly affect wilt development, and also did not contribute to higher wilt in plants grown in infested soil. When comparing *V. dahliae* seed-tuber infections (low vs. high) and potato rotations (5 vs. 30 or more years), potato rotation was more closely related to pathogen colonization, foliar disease, infection in daughter tubers and yield than was the amount of infection in seed-tubers (Dung et al., 2012). In most instances, the level of seed-tuber inoculum did not affect disease progression.

Vegetative compatibility groups. *V. dahliae* reproduces asexually and has no known teleomorphic stage (Klosterman et al., 2009). However, genetic recombination via hyphal anastomosis and the formation of a heterokayon, has been demonstrated (Puhalla and Mayfield, 1974). The first evaluation of *V. dahliae* VCGs sub-divided 19 isolates, either defoliating or non-defoliating on cotton, into four groups (P1-P4) (Puhalla, 1979). Later, related work divided 96 isolates from 38 hosts into 16 groups (P1-P16), but nine groups contained only a single isolate (Puhalla and Hummel, 1983). Both of these evaluations were performed using UV induced

mutant isolates which formed albino and brown microsclerotia. Later studies, using complementary auxotrophic nitrate-nonutilizing (nit) mutants, reported four VCGs, which did not correspond to the existing 16 that had been previously identified (Joaquim and Rowe, 1990). Based on reactions of 183 and 47 *V. dahliae* isolates from potatoes in Ohio, and other potato growing states, respectively, only 3 VCGs were identified, and most isolates belonged to VCG4. Additionally, VCG4 was sub-divided further into VCG4A and VCG4B (Joaquim and Rowe, 1991). These sub-divisions were supported by pathogenicity experiments performed on potatoes, in which VCG4A was observed to be more virulent than VCG4B and VCG2. Subsequent studies comparing disease progress, determined there was no difference between isolates VCG4A and VCG4B when inoculated alone (Botseas and Rowe, 1994). However, when soil also was infested with root lesion nematodes, VCG4A resulted in significantly higher wilt progress and lower tuber yields when compared to infestations with isolates of VCG4B, or non-infestation. While several VCGs are known to colonize potato tissue, most pathogenic infections are attributed to VCG4 (Davis and Huisman, 2001).

Symptoms of Verticillium wilt of potato. General symptoms of Verticillium wilt include overall plant wilting, premature vine death, foliar chlorosis and necrosis, as well as vascular discoloration. Symptoms resembling natural plant senescence begin in the lower canopy and move upward in three stages as the season progresses (Bowden and Rouse, 1991; Powelson and Rowe, 1993; Rowe, 1985). The first stage is the latent phase where *V. dahliae* can be detected, but no visual symptoms are present (Bowden and Rouse, 1991). In the second, or local, stage the base of the plant begins to senesce, moving upward in the final systemic phase. Because individual vascular bundles may become colonized at different times, infection by *V. dahliae* can result in a characteristic unilateral wilting of the leaflets (Davis and Huisman, 2001; Rowe,

1985). Discoloration of affected vascular tissue can be observed in lower stems when cut longitudinally. The tendency for plants to die erect can distinguish Verticillium wilt from other wilt diseases caused by pathogens including *C. coccodes* and *Fusarium* ssp. (Rowe, 1985). Vascular discoloration also can be evident in tuber tissue, especially on the stem end, resulting in browning of processed products.

The importance of Verticillium wilt of potato. References to Verticillium wilt of potato go back to as early as the 1920's in the United States (McKay, 1926). At this time, wilt caused by *Verticillium* spp., as well as *Fusarium* spp., was noted as important in Oregon potato production due to reductions in yield (McKay, 1926). More recently, during a time when yields were increasing due to advances in new high-yielding cultivars, cultural practices and the control of weed, insect and disease pests, Verticillium wilt was responsible for underlying yield reductions that were commonly not noticed by producers (Powelson and Rowe, 1993). This issue was not recognized as readily in areas with long histories of potato production, but in newer areas, particularly Oregon and Washington, where high yields had been obtained for the first 1 to 2 crops and then sharply declined, it was difficult to ignore (Powelson and Rowe, 1993). Surveys of growers taken in the late 1980's indicated that Verticillium wilt was the most costly problem facing the potato industry (Powelson and Rowe, 1993). Several factors have led to the increased importance of Verticillium wilt including higher value placed on tuber quality, as well as an increase in use restrictions of the soil fumigant metam sodium (MacRae and Noling, 2010; Rowe and Powelson, 2002). Losses due to Verticillium wilt are the result of yield reductions as high as 50%, reductions in tuber size and quality, and the cost of soil fumigation (Rowe and Powelson, 2002). Soil fumigation with metam sodium can cost as much as $750/ha, depending on the rate and type of application (Rowe and Powelson, 2002). In the absence of cultivars with resistance

to *V. dahliae* and acceptance by the marketplace, soil fumigation is the most effective method by which growers can control Verticillium wilt (Powelson et al., 1993; Rowe and Powelson, 2002). To exacerbate the problem further, soil fumigants such as methyl bromide have been removed from the market by the Environmental Protection Agency (EPA), and restrictions have been placed on the application of metam sodium which will make it increasingly more difficult for growers to effectively use this fumigant (MacRae and Noling, 2010).

Disease management. The level of microsclerotial inoculum of *V. dahliae* in the soil is affected by crop rotation, soil type, irrigation and other management practices (Ben-Yephet and Szmulewich, 1985; Evans et al., 1967; Joaquim et al., 1988; Rowe and Powelson, 2002; Taylor et al., 2005). Concomitantly, the level of damage caused has been related to pathogen levels, but also host susceptibility, temperature, organic matter, moisture and fertility, among other factors (Ben-Yephet and Szmulewich, 1985; Davis, 1985; Davis and Everson, 1986; Nicot and Rouse, 1987b; Nnodu and Harrison, 1979). As few as 3.15 propagules of *V. dahliae* in 5 grams of soil were sufficient to cause severe wilting under conditions of high temperatures in Israel (Ben-Yephet and Szmulewich, 1985). An economic threshold of 10 cfu/g and 18 to 23 cfu/g of soil was determined under Idaho and Colorado growing conditions, respectively (Davis, 1985; Nnodu and Harrison, 1979). A relationship was observed between the level of *V. dahliae* in the soil and stem colonization of cv. Russet Burbank (Nicot and Rouse, 1987b). When temperature and overall conditions were favorable for the pathogen, a significant inverse relationship also was observed between *V. dahliae* propagules and yield in cvs. Norgold and Norchip grown in Colorado (Nnodu and Harrison, 1979). However, no consistent relationship was observed in Idaho, and rather, relationships existed between irrigation method as well as fertility (Davis and Everson, 1986). Overall, it is widely accepted that accurate quantification of *V. dahliae*

propagules in the soil is important for the selection of appropriate control measures and to determine the efficacy of these control measures (Mahuku and Platt, 2002; Mpofu and Hall, 2003). Traditionally microsclerotial quantification in soil has been determined indirectly via the use of indicator plants or directly using various methods of plating soil on selective media. Extensive research has been devoted to optimizing these methods, but results remain less than ideal (Goud and Termorshuizen, 2003; Lopez-Escudero and Blanco-Lopez, 2005). More recently, several conventional and real-time PCR assays have attempted to replace soil plating methods (Bilodeau et al., 2012; Mahuku and Platt, 2002; Mahuku et al, 1999; Perez-Artes et al., 2005). The main obstacles for the success of these molecular methods have been rupturing microsclerotia to extract DNA and overcoming inhibitors from the soil co-extracted with target DNA (Bilodeau et al., 2012; Cullen and Hirsch, 1998).

No singular disease management tactic available will completely eliminate *V. dahliae* microsclerotia from the soil, and therefore, completely control Verticillium wilt (Powelson and Rowe, 1993). Control measures for *V. dahliae* are very similar across the herbaceous hosts and include using clean seed or planting stock, crop rotation, deep plowing, flaming crop debris, soil fumigation, or solarization, and the use of resistant cultivars. Even though microsclerotia of *V. dahliae* can persist in the soil for 15 years, crop rotation can aid in reducing the level of pathogen in the soil. It has been reported that the breakdown of crop debris resulted in release of microsclerotia and a subsequent significant increases in soil levels (Ashworth et al., 1974; Ben-Yephet and Szmulewich, 1985; Evans et al., 1967; Joaquim et al., 1988; Taylor et al., 2005). After the breakdown of debris, soil pathogen levels began to decline significantly (Ben-Yephet and Szmulewich, 1985; Joaquim et al., 1988; Taylor et al., 2005).

Soil fumigation using metam sodium has proven very effective at reducing the number of microsclerotia in the soil. Unfortunately, this method of disease control is expensive and has unfavorable consequences to the environment (Davis, 1985; MacRae and Noling, 2010; Powelson et al., 1993). EPA restrictions on the use of this chemical have begun, and will continue to increase in the coming years, limiting the availability of this control measure for many growers (MacRae and Noling, 2010). While the use of solar energy was used to disinfest soil by ancient civilizations, soil solarization as we know it today was first presented as a method to disinfest soil in 1976 (Katen et al., 1976; 1987). Under desert conditions of Israel, soil artificially inoculated with *V. dahliae* was pathogen-free at depths up to 25 cm after coverage by polyethylene sheets for 2 weeks, and significant reductions in disease severity were observed in naturally infested fields (Katen et al., 1976). Soil solarization since has been evaluated to reduce infestation levels in many soil-borne pathogens in more than 24 countries in both hot arid climates as well as more northern locations including the Midwestern United States (Katen et al., 1987). Significant reductions in Verticillium wilt, stem vascular discoloration and colonization, as well as yield increases of 46% were observed in moderately susceptible cv. Russet Burbank in Idaho (Davis and Sorenson, 1986). However, this management tactic is not widely used in potato production in the United States today. Organic soil amendments including liquid swine manure, meat and bone meal and green manure successfully have been utilized to inactivate and kill microsclerotia of *V. dahliae* (Conn, et al., 2005; Lazarovits et al., 2001; Tenuta and Lazarovits, 2004). The accumulation of ammonia or nitrous acid via the addition of liquid swine manure or meat and bone meal to the soil is responsible for inactivating or killing microsclerotia of *V. dahliae* (Conn, et al., 2005; Lazarovits et al., 2001; Tenuta and Lazarovits, 2004). While mechanisms differ based on soil pH, liquid swine manure was reported to significantly reduce

microsclerotial germination in both acidic and basic soils (Conn, et al., 2005; Lazarovits et al., 2001). Similarly, mechanisms of meat and bone meal amendments have been reported to be effective in sandy soil, but not loam soil (Tenuta and Lazarovits, 2004). Incorporation of green manure crops including Sudan grass, Australian winter pea, broccoli, rape, oat and corn, also has shown promise for Verticillium wilt management (Davis et al., 1996; Goicoechae, 2009; Ochiai et al., 2007). In field trials conducted in Idaho using potato cv. Russet Burbank, significant yield increases were observed after the incorporation of Sudan grass and corn, but not with Austrian winter pea, oat, rye, or Dwarf Essex and Bridger rape (Davis et al., 1996). These yield increases were not attributed solely to a reduction in soil inoculum, but possibly changes in the soil micro flora. Later studies conducted in Oregon also using cv. Russet Burbank, resulted in reductions in wilt severity depending on amendment (Austrian winter pea, broccoli, Sudan grass) and rate (6 mg/ha, 12 mg/ha, 24 mg/ha) used, but no yield increases were observed with any treatment (Ochiai et al., 2007). Additionally, while *V. dahliae* inoculum density was reduced by many treatments, root infection was not. The incorporation of green manure has shown promise in many situations, but due to variability in control due to differences in soil type and climate, use may be limited to integration into a multi-faceted management system (Ochiai et al., 2007).

Cultural practices including tillage, water and fertility management can reduce Verticillium wilt severity (Ben-Yephet and Szmulewich, 1985; Cappaert et al., 1992; 1994; Taylor et al., 2005). Tillage practices that invert the soil layers and bury crop debris have been used to reduce soil infestation levels in the upper soil strata (Ben-Yephet and Szmulewich, 1985; Taylor et al., 2005). Water management can reduce the effects of Verticillium wilt in two ways (Cappaert et al., 1992; 1994). Early in the growing season, before tuber initiation, slightly dry conditions help to limit microsclerotial germination and, therefore, limit plant infections

(Cappaert et al., 1992; 1994; Davis et al., 1990). Later, when tubers are bulking, providing adequate water so that plants are not stressed diminishes the effects plant infections have on plant wilting (Davis et al., 1990). In addition to the amount of irrigation, the type of irrigation also can affect severity of Verticillium wilt (Davis and Everson, 1986). Studies indicate that disease severity is greater under furrow-irrigation when compared to sprinkler irrigation, possibly due to the distribution and subsequent availability of nitrogen throughout the root zone. Similarly, fertility management that reduces stress to plants has been reported to limit colonization and concomitant damage by the pathogen (Davis, 1985; Davis and Everson, 1986; Davis et al., 1990). A relationship was reported between the availability of nitrogen and colonization by *V. dahliae,* as well as wilt development, tuber yield and size in cv. Russet Burbank (Davis and Everson, 1986). The availability of nitrogen displayed synergistic affects when combined with fumigation treatments, resulting in decreases in colonization and increases in wilt (Davis, 1985). In addition to nitrogen, adequate levels of potassium and phosphorus also appear to diminish the effects of *V. dahliae* colonization (Davis and Everson, 1986).

Breeding for resistance to *V. dahliae*. While some cultural management practices including fertility management, crop rotation and the use of green manure crops can aid in decreasing soil infestation levels, these measures alone are not adequate to reduce disease levels to an acceptable level because of the general lack of resistance in commonly grown cultivars (Rowe et al., 1987). Several studies have demonstrated that resistance to Verticillium wilt is heritable and stable (Corsini et al., 1985; 1990; Hoyos et al., 1993; Hunter et al., 1968). Sources of resistance to *V. dahliae* have been identified in wild Solanum spp. including *S. gourlayi, S. chacoense,* and *S. tarijense,* as well as breeding clones (Concibido et al., 1994; Corsini et al., 1988; Davis, 2009; Davis et al., 1983; Jansky and Rouse, 2000; 2003). Resistance to *V. dahliae*

also has been reported in commercially available cultivars, but the majority of these cultivars no longer are grown (Rich, 1983). Breeding resistance to *V. dahliae* into cultivars and parental lines for the potato chip market was initiated in Wisconsin in 1957 (Hunter et al., 1968). What the authors refer to as resistance was identified more frequently in clones with at least one resistant parent. However, this resistance claim was based solely on the identification of visual symptoms of Verticillium wilt, not the recovery of the pathogen (Hunter et al., 1968). More recently, several cultivars have been reported to exhibit resistance to Verticillium wilt, but no attempts were made to quantify the level of pathogen present to determine if the host response was one of true resistance or tolerance. Among these are russet-skinned cultivars with some level of purported resistance to Verticillium wilt include Goldrush (Johansen et al., 1994), Century Russet (Mosely et al., 2000b), Umatilla Russet (Mosely et al., 1999), Bannock Russet (Novy et al., 2002), Gem Russet (Love et al., 2002), Alturas (Novy et al., 2003), Summit Russet (Love et al., 2005), GemStar Russet (Love et al., 2006), Defender (Novy et al., 2006), Premier Russet (Novy et al., 2008), Highland Russet (Stark et al., 2009), and Clearwater Russet (Novy et al., 2010).

In planta quantification of *V. dahliae*. As a result of increased breeding efforts for resistance to Verticillium wilt caused by *V. dahliae*, there has been an increased need for accurate and rapid assays to detect the pathogen in host tissue in order to define the exact nature of the host:parasite interaction (Corsini et al., 1985; Hoyos et al., 1991). Procedures for quantifying *V. dahliae* in plant tissue have taken many forms including estimating microsclerotia on plant tissue with the aid of photographs and counting microsclerotia sieved from air-dried or fresh ground plant tissue (Evans et al., 1966; Isaac et al., 1971; Tsai and Erwin, 1975). Subsequent efforts to attempt to simplify the process of microsclerotial quantification included

air-drying stems and plating using an Anderson sampler or directly plating sap from fresh stems onto semi-selective media (Davis et al., 1983; Hoyos et al., 1991). The procedure for plating air-dried stems was developed following similar assays for quantifying *V. dahliae* in soil (Davis et al., 1983). The number of propagules recovered from fresh stem tissue that had been dried and ground before plating was correlated to percent wilt and yield (Davis et al., 1983). A procedure for plating sap extracted from stem tissues was developed to evaluate Verticillium resistance in breeding clones from several breeding programs across the United States (Hoyos et al., 1991). Sap was extruded from the basal portion of stem tissue and plated to semi-selective media. The number of colony forming units in plant sap was well correlated with Verticilllium wilt (Hoyos et al., 1991). Variations and combinations of the fresh and dried plating techniques have been used to evaluate resistance in breeding selections and cultivars on several occasions (Concibido et al., 1994; Corsini et al., 1985; 1990; Davis et al., 1983; Hoyos et al., 1993; Jansky, 2009; Jansky and Miller, 2010; Jansky and Rouse, 2000; 2003; Jansky et al., 2004; Mohan et al., 1990). These methods also have been utilized to evaluate the effects of soil inoculum (Nicot and Rouse 1987a). Recent research comparing traditional plating methods of pathogen quantification to visual wilt symptoms again confirms the accuracy and reliability observed in previous research, but these methodologies are time and labor intensive (Jansky, 2009). A quantitative real-time PCR assay would reduce drastically the time and labor required to quantify the *S. tuberosum*:*V. dahliae* interaction.

Countless PCR assays have been developed for use in plant pathogen detection and/or quantification. Among the first fungal plant pathogens to be evaluated using DNA amplification technology were *Verticillium* spp. (Henson and French, 1993). Since that time, several PCR assays have been developed to detect, and in some instances, quantify *V. dahliae in planta*

(Atallah et al., 2007; Dan et al., 2001; Hu et al., 1993; Li et al., 1999; Mahuku et al., 1999; Mercado-Blanco et al., 2001; 2002; 2003; Nazar et al., 1991; Perez-Artez et al., 2000). The first PCR primers were designed to differentiate *V. dahliae* from *V. albo-atrum* utilizing polymorphisms in the ITS region of these fungi (Nazar et al., 1991). Many subsequent applications for, and adaptations to, this assay were investigated including, quantifying *V. albo-atrum* in alfalfa and *V. dahliae* in sunflowers (Hu et al., 1993). In later work using environmental samples, the PCR assay using the ITS1/ITS2 primers was compared to media plating for the identification of *Verticillium* species in field grown potatoes and soil (Mahuku et al., 1999) and quantification of fungal biomass from potato breeding clones and cultivars (Dan et al., 2001). The information generated from these and other research projects using the ITS1/ITS2 primers advanced research on *Verticillium* species tremendously in many host systems. Subsequently, two pairs of PCR primers, D-1/D-2 and ND-1/ND-2, were generated from RAPD polymorphisms to characterize cotton defoliating and non-defoliating pathotypes of *V. dahliae* (Perez-Artez et al., 2000). The detection of symptomless infections of olive trees was improved over previous single-step PCR assays using the primers developed for defoliating and non-defoliating pathotypes of cotton, and additional primers developed in the same regions of the genome (Mercado-Blanco et al., 2001; 2002; 2003). PCR primers, VDS1/VDS2, were developed from a unique RAPD band to quantify *V. dahliae* in potatoes using a competitive internal control in a classical endpoint PCR platform (Li et al., 1999). The first real-time quantitative PCR assay was developed to quantify *V. dahliae* in olive using primer pairs DB19/DB22 and internal control primers in a SYBR-Green format (Mercado-Blanco et al., 2003) and potato using VertBt-F/VertBt-R primers developed to the *β-tubulin* gene in a Plexor Q-PCR format (Atallah et al., 2007). The VertBt assay was used to compare colonization of

resistant cv. Ranger Russet to susceptible cv. Russet Norkotah (Bae et al., 2007). While these assays have shown some utility, none of them have been adopted with wide-spread use in the industry.

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CHAPTER ONE. EFFECT OF INOCULUM SOURCE ON *COLLETOTRICHUM COCCODES* INFECTION IN POTATO[1]

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Abstract

Colonization of potato (*Solanum tuberosum*) tissue, including roots, stolons, and above and below ground stems, by *Colletotrichum coccodes*, the causal agent of black dot, was evaluated following soil infestation, inoculation of seed tubers and foliage, and every combination thereof, in field trials over two growing seasons in North Dakota and Minnesota. A total of 107,520 isolations for *C. coccodes* performed across four site-years allowed for an extensive comparison of fungal colonization of the host plant and disease severity. The black dot pathogen was detected in potato stems at the first sampling date in all four site-years, as early as 14 days prior to emergence. Colonization of above and below ground stems occurred at a higher frequency than in roots and stolons in all four site-years, resulting in significantly higher relative area under the colonization progress curves (RAUCPCs) ($\alpha = 0.05$). Although fungal colonization and disease incidence were higher in inoculated and/or infested treatments, sufficient natural inoculum was present to result in substantial levels of disease in noninoculated and noninfested plots. However, noninoculated and noninfested plots displayed the lowest RAUCPC values across three of four site-years and those treatments with multiple inoculation events tended to have higher RAUCPC values. Isolates belonging to vegetative compatibility

[1]The material in this chapter was co-authored by Julie S. Pasche, Raymond J. Taylor, and Neil C. Gudmestad. J. S. Pasche had primary responsibility for data collection and analysis, while R. J. Taylor was responsible for collection of a portion of the data. J. S. Pasche drafted and revised all versions of this chapter and was the primary developer of the conclusions. N. C. Gudmestad and R. J. Taylor served as proofreaders and provided comments on the content.

group (VCG)2 and -5 were recovered from plants sampled in 2004 more frequently than isolates belonging to VCG1 and -3. A significant difference in disease incidence on stems was observed only in North Dakota in 2004 and Minnesota in 2003 ($\alpha = 0.05$). Noninoculated and noninfested plots displayed the lowest disease incidence, whereas those treatments with more than one inoculation and/or infestation event tended to have higher disease incidence. Results of this study, including the disease severity and yield data, provide a better understanding of colonization of potato plants by *C. coccodes* and its impact.

Introduction

Black dot, caused by *Colletotrichum coccodes* (Wallr.) S. Hughes, is a disease that occurs wherever potato crops (*Solanum tuberosum* L.) are grown. Although the most economically important hosts of *C. coccodes* are potato, tomato, and pepper, this pathogen is able to infect a wide range of plant species, a majority of them members of the Solanaceae family. Several weed species, including some *Solanaceous* species, also have been identified as hosts (Nitzan et al., 2006b; Raid and Pennypacker, 1987). On potato, black dot generally is considered to be primarily a tuber blemish disease resulting in symptoms similar to silver scurf caused by *Helminthosporium solani* Durieu & Mont.; however, the pathogen also can infect roots, stolons, stems, and foliage (Andrivon et al., 1998; Barkdoll and Davis, 1992; Dickson, 1926; Johnson, 1994; Mohan et al., 1992; Pavlista and Kerr, 1992; Tsror (Lahkim) et al., 1999). Infected areas of tubers are silver to brown in color with microsclerotia present. Deep sunken lesions (Glais and Andrivon, 2004) and tuber shrinkage (Hunger and McIntyre, 1979) also have been noted with severe infections. Dark lesions form on infected leaf and stem tissue and contribute to wilting and defoliation (Davis and Johnson, 2001; Johnson and Miliczky, 1993b). In severe cases, the cortical tissue of infected below ground stems and stolons may slough off,

resulting in a frayed or stringy appearance (Davis and Johnson, 2001). Upon vine desiccation and disintegration of the cortex, an area of amethyst coloration may be observed in association with the remnants of the vascular bundles (Dickson, 1926).

Black dot has been recognized as a disease of potato since the early part of the 20th century (Dickson, 1926). It generally had been considered to be of minor importance (Komm and Stevenson, 1978; Otazu et al., 1978; Rich, 1983; Stevenson et al., 1976; Thirumalachar, 1967), having little impact on commercial potato production in most growing areas. Several factors have contributed to this view, many of which may be related to misdiagnosis. As with other typical anthracnose pathogens, *C. coccodes* often infects the host early and symptoms are not expressed until much later in the growing season (Andrivon et al., 1998), at which point they are often mistaken for normal plant senescence and saprophytic colonization. *C. coccodes* also can cause early dying in potato similar to other diseases such as Verticillium wilt, caused by *Verticillium dahliae* Kleb. and V. *albo-atrum* Reinke & Berthold, as well as early blight, caused by *Alternaria solani* Sorauer, and often occurs in conjunction with these diseases. Co-infection with *V. dahliae* has been shown to result in greater reductions than observed with either pathogen alone (Tsror (Lahkim) and Hazanovsky, 2001). Although yield losses of up to 30% due to black dot have been documented, it has proven difficult to reproduce these losses across growing seasons under field conditions, even when differences in black dot symptoms were noted among treatments (Mohan et al., 1992; Nitzan et al., 2005; Tsror (Lahkim) et al., 1999). Yield losses due to black dot also have been documented in the absence of symptom expression (Barkdoll and Davis, 1992).

Because of the aforementioned yield and quality losses, as well as losses reported by commercial potato growers, black dot research has garnered renewed interest in recent years as a

developing threat to potato production and crop quality (Lees and Hilton, 2003; Tsror (Lahkim) and Johnson, 2000). The apparent emergence of black dot as an important disease of potato simply may be due to increased awareness of the factors outlined above. Changes in tillage and other cropping practices during the later part of the past century also may have promoted the accumulation of soilborne sclerotial inoculum (Denner et al., 2000; Rothrock, 1992; Taylor et al., 2005), because the pathogen's longevity in the soil has been demonstrated to extend 5 to 13 years in the absence of a potato crop (Cullen et al., 2002; Dillard and Cobb, 1998). Although crop rotation may be successful in reducing soil inoculum, the wide host range of *C. coccodes*, including both weed and rotational crop hosts, as well as the longevity of the microsclerotia render crop rotation a fairly impractical control measure (Davis and Johnson, 2001; Eberlein et al., 1991; Nitzan et al., 2006b; Raid and Pennypacker, 1987). Additionally, the large, heavily melanized microsclerotia of *C. coccodes* are not killed effectively by currently registered soil fumigants (Davis et al., 1998; Stevenson et al., 1976; Tsror (Lahkim) and Johnson, 2000). Although seed treatment and in-furrow fungicide applications have not been successful at reducing black dot incidence or increasing yield in infested soil, foliar fungicide applications of the QoI fungicide azoxystrobin have proven effective in reducing black dot severity on stems and progeny tubers and increasing yield in the Columbia Basin of the United States (Cummings and Johnson, 2008; Nitzan et al., 2005). Long-term survival of the pathogen in the soil, limited number of useful fungicides, a long latent period, a wide host range, varying and unpredictable effects of the disease, and confusion regarding disease etiology illustrate why effective control of black dot can be difficult.

A more complete understanding of disease epidemiology and etiology is needed to develop successful strategies for effective management of black dot of potato. The pathogen

typically is introduced into noninfested soils via contaminated seed tubers, becomes established on the current season crop, and subsequently builds up in the soil on infected plant debris (Barkdoll and Davis, 1992; Komm and Stevenson, 1978; Read and Hide, 1988). Soilborne inoculum may infect tubers, stolons, roots, and below ground stems (Read and Hide, 1988; Tsror (Lahkim) et al., 1999; Tsror (Lahkim) and Hazanovsky, 2001). The airborne phase of *C. coccodes* also can cause above ground stem and foliar infections via windblown inoculum originating from the soil, debris of previously infected plants, or current season foliar infections, often exacerbated by wounds caused by windblown soil (Barkdoll and Davis, 1992; Johnson, 1994; Johnson and Miliczky, 1993b; Mohan et al., 1992). Although considerable research has been performed comparing the effects of some inoculum sources, this research has not taken into account all potential infection sites and the importance, frequency, and timing of both above and below ground host tissue colonization (Andrivon et al., 1998; Dashwood et al., 1992; Davis et al., 1998; Johnson, 1994; Mohan et al., 1992; Nitzan et al., 2005; Read and Hide, 1988; Read and Hide, 1995). Additionally, much of the early research was performed before differences in aggressiveness were characterized among vegetative compatibility groups (VCGs) (Aqeel et al., 2008; Heilmann et al., 2006; Nitzan et al., 2006a; Nitzan et al., 2002; Nitzan et al., 2006c). As a result, there are gaps in knowledge concerning the influence, significance, and relative importance of individual or combinations of infection courts upon pathogen colonization and disease development in various host tissues.

Yield losses are known to occur as a result of *C. coccodes* infections (Barkdoll and Davis, 1992; Johnson, 1994; Mohan et al., 1992; Tsror (Lahkim) et al., 1999), but the relationship among disease severity, particularly with infection of specific host tissues, inoculation and/or infestation sites, the extent of these losses, and the interactions among these

factors has not been investigated. The objectives of this research were to determine infection frequency of *C. coccodes* in specific plant tissues as affected by the site of inoculation and/or infestation, and to determine the effects of such infections on black dot disease severity, as well as yield and market value of the potato crop.

Materials and Methods

Field trials. Field trials were conducted in 2003 and 2004 at the Northern Plains Potato Growers Association Irrigated Research Site in central North Dakota. The plot area in 2003 had been pasture land with no previous potato crop, but in 2004, the experiment was the second crop of potato in 3 years. The same trial also was conducted both years in commercial potato fields in west-central Minnesota which previously had been cropped to potato and were presumed to have indigenous levels of the black dot fungus. Trials were planted on 24 April and 29 May 2003 and 15 and 29 April 2004 in Minnesota and North Dakota, respectively. Certified seed tubers of cv. Russet Burbank were used in all four site-years, with the same lot used at both sites in a given year. The trials were managed using standard agronomic practices employed in each region. Fungicides, including chlorothalonil, ethylenebisdithiocarbamates, and fluazinam, were applied to the entire trial as a foliar spray to prevent late blight (*Phytophthora infestans*, (Mont.) de Bary) and to minimize development of early blight (*A. solani*). All trials were conducted using overhead irrigation and water was applied at intervals necessary to meet the evapotranspirational demands of the crop. Treatments consisting of four-row blocks were arranged in a randomized complete block design with four replications. The distance between rows was 0.91 m, in-row seed tuber spacing was 0.3 m, and row length was 12.2 and 13.7 m in 2003 and 2004, respectively.

Quantification of *C. coccodes* in soil. The indigenous level of *C. coccodes* in the soil each year at the central Minnesota site was quantified using dilution plating techniques as previously described (Davis et al., 1994), with the following modifications. Soil cores were removed at 0- to 20-cm depths in a grid pattern (equidistance within the trial border), air dried and ground before being combined, and mixed thoroughly. In total, five 5-µg subsamples each of nondiluted and diluted (1:10 with sterile soil) cores were evenly dispersed onto Sorenson's NP-10 semiselective medium (Farley, 1972). Plates were incubated at 25 ± 2°C for 14 days in the dark, soil particles were washed from the plates under running tap water, and colonies were counted using a stereomicroscope at ×65 magnification.

C. coccodes inoculations. Eight isolates of *C. coccodes* collected from tubers, stems, roots, and stolons of commercial potato plants from across the United States were used to infest soil and inoculate seed tubers and foliage for each of four site-years. In 2003, VCG designation was not known prior to performing the trial and, therefore, each *C. coccodes* VCG is not equally represented. Subsequent testing revealed that, among these eight isolates, two belonged to VCG1, two to VCG2, one to VCG5, and three to VCG6 (Heilmann et al., 2006). In 2004, isolates were specifically chosen representing VCG1 to -5: two isolates each of VCG1, 2, and 5 and one each of VCG3 and 4. In either case, each VCG was equally represented in the inoculum mixture; that is, twice the volume was added for each of the single isolates of VCG3 and -4 compared with the two isolates which were used for each of the other VCGs.

The importance of inoculation and/or infestation site was examined in a similar manner at all four site-years. Soil infestations, seed tuber, and foliar inoculations were performed individually and in every combination thereof, resulting in eight treatments, including a noninoculated and noninfested control. In the Minnesota 2003 trial, soil was infested with *C.*

coccodes colonized rye seed (Nitzan et al., 2006c). *C. coccodes* was grown on solid 10% clarified V8 juice (CV8) medium (Miller, 1955) for 7 to 9 days in the dark at 25 ± 2°C. Conidia and microsclerotia were scraped from cultures in sterile water and used to inoculate sterile rye seed. The rye was incubated in the dark at 25 ± 2°C for 4 weeks, air dried for 6 days, and subsequently placed in furrow at planting (IFAP) at a rate of 1.9 g/m. In the remaining three site-years, a *C. coccodes* infested agar slurry was utilized to inoculate the soil. Isolates of *C. coccodes* were grown on CV8 for 2 to 3 weeks in the dark at 25 ± 2°C. Agar cultures were homogenized in a blender and the microsclerotial concentration of each isolate was standardized to 102 CFU/ml. In 2003 in North Dakota, 4 liters of microsclerotia–agar suspension was mixed with 22 liters of vermiculite and applied IFAP at a rate of 164 ml/m of row. At both sites in 2004, the ensuing fungal slurry was applied directly to the field at a rate of 80 ml/m^2 and tilled into the soil at a depth of 7.5 to 10.0 cm prior to planting.

Inoculum applied to seed tubers was prepared by growing isolates in 10% CV8 liquid medium for 2 to 3 weeks. Fungal cultures were centrifuged at 5,000 rpm for 5 to 7 min and resuspended to an adjusted concentration of 10^2 spores/ml in a 0.25% gelatin solution to aid in spore adhesion and prevent desiccation. This suspension was sprayed onto suberized seed tubers until each tuber was coated (approximately 60 ml per 450 g). Noninoculated tubers were sprayed with a sterile solution of 0.25% gelatin. The tubers were air dried for 5 to 10 min, placed in paper bags, and stored at 12 ± 2°C and 80 to 85% relative humidity (RH) for no longer than 24 h prior to planting. Foliar inoculations were conducted using a microsclerotial suspension prepared utilizing the same procedure as was described for soil inoculations in 2004. At 6 to 8 weeks after planting, the basal portion of plants in each row of the four-row plot, for applicable treatments, were sandblasted with silica sand at 245 kPa of pressure to create wounds for infection (Johnson

and Miliczky, 1993b). A 102 microsclerotia/ml suspension at 15 ml/m was applied to the resulting wounded portion of the canopy using a hand sprayer at 137 kPa of pressure.

Tissue colonization. In all four site-years, the frequency of colonization of *C. coccodes* was determined throughout the growing season by destructively sampling five plants from each treatment–replication combination at approximately 7-day intervals. The process was initiated 7 days post emergence and continued for 12 weeks in 2003 and 14 days preemergence and continued for 16 weeks in 2004. Three stems per hill (stems originating from a single seed tuber) were assayed on each sampling date by excising a 2- to 3-mm stem segment approximately 10 cm above and below the soil line. A single stolon and root segment, 5 to 7 mm in length, also was collected from each stem. In total, 46,080 and 61,440 isolations for *C. coccodes* were made in 2003 and 2004, respectively. All tissue samples were placed onto culture plates containing solid Sorenson's NP-10 medium. Cultures were examined for the presence of *C. coccodes* after 3 to 4 weeks of incubation at 25 ± 2°C in the dark. The number of infections per tissue segment was recorded and infection frequency was expressed as percentage per stem. The area under the colonization progress curve (AUCPC) was calculated using weekly colonization data (Shaner and Finney, 1977):

$$AUCPC = \sum_{i=1}^{n} [(W_{i+1} + W_i)/2](t_{i+1} - t_i)$$

where W_i = percentage of *C. coccodes* colonization at the *i*th observation, t_i = time in days at the *i*th observation, and n = total number of observations. AUCPC values were standardized to enable comparisons among site-years. Standardization was achieved by dividing the AUCPC values for each treatment of the replicated trials from each site-year by the total area of the graph, resulting in relative area under the colonization progress curve (RAUCPC).

Presumptive VCG analysis. Monoconidial isolates collected from all tissues and treatments at both sites in 2004 were selected for presumptive VCG analysis using amplified fragment length polymorphism (AFLP) markers (Heilmann et al., 2006). Sections of *C. coccodes* grown from tissues sampled at week 1, 2, 3, 7, and 8 were transferred to solid media containing 1.5% agar for hyphal tip or monoconidial isolation by micromanipulation. Permanent cultures were established on silica gel crystals stored at –80°C in a 7.5% skim milk solution using microsclerotia scraped from homogeneous cultures of *C. coccodes* grown on CV8 medium amended with ampicillin at 50 mg/ml for 5 to 7 days (Smith, 1984).

Disease incidence. Black dot incidence on stems was assessed visually throughout the growing season. The number of stems in the center two rows of each four-row plot was recorded approximately 3 weeks after emergence. Incidence of black dot infection was assessed by determining the number of infected, wilted, or dead stems with obvious microsclerotial formation characteristic of *C. coccodes* commencing 62 to 115 days after planting (DAP) and continuing for 1 to 3 and 5 to 11 weeks in 2003 and 2004, respectively. Incidence was expressed as the percentage of stems exhibiting black dot disease symptoms.

Assessment of tuber yield and quality. The center two rows of each replicated treatment, 9.1 m in length at all four site-years after destructive sampling was completed, were harvested between 125 and 160 DAP. Total yield and United States Department of Agriculture grade data were collected at the end of each growing season for each treatment. In 2004, French fry color and quality ratings also were performed on 25 arbitrarily selected tubers per replicate.

Statistical analysis. Two-factor analyses of variance (ANOVA) were performed on RAUCPC generated from in vitro tissue assays within each site-year using Proc GLM of SAS (version 9.1; SAS Institute, Cary, NC) with tissue type assayed and inoculation and/or infestation

site as main effects. One-factor ANOVAs were performed on black dot stem incidence as well as yield grade and processing data, when applicable, across each site-year. In all instances, means were differentiated using Fisher's protected least significant difference (LSD) test ($\alpha = 0.05$). Pearson's correlation was utilized to show relationships among combinations of *C. coccodes* colonization at the point in the growing season when frequency was approximately 40 to 50% at each site-year, black dot stem incidence at the final data collection date at each of the four site-years, and total yield. Then, $\chi 2$ tests of homogeneity were performed to evaluate the frequency of presumptive VCG recovery across sites (Minnesota and North Dakota), tissues (above and below ground stems, roots, and stolons) and weeks (1, 2, 3, 7, and 8) during which *C. coccodes* isolates were obtained, as well as across all eight treatments ($\alpha = 0.05$). Fisher's exact tests were performed when underlying assumptions of the $\chi 2$ test were not met ($\alpha = 0.05$).

Results

Quantification of *C. coccodes* in soil. In Minnesota in 2003, the indigenous *C. coccodes* population was 69 propagules per gram (ppg) dry weight of soil and, in 2004, the population was less than 1 ppg dry weight. Levels of indigenous *C. coccodes* in the soil were not determined for either year at the North Dakota site because it was a newly developed potato research site.

C. coccodes tissue colonization. *C. coccodes* was detected in stems of potato plants at the first sampling date in all four site-years, including 14 days prior to emergence at both sites in 2004. The progression of *C. coccodes* colonization was variable, in some cases substantially, in noninoculated and noninfested plants among the four site-years when this study was performed (Fig. 1.1). At the North Dakota site in 2003, the frequency of colonization remained relatively low and unchanged until the last three collection dates of the season. Similar trends were observed at this site in 2004 but *C. coccodes* colonization frequencies began to increase earlier

and were higher at the end of the growing season compared with 2003. *C. coccodes* colonization frequency was highest and progressed more rapidly in Minnesota in 2003 when compared with the other three site-years. At this site, *C. coccodes* colonization of noninoculated and noninfested plants was nearly 40% at 28 days after emergence (DAE) and exceeded 80% at 49 DAE compared with between nearly 0 and 50% during that same time period in the other three site-years. Colonization by *C. coccodes* at the Minnesota site in 2004 was similar to that of the North Dakota site that same year.

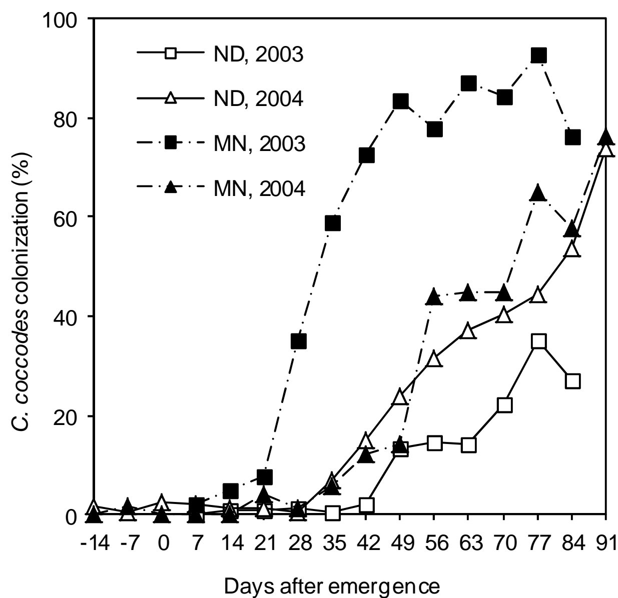

Figure 1.1. Percentage of *Colletotrichum coccodes* colonization assayed in vivo from tissue of noninoculated potato plants grown in field trials performed in North Dakota (ND) and Minnesota (MN) in 2003 and 2004. Colonization frequency represents the mean of above and below ground stem, root, and stolon tissue.

A significant interaction was observed between the main effects of inoculation and/or infestation site and tissue sampled in colonization rate as expressed by the RAUCPC in the 2004 North Dakota trial ($P < 0.0001$) but not in the 2003 North Dakota trial ($P = 0.065$) or the Minnesota trial in 2003 ($P = 0.748$) and 2004 ($P = 0.998$). The interaction at the 2004 North Dakota trial was due, in part, to those treatments with inoculated seed tubers having higher *C. coccodes* colonization frequencies of roots than stolons while those treatments without seed tuber

inoculation had higher colonization frequencies of stolons compared with roots (data not shown). There also were significant differences among the main effects of inoculation and/or infestation site, as well as tissues sampled in all four site-years (Tables 1.1 and 1.2). *C. coccodes* colonization frequencies measured by RAUCPC were significantly different in all site-years among inoculation and/or infestation treatments (Table 1.1). Similar patterns of tissue colonization were observed in both years in North Dakota. At this site, RAUCPC values of noninoculated and noninfested controls were significantly lower than nearly all inoculated and/or infested treatments. Treatments with multiple inoculation and/or infestation sites also tended to have significantly higher RAUCPC values than those treatments with single inoculation or infestation events. Plants from soil infested + seed tuber + foliar inoculated treatments displayed the highest level of colonization, although not always significantly so. When comparing multiple inoculation and infestation events, plants from treatments in which seed tubers were inoculated, in combination with either soil infestation or foliar inoculation, tended to have higher colonization levels compared with the combination of soil infestation and foliar inoculation. Again, these differences were not always significant. In Minnesota in 2003, although differences among RAUCPC values were significant, the range of these values was small (Table 1.1). The noninoculated and noninfested control did not display the lowest RAUCPC values, and additional inoculation and/or infestation events did not consistently increase colonization as was observed in North Dakota, presumably due to high indigenous soil populations present that year (69 ppg of soil). However, at this site in 2004, with relatively low indigenous soil populations (<1 ppg of soil), trends were similar to those observed in North Dakota.

Table 1.1. Relative area under the *Colletotrichum coccodes* colonization progress curve (RAUCPC) among inoculation/infestation sites across all potato tissues sampled.

	North Dakota		Minnesota	
Site of inoculation/infestation	2003	2004	2003	2004
No inoculation/infestation	0.10 f	0.20 f	0.57 bc	0.22 d
Seed tuber inoculation	0.11 ef	0.25 bc	0.59 ab	0.25 bcd
Soil infestation	0.12 de	0.21 ef	0.57 bc	0.24 cd
Foliar inoculation	0.15 bc	0.22 de	0.58 b	0.27 bc
Soil infestation + seed tuber inoculation	0.14 bc	0.27 ab	0.59 ab	0.26 bc
Seed tuber + foliar inoculation	0.16 bc	0.28 a	0.62 a	0.31 a
Soil infestation + foliar inoculation	0.13 cd	0.24 cd	0.57 bc	0.28 ab
Soil infestation + seed tuber + foliar inoculation	0.19 a	0.28 a	0.55 c	0.31 a
P value[a]	<0.0001	<0.0001	<0.0001	<0.0001

[a]Values in a column followed by the same letter are not statistically different based on Fisher's protected least significant difference ($\alpha = 0.05$). *P* value represents the probability of observing a greater value in the F test.

Table 1.2. Relative area under the *Colletotrichum coccodes* colonization progress curve (RAUCPC) among potato tissues sampled across all non-inoculated/non-infested and inoculated/infested treatments.

	North Dakota		Minnesota	
Tissue	2003	2004	2003	2004
Above ground stem	0.16 b	0.34 a	0.75 a	0.28 a
Below ground stem	0.20 a	0.34 a	0.63 b	0.30 a
Roots	0.07 d	0.13 c	0.42 d	0.25 b
Stolons	0.12 c	0.15 b	0.52 c	0.24 b
P value[a]	<0.0001	<0.0001	<0.0001	<0.0001

[a]Values in a column followed by the same letter are not statistically different based on Fisher's protected least significant difference ($\alpha = 0.05$). *P* value represents the probability of observing a greater value in the F test.

Across site-years, colonization was detected at the first sampling date but progressed more quickly in above and below ground stem tissue than in roots and stolons, resulting in significantly higher RAUCPC values for these tissues (Figs. 1.2A–D and Table 1.2). In North Dakota in 2003, below ground stem tissue was infected at significantly higher frequencies than above ground stem tissue, while colonization frequencies of above and below ground stems were the same in 2004 at this site (Figs. 1.2A and B and Table 1.2). In both years in North Dakota, colonization of stolon tissue was significantly greater than that of root tissue (Figs. 1.2A and B

and Table 1.2). In Minnesota in 2003, differences in colonization were significantly different among all tissues, with above ground tissue colonization greatest, followed by below ground stems, stolons, and roots (Fig. 1.2C and Table 1.2). In 2004 at this same site, there was no significant difference between above and below ground stem colonization or between roots and stolons; however, stems were colonized at significantly higher frequencies than roots and stolons (Fig. 1.2D and Table 1.2).

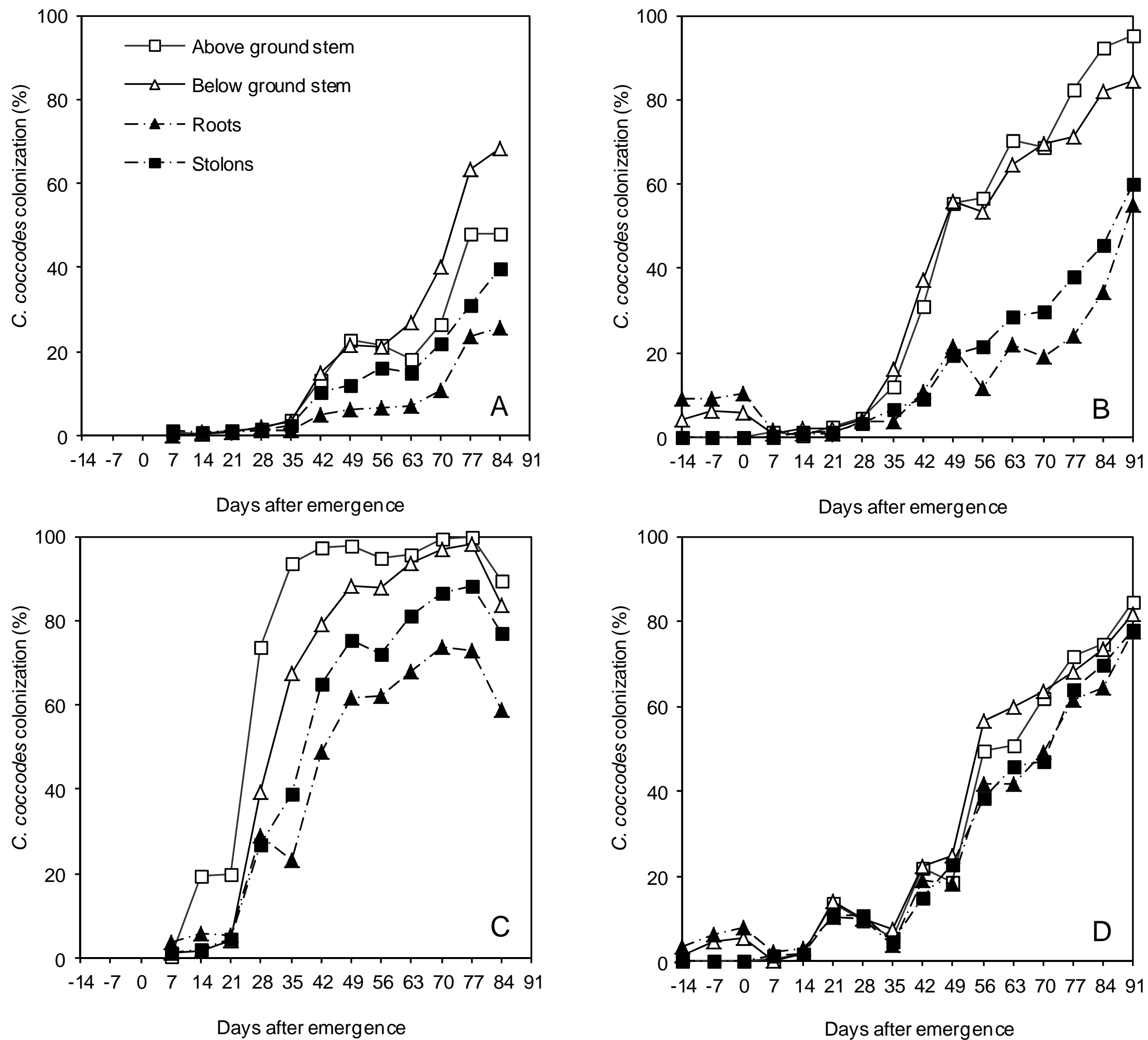

Figure 1.2. Percentage of *Colletotrichum coccodes* colonization assayed in vivo from above and below ground stem, root, and stolon tissue of potato plants grown in field trials performed in (A and B), North Dakota and (C and D), Minnesota in (A and C), 2003 and (B and D), 2004. Colonization frequency represents the mean of all plants in noninoculated and noninfested and inoculated and/or infested treatments.

Presumptive VCG analysis. In total, 91 *C. coccodes* isolates collected from both sites in 2004 were evaluated via AFLP analysis to determine a presumptive VCG (Heilmann et al., 2006). AFLP analysis is not able to differentiate the uncommon VCG4 from other VCGs; therefore, only VCG1, -2, -3, and - 5 were detected among isolates collected from sample plants. Across all isolates, the frequency of recovery was not evenly distributed among these four VCGs. Overall, the frequency of recovery of isolates from VCG2 and -5 (28 and 40%, respectively) was substantially greater than that of VCG1 and -3 (10 and 13%, respectively). However, $\chi 2$ and Fisher's exact analyses revealed that there was no significant difference in the frequency of recovery of each VCG across the two sites ($P = 0.1371$; $P = 0.1106$), across the 5 weeks isolates were characterized ($P = 0.1970$; $P = 0.3369$), or among above and below ground stems, stolons, or roots ($P = 0.8988$; $P = 0.9719$). A significant difference was observed in the frequency of isolates recovered from each of the eight treatments using both analyses ($P = 0.0008$; $P = 0.0001$) (Figs. 1.3A–D). Interestingly, although the number of isolates recovered per treatment–VCG combination was low, the noninoculated and noninfested control was the only treatment from which no isolates belonging to VGC5 were recovered (Fig. 1.3D). The mean of isolates belonging to VCG5 recovered from treatments containing inoculated seed tubers, alone or in any combination, ranged from 46 to 73%, whereas those with infested soil and inoculated foliage alone had a mean of 17 and 9%, respectively. Similarly, the treatment containing the combination of soil infestation and foliar inoculation yielded 29% of isolates belonging to VCG5. The inverse was true for isolates belonging to VCG2 (Fig. 1.3B). The frequency of *C. coccodes* isolates belonging to VCG2 recovered from treatments with inoculated seed tubers was much lower (mean of 13%) than the frequency of this VCG recovered from infested soil or foliar inoculated treatments at 67 and 82%, respectively. The combination of soil infestation and foliar

inoculation yielded 71% of isolates belonging to VCG2. Isolates belonging to VCG1 and -3 represented less than 25% of the total for any individual treatment (Figs. 1.3A and C).

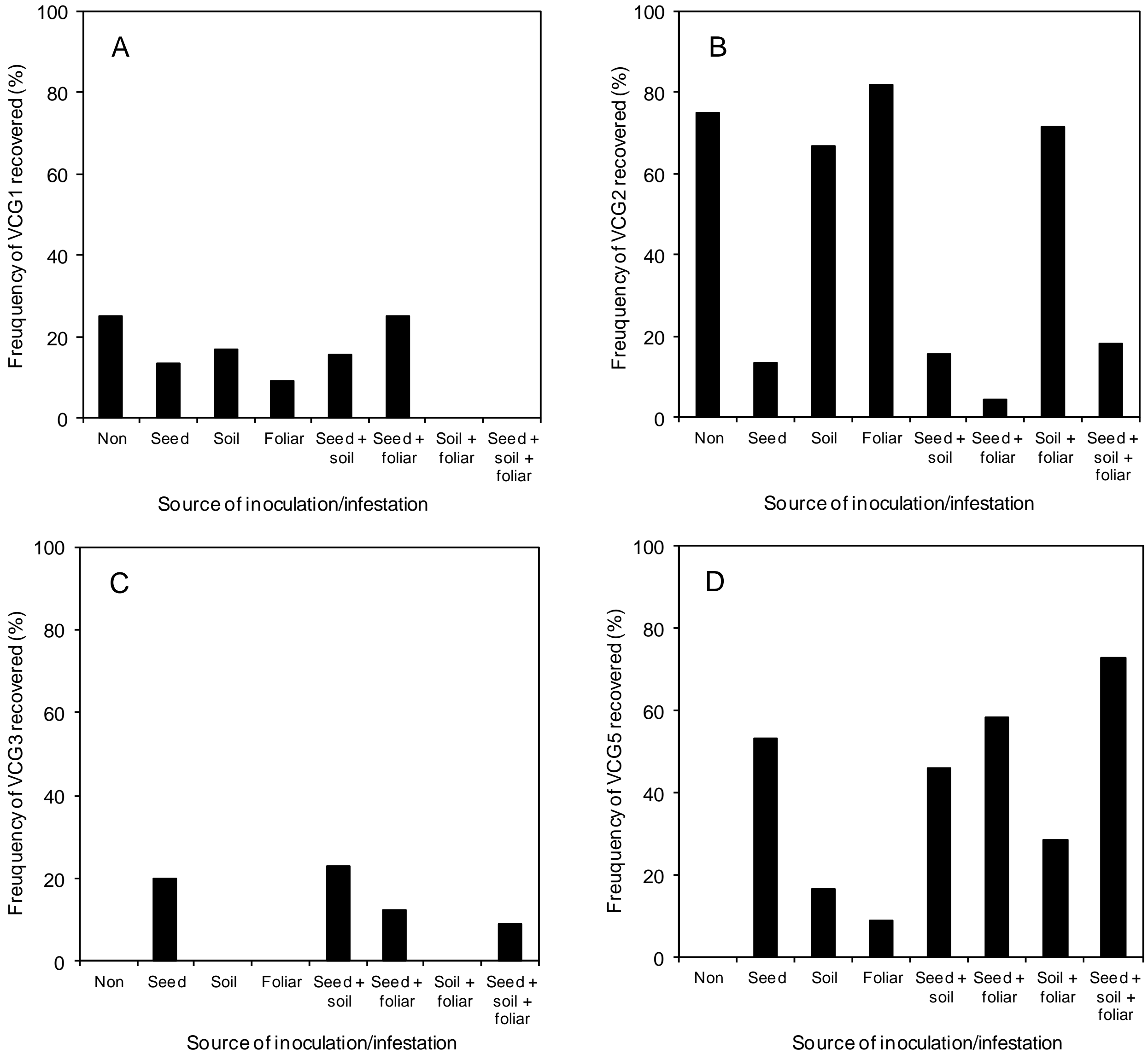

Figure 1.3. Frequency of recovery of *Colletotrichum coccodes* isolates belonging to (A) vegetative compatibility group (VCG)1; (B) VCG2; (C) VCG3; and (D) VCG5 among sources of inoculation and/or infestation. Isolates were recovered from potato plants produced in North Dakota and Minnesota trials in 2004 from above and below ground stem, root, and stolon samples taken at five sampling dates across the season.

Black dot disease incidence. Black dot disease incidence on stems tended to be significantly different only among inoculation and/or infestation treatments when disease incidence was high (Figs. 1.4A–D). In central North Dakota in 2003, black dot disease incidence ranged from 9.6 to 18.5% at the final data collection date 113 DAP (Fig. 1.4A). Although nearly twice as much black dot was observed in soil infested + seed tuber + foliar-inoculated plots compared with noninoculated and noninfested plots, there was no significant difference among treatments at any of the four data collection dates. In 2004 at this site, a significant difference was observed among treatments at the last data collection date 138 DAP, with disease incidence ranging from 37.9 to 55.4% (Fig. 1.4B), substantially higher than that observed in 2003. The noninoculated and noninfested control displayed the least amount of black dot stem incidence, although not significantly different from all inoculated and/or infested treatments.

In the 2003 Minnesota trial, a significant difference was observed among treatments in black dot stem incidence at both data collection dates. Disease incidence ranged from 24.9 to 47.2% at the last data collection date 119 DAP, and the noninoculated and noninfested treatment had significantly lower disease incidence than inoculated and/or infested treatments with the exception of seed tuber inoculation (Fig. 1.4C). There were no significant differences observed among any of the inoculated and/or infested treatments (Fig. 1.4C). At this site in 2004, no significant differences were observed among treatments at any data collection date, and black dot disease incidence was low, ranging from 7.7 to 14.7% at the last data collection date at 139 DAP, similar to what was observed in North Dakota in 2003 (Fig. 1.4D).

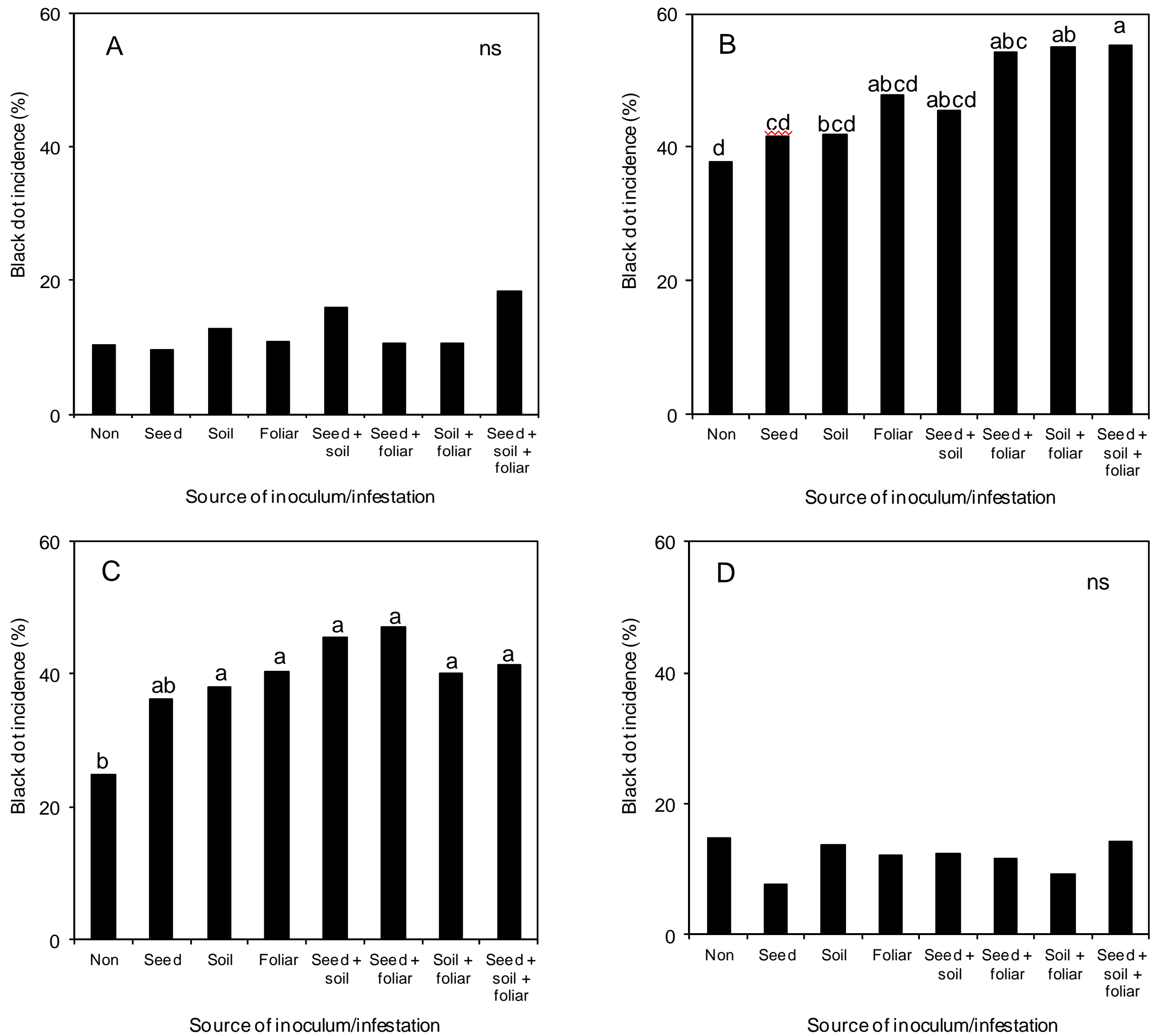

Figure 1.4. Incidence of black dot symptoms in potato stems on the final data collection date for each trial: (A) 113 days after planting (DAP) at the North Dakota 2003 trial; (B) 138 DAP at the North Dakota 2004 trial; (C) 119 DAP at the Minnesota 2003 trial; and (D) 139 DAP at the Minnesota 2004 trial. Bars with the same letter are not statistically different based on Fisher's protected least significant difference ($\alpha = 0.05$).

Yield and tuber quality assessments. No significant differences in total yield were observed among sites of inoculation and/or infestation in either year at the North Dakota site (Table 1.3). A significant difference in total yield was observed only among site of inoculation and/or infestation at the 2003 Minnesota site. Although the noninoculated and noninfested

treatment did result in the highest total yield, these differences were significant only when compared with treatments with more than one site of inoculation and/or infestation, with the exception of the soil infested + seed tuber + foliar-inoculated treatment. However, differences in total yield were not significantly different in Minnesota in 2004, and no significant differences were observed in marketable yield, including tuber size and quality, or French fry quality among the four site-years (data not shown).

Table 1.3. Total Yield (mT/ha) among *Colletotrichum coccodes* inoculation/infestation sources.

	North Dakota		Minnesota	
Site of inoculation/infestation	2003	2004	2003	2004
No inoculation/infestation	7.94	7.26	9.05 a	8.84
Seed tuber inoculation	8.08	7.45	8.40 ab	9.33
Soil infestation	7.98	7.78	8.65 ab	9.38
Foliar inoculation	7.60	6.95	7.94 bc	9.16
Soil infestation + seed tuber inoculation	8.16	7.09	7.99 bc	9.00
Seed tuber + foliar inoculation	8.17	6.51	7.47 c	9.42
Soil infestation + foliar inoculation	7.98	7.66	7.98 bc	9.21
Soil infestation + seed tuber + foliar inoculation	7.86	7.46	8.35 ab	9.37
P value[a]	0.895	0.064	0.012	0.847

[a]Values in a column followed by the same letter are not statistically different based on Fisher's protected least significant difference (α = 0.05). *P* value represents the probability of observing a greater value in the F test.

The relationship among black dot stem incidence at the last data collection date, *C. coccodes* colonization, and total yield was variable among site-years, according to Pearson’s correlation analyses (Table 1.4). A consistent and significant correlation was observed among all three comparisons only at the 2003 Minnesota site. At this site, black dot stem incidence at 122 DAP and colonization frequency at 60 DAP (r = 0.83; *P* = 0.012), black dot stem incidence and total yield (r = –0.88; P = 0.004), and colonization frequency and yield (r = –0.76; *P* = 0.030) all had a highly significant relationship. A significant negative relationship was also observed between *C. coccodes* colonization frequency 96 DAP and total yield (r = –0.64; *P* = 0.087) at the

North Dakota 2003 site, as well as black dot stem incidence at 138 DAP and *C. coccodes* colonization frequency 89 DAP ($r = 0.84$; $P = 0.010$) at the North Dakota 2004 site. Although some trends were observed among these variables at other site-years, none were significant (Table 1.4).

Table 1.4. Relationship between black dot stem incidence, *Collototrichum coccodes* colonization frequency and total yield of potato as determined by Pearson's correlation coefficient.

Comparison parameters	North Dakota		Minnesota	
	2003	2004	2003	2004
Black dot incidence[a] vs. *C. coccodes* colonization frequency[b]	r = 0.04[c]	r = 0.84*	r = 0.83**	r = 0.38
Black dot incidence vs. total yield	r = -0.01	r = -0.22	r = -0.88**	r = -0.28
C. coccodes colonization frequency vs. total yield	r = -0.64*	r = -0.42	r = -0.76**	r = 0.37

[a]Black dot stem incidence was evaluated at 113, 138, 122, and 139 days after planting for the North Dakota 2003 and 2004, and Minnesota 2003 and 2004 trials, respectively.

[b]*C. coccodes* colonization frequency was evaluated at 96, 89, 60, and 101 days after planting for the North Dakota 2003 and 2004, and Minnesota 2003 and 2004 trials, respectively.

[c]Pearson correlation coefficients were siginificant at the $\alpha = 0.05$ (**) and $\alpha = 0.10$ (*) levels, n = 8.

Discussion

Although several previous research studies have examined *C. coccodes* colonization and the development of black dot symptoms in potato (Andrivon et al., 1998; Dashwood et al., 1992; Davis and Everson, 1986; Johnson and Miliczky, 1993a; Nitzan et al., 2006a; Read and Hide, 1995; Tsror (Lahkim) et al., 1999; Tsror (Lahkim) and Hazanovsky, 2001), the results reported here provide a comprehensive comparison of colonization, disease development, and yield. Some of the previous studies concentrated on the development of disease without evaluating the frequency of *C. coccodes* colonization (Andrivon et al., 1998; Read and Hide, 1995). Research conducted with soil infestations of *C. coccodes* on two cultivars commonly grown in the United Kingdom demonstrated that black dot symptoms appeared at a high rate in root tissue (60 to 90%) at the first assessment date 5 weeks after planting regardless of inoculum level (low versus high), but little or no disease was visible on below ground stems (Read and Hide, 1995). Similar research focused on tuber-borne inoculum determined that symptoms on roots and stolons could be detected within 1 week after inoculating seed tubers, around the time of emergence, whereas symptoms on stems did not appear until approximately 7 to 10 weeks after inoculation (Andrivon et al., 1998).

Among studies that have examined *C. coccodes* colonization of host tissue, in plants assayed from 37 commercial potato fields in Idaho, colonization of both basal and apical stem sections by *C. coccodes* was correlated with the amount of pathogen recovered from the soil (Davis and Everson, 1986). However, subsequent research determined that, under growth-chamber conditions, colonization by *C. coccodes* at the base of the stem was not affected by soil inoculum density (Tsror (Lahkim) and Hazanovsky, 2001). Research performed under commercial growing conditions in the Columbia Basin of central Washington reported that *C.*

coccodes was isolated at the first sampling date, as early as 15 days after emergence in above ground stems, and later, 22 days after emergence, in below ground stems; however, a larger number of CFU typically were isolated from below ground stems on subsequent sampling dates (Johnson and Miliczky, 1993a). More recent research performed under greenhouse conditions by the same group determined that the pathogen moved more quickly downward from a single inoculation point on the above ground stem, than toward the apex of the plant (Nitzan et al., 2006a). Root and stolon tissue was not assayed in any of these studies. Under field trial conditions in Scotland, *C. coccodes* colonization of root tissue produced from disease-free micropropagated plants was similar to that in roots produced from both visually blemish-free and blemished seed tubers when evaluated early in the growing season, but was substantially lower at later sampling dates (Dashwood et al., 1992). One study has evaluated *C. coccodes* colonization in roots, as well as above and below ground stem tissue in inoculated plants under field conditions, but did so only once during the growing season at 90 DAP (Tsror (Lahkim) et al., 1999). At that point, no differences in colonization frequency among these plant tissues were apparent across the five cultivars evaluated in five trials. To our knowledge, the studies reported here represent the first attempt to evaluate colonization of potato tissue by *C. coccodes* using multiple inoculation and/or infestation sites and all affected tissues, including roots and stolons as well as above and below ground stems, across the entire growing season.

The results reported here illustrate a different picture of tissue colonization than previously has been described. Colonization of stem tissue by *C. coccodes* above and below ground was higher than the colonization frequency of stolons and roots at all four site-years of the study. This trend was true regardless of whether the infection originated from soil infestation, seed tubers, or foliar inoculation. This is in contrast to previous studies which have demonstrated

that black dot disease symptoms can be detected first in root tissue, compared with other plant tissues evaluated (Andrivon et al., 1998; Read and Hide, 1995). Both of these studies evaluated symptom expression, and not tissue colonization of the fungus, the most likely reason for the discrepancies. It commonly is accepted that infections by *C. coccodes* remain latent for an extended time period, and this may be more evident in thicker stem tissue than in finer root and stolon tissue. Among the previous research which evaluated colonization, comparisons in timing of colonization were made only between above and below ground stems, and indicated that above ground stems were colonized approximately a week earlier than below ground stem tissue (Johnson and Miliczky, 1993a). In the first year of this study, colonization was detected in all tissues sampled at the Minnesota site and in above ground stems and stolons at the North Dakota site at the first sampling date 14 days after emergence. Because the point at which initial infection had taken place presumably was missed, sampling was initiated earlier in the second year of the study. Again, *C. coccodes* stem colonization was detected at both sites in both below ground stems and roots approximately 14 days prior to emergence, which never has been reported previously. Also, colonization was recorded at the first sampling date in all tissues sampled in three of the four site-years. The contrast in these results from previous studies might be attributed to differences in the levels of seed tuber inocula and soil infestation (Barkdoll and Davis, 1992; Davis et al., 1998; Dillard and Cobb, 1998; Farley, 1976), cultivar susceptibility (Andrivon et al., 1998; Mohan et al., 1992; Thirumalachar, 1967; Tsror (Lahkim) et al., 1999; Tsror (Lahkim) and Hazanovsky, 2001), or environmental factors (Read and Hide, 1988; Stevenson et al., 1976; Tsror (Lahkim) et al., 1999).

These studies also corroborate previous work indicating that soil infestations and foliar and seed tuber inoculations are all capable of initiating *C. coccodes* infections. Previous research

conducted by inoculating foliage under greenhouse conditions found a correlation between leaf lesions and wilt, as well as wilt and yield for plants, but seed tuber inoculations or soil infestations were not evaluated (Barkdoll and Davis, 1992). The effect of foliar inoculations and soil infestations have been investigated individually in the field and greenhouse (Mohan et al., 1992; Tsror (Lahkim) et al., 1999). Although some significant differences in stem death and wilting were observed between foliar and noninoculated plants under field conditions as well as between soil and noninfested plants in the greenhouse (Mohan et al., 1992), greater stem colonization occurred with foliar inoculations compared with soil infestations under field conditions (Tsror (Lahkim) et al., 1999); however, these sources were not evaluated in the same trial and direct comparisons are not possible.

Previous studies which compared the effect of soil and seedborne inoculum indicated that soil inoculum may cause more black dot than seedborne inoculum (Davis et al., 1998; Nitzan et al., 2005; Nitzan et al., 2008; Read and Hide, 1995). Under field conditions in the United Kingdom, varying levels of inoculum applied to seed tubers resulted in increases in black dot infection on stem bases and roots, but not consistently across seed tuber disease levels and cultivars, whereas soil infestation more consistently increased black dot infection (Read and Hide, 1995). Seed tuber and soilborne inoculum was investigated in individual field trials which did not allow for direct comparisons to be made, and no combination of the inoculation and/or infestation sources was evaluated (Read and Hide, 1995). Also, under field conditions, soil infestation was reported to result in decreased yield and increased black dot incidence on progeny tubers, compared with either light or severe seedborne pathogen levels (Davis et al., 1998) but no wilt severity or *C. coccodes* colonization levels were examined. More recently, where similar levels of natural soil infestations were present, progeny tubers from seed tubers

with low levels of black dot displayed more black dot symptoms than tubers produced from seed tubers with higher levels of black dot (Nitzan et al., 2005). This indicates that soil inoculum may cause more infections in progeny tubers than seedborne inoculum. Finally, soil infestations performed in the greenhouse were determined to result in increased sclerotial development on roots and stems when compared with seedborne inoculum (Nitzan et al., 2008). However, differences in research methods, as well as the type of data collected in the aforementioned research, make comparisons between studies difficult. Therefore, gaps remain in our understanding of *C. coccodes* colonization of potato plants.

In all four site-years, sufficient indigenous inoculum was available to establish substantial disease levels in noninoculated and noninfested treatments. Despite this, at three of the four site-years, lower colonization frequency was observed in noninoculated and noninfested treatments compared with inoculated and/or infested treatments, indicating that the inoculations and/or infestations were effective in increasing black dot colonization to varying degrees. Levels of *C. coccodes* in the soil were evaluated prior to planting the trial in both years at the central Minnesota site. Although colonization frequencies were affected by inoculation and/or infestation in both years, no consistent trends were observed in 2003, likely due to the relatively high level of naturally occurring inoculum (approximately 69 ppg of soil). However, in 2004, when the indigenous soil inoculum was <1 ppg, plants from noninoculated and noninfested treatments displayed the lowest levels of colonization, followed by plants from single inoculation or -infestation and multiple inoculation and/or -infestation treatments, respectively. Interestingly, it is apparent from these data that inoculum potential <1 ppg of soil was sufficient to establish stem infections as high as 50% midway through the season, while higher levels of inocula present the previous year were effective in raising infection frequencies to nearly 95% at the

same time in the growing season. Although levels of soilborne inoculum in naturally occurring infestations of *C. coccodes* and their relationship to disease development have not been examined in detail, increasing soilborne inoculum was reported to increase black dot disease severity, including foliar necrosis and chlorosis as well as sclerotial development on roots and stems, under greenhouse conditions (Nitzan et al., 2008). Also, among 37 potato fields in Idaho, the levels of *C. coccodes* in the soil were highly correlated with both basal and apical stem colonization (Davis and Everson, 1986). A later survey of Idaho potato fields confirmed these reports. *C. coccodes* levels ranged from 0.2 to 211 ppg of soil and tuber tissue infection was highly correlated with the field soil inoculum levels (Barkdoll and Davis, 1992). Soil infestation levels at the central Minnesota trial site in the present study fall within the range documented above.

Inconsistencies in the ability of *C. coccodes* to affect yield or cause disease are not unexpected and have been reported on numerous occasions with black dot greenhouse and field research (Barkdoll and Davis, 1992; Davis and Everson, 1986; Johnson, 1994; Johnson and Miliczky, 1993a; Mohan et al., 1992; Nitzan et al., 2005; Read and Hide, 1995; Scholte et al., 1985; Stevenson et al., 1976; Tsror (Lahkim), 2004; Tsror (Lahkim) et al., 1999; Tsror (Lahkim) and Hazanovsky, 2001; Tsror (Lahkim) and Johnson, 2000). Variable results in yield reduction were reported between field experiments performed over 2 years in Idaho when comparing cultivar reaction to foliar inoculations (Mohan et al., 1992). A later study successfully demonstrated that *C. coccodes* infections significantly reduced yield under both greenhouse and field conditions and that these yield losses could be correlated to wilt, although asymptomatic *C. coccodes* infections also led to yield reductions (Barkdoll and Davis, 1992). In field experiments examining soil infestations and seedborne inoculum on black dot development in two cultivars,

significant reductions in total tuber yield were observed in only 1 year of this study, when the crop was planted with seed tubers severely infected with *C. coccodes*, even though plants were noticeably colonized by *C. coccodes* (Read and Hide, 1995). Differences in tuber weight reductions were reported under greenhouse conditions when comparing foliar to root inoculations (Aqeel et al., 2008). In that study, root inoculations reduced tuber weights more than foliar inoculations, but disease progression and colonization were not evaluated. In the current study, root colonization by *C. coccodes* generally lagged behind above ground infections; therefore, it is clear why yield reductions were not detected in most site years. In contrast, foliar inoculations decreased yield more than both seed tuber inoculation and soil infestation, although these differences were significant at only one site-year. This disparity is most likely due to the presence of natural inoculum, differences in inoculation methods, and environmental factors (which were controlled under greenhouse conditions), in addition to difficulties measuring yield reductions caused by this pathogen.

Differences in aggressiveness among VCGs of *C. coccodes* (Aqeel et al., 2008; Heilmann et al., 2006; Nitzan et al., 2002; Nitzan et al., 2006c) may play a role in these inconsistencies because most studies involving the impact of black dot on yield of potato were performed before vegetative compatibility was reported in this fungus. One recent research study has taken into account *C. coccodes* VCGs (Nitzan et al., 2008). Two isolates of VCG2 led to higher disease incidences when originating from soil than from seed tubers, whereas the opposite was true for a third isolate belonging to VCG1. These results support the findings reported here, in which higher frequencies of isolates of VCG2 were recovered from plants that had grown in infested soil when compared with those from inoculated seed tubers. Some of the past contradictory yield results also may be attributed to differences in cultivar susceptibility. Data generated in both

field and greenhouse trials demonstrated that later maturing cultivars are more likely to suffer yield reductions than earlier-maturing cultivars (Aqeel et al., 2008; Mohan et al., 1992). Also, recently reported results of colonization of control cultivars and breeding selections by *C. coccodes* grown in naturally infested soil indicated that differences among cultivars or selections exist and that these differences were significantly affected by environmental conditions (Nitzan et al., 2009). Although there are other examples of contradictory reports concerning the effect of *C. coccodes* on yield of potato, the aforementioned research results provide an ample illustration of the difficulties that lie in quantifying direct effects of this pathogen. The effects of black dot on yield reported here are consistent with the observations made in the several earlier studies. The Minnesota 2003 site was the only one to have significant yield loss compared with the noninoculated and noninfested control. At this site, the significant negative correlation between black dot incidence at the time of haulm desiccation on 22 August and yield in the late maturing cv. Russet Burbank may provide some indication of yield loss. A similar comparison was made with soil inoculum, *C. coccodes* colonization levels, and wilt on 23 August in previous research performed in Idaho (Davis and Everson, 1986). An association of this type potentially may act as a predictor of season-end black dot incidence and, ultimately, effect on tuber yield. These relationships clearly should be investigated in further studies.

C. coccodes still often is considered to be a weak pathogen, attacking plants following periods of stress or causing blemishes on tubers; however, results reported here and from similar work demonstrate that the picture is much broader. The effects of *C. coccodes* on yield and tuber quality ultimately will be tied to a variety of factors, such as inoculum potential, environmental conditions, cultural practices, cultivar, and pathogen VCG. Although black dot may not cause reductions in yield and tuber quality in all instances, *C. coccodes* remains a serious threat to

commercial potato production and the seed potato industry, particularly in areas where Verticillium wilt is a concern and interactions between the two pathogens occur (Davis and Howard, 1976; Otazu et al., 1978; Rowe et al., 1987; Scholte et al., 1985; Tsror (Lahkim) and Hazanovsky, 2001). The data reported here may be useful in establishing the proper timing of fungicides such as azoxystrobin, which is highly efficacious (Nitzan et al., 2005). Because the infections that are most likely to become symptomatic occur early in the growing season, fungicide applied immediately following emergence may be the most effective.

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CHAPTER TWO. DEVELOPMENT AND VALIDATION OF A REAL-TIME PCR ASSAY FOR THE QUANTIFICATION OF *VERTICILLIUM DAHLIAE IN PLANTA*

Abstract

An increase in the stringency for higher quality tubers and restrictions on the use of soil fumigants, among other factors, has garnered renewed interest in Verticillium wilt, particularly in russet-skinned cultivars grown for processing. In response to the needs of producers, breeders have increased efforts in the development of cultivars with resistance to *Verticillium dahliae* Kleb., the primary cause of Verticillium wilt. These efforts have resulted in the release of numerous russet-skinned cultivars with purported resistance to the pathogen. However, because efficient methods to screen germplasm for true resistance do not exist, breeders typically have reported resistance based on the development of wilt symptoms alone. The studies reported here demonstrate the efficiency and practicality of a QPCR method for quantification of *V. dahliae* in stem tissue. This method, developed to detect the target trypsin protease gene of the pathogen, was compared to traditional methods for *V. dahliae* quantification which involve plating stem tissue or sap onto semi-selective media. The QPCR assay was demonstrated to be sensitive to 250 fg of DNA and specific to *V. dahliae*. Use of the duplex real-time PCR assay utilizing the potato actin gene to normalize quantification, resulted in clearer differentiation of levels of resistance among eight russet-skinned cultivars inoculated in greenhouse trials. Relative levels of resistance among cultivars were similar between traditional plating and QPCR methods, resulting in correlation coefficients greater than 0.93. The QPCR assay developed demonstrates rapid, efficient and accurate quantification of *V. dahliae,* providing a tool amenable for use by breeding programs on large numbers of clones and selections, and will aid researchers evaluating other control strategies for Verticillium wilt.

Introduction

Verticillium wilt in United States potato production is caused largely by *Verticillium dahliae* Kleb*,* although *V. albo-atrum* Reinke & Berthold may be associated with the disease in cooler climates (Davis and Huisman, 2001; Powelson and Rowe, 1994; Rowe et al., 1987). *V. dahliae*, either alone or in conjunction with other fungal pathogens, including *Fusarium* spp., and *Colletotrichum coccodes* (Wallr.) S. J. Hughes, and the root-lesion nematode *Pratylenchus penetrans* (Cobb) Sher and Allen, may be the primary cause of economic losses in the potato industry in the United States (Davis et al., 2001; Davis 1985; Nicot and Rouse, 1987; Otazu et al., 1978; Powelson and Rowe, 1993). This is due not only to yield reductions, but also losses in tuber size and quality from vascular discoloration, as well as losses from costs of control measures, primarily soil fumigation using metam sodium (Davis 1985; Hunter et al., 1968).

Symptoms of Verticillium wilt begin to appear at the base of the plant and move acropetally as the season progresses (Bowden and Rouse, 1991; Powelson and Rowe, 1993; Rowe, 1985). These symptoms of general plant wilting essentially can be impossible to differentiate from natural senescence. However, unilateral wilting, the result of differential colonization of individual vascular bundles, and the erect posture of dead plants infected with *V. dahliae* are diagnostic of Verticillium wilt (Davis and Huisman, 2001; Rowe, 1985). Discoloration often is present in stem, as well as tuber vascular tissue.

Microsclerotia are the main infective propagules of *V. dahliae* under field conditions and are reported to survive in the soil for up to 14 years (Wilhelm, 1955). Microsclerotia germinate in the presence of susceptible host plants to infect near the zone of elongation of root tips (Bowers et al., 1996; Huisman and Gerik, 1989). Any movement of soil into a field subsequently may result in the introduction of the pathogen into non-infested soil (Rowe, 1985; Powelson et

al., 1993). Initial introductions of *V. dahliae* into fields often are isolated, but when susceptible potato cultivars are grown, infected stem tissue serves as inoculum for subsequent crops, returning microsclerotia to the soil after harvest (Rowe, 1985). In potatoes, 100% stem infection was recorded at soil infestation levels of 6-10 microsclerotia/g in Wisconsin (Nicot and Rouse, 1987), whereas, 8 and 18 to 23 microsclerotia/g of soil have been documented as the economic thresholds in Idaho and Colorado, respectively (Davis and Sorenson, 1986; Nnodu and Harrison, 1979). While the relationship between disease incidence and microsclerotial concentration differ among cultivars, environmental conditions, and cropping practices, the reduction of microsclerotia in the soil is paramount in mitigating the effects of this monocyclic pathogen in the absence of genetic resistance (Berbegal et al., 2007; Xiao and Subbarao, 1998).

Several factors have led to the increased importance of Verticillium wilt including reduced tillage and shorter rotations between potato (*Solanum tuberosum* L.) crops, higher value placed on tuber quality, and an increase in use restrictions of soil fumigants, most recently, metam sodium (Davis, 1985; MacRae and Noling, 2010; Rowe and Powelson, 2002). Soil fumigation using metam sodium is effective and widely used, but also is expensive and detrimental to the environment (Davis, 1995; MacRae and Noling, 2010; Powelson et al., 1993). Environmental Protection Agency (EPA) restrictions on the application of metam sodium are mounting and will result in limiting usage by growers (MacRae and Noling, 2010). Verticillium wilt management also involves reducing soil pathogen populations, as well as limiting the affect the pathogen has on the host. Control measures which focus on reducing populations in the soil, in addition to soil fumigation, include crop rotation, tillage, and solarization (Ben-Yephet and Szmulewich, 1985; Davis, 1985; Davis and Sorenson, 1986; Joaquim et al., 1988; Powelson et al., 1993; Rowe and Powelson, 2002; Taylor et al., 2005). Control measures that focus on

minimizing infection and the effects of the pathogen on the host include nutrient and water management, as well as the use of resistant cultivars (Cappaert et al., 1992; 1994; Davis, 1985; Davis and Everson, 1986; Davis et al., 1990; Powelson et al., 1993). Because of the general lack of resistance in commonly grown cultivars, some cultural management practices can aid in decreasing the severity Vertcillium wilt, but these measures are not adequate to reduce disease to acceptable levels (Jansky et al., 2004; Jansky and Rouse, 2000; Pegg and Brady, 2002). *V. dahliae* resistance has been identified in wild *Solanum* spp., breeding clones and commercial cultivars (Concibido et al., 1994; Corsini et al., 1988; Davis, 2009; Davis et al., 1983; Jansky and Rouse, 2000; 2003). Unfortunately, few cultivars exhibiting *V. dahliae* resistance currently meet industry standards, and therefore, growers rely heavily on soil fumigation for effective control of Verticillium wilt (Powelson et al., 1993; Rowe and Powelson, 2002).

Several recently released cultivars have been reported to exhibit resistance to Verticillium wilt based on symptom expression, but in many instances, no attempts were made to quantify the level of pathogen present to determine if the host response was one of true resistance or tolerance (Johansen et al., 1994; Love et al., 2002, 2005, 2006; Mosley et al., 1999, 2000, 2001; Novy et al., 2002, 2003, 2006, 2008, 2010; Stark et al., 2009). Additionally, symptom expression can be very limited and difficult to discern from natural senescence, often resulting in incorrect diagnoses (Davis, 1985). Several research studies have described the use of fresh and dried plating techniques to evaluate *V. dahliae* resistance and control strategies (Concibido et al., 1994; Corsini et al., 1985; 1990; Davis et al., 1983; Hoyos et al., 1993; Jansky, 2009; Jansky and Miller, 2010; Jansky and Rouse, 2000; 2003; Jansky et al., 2004; Mohan et al., 1990; Nicot and Rouse, 1987). While recent research comparing traditional plating methods of pathogen quantification to visual wilt symptoms appears to be accurate and reliable, these methodologies

are time and labor intensive (Bae et al., 2008; Jansky, 2009; Jansky and Miller, 2010). This, in turn, represents an increase in the need for accurate and rapid assays to detect the pathogen in host tissue in order to define the exact nature of the host:parasite interaction. A quantitative real-time PCR assay would significantly reduce the time and labor required to quantify the *S. tuberosum*:*V. dahliae* interaction.

PCR assays including traditional endpoint detection and quantification as well as real-time quantification have been developed for *V. dahliae* (Atallah et al., 2007; Dan et al., 2001; Hu et al., 1993; Li et al., 1999; Mahuku et al., 1999; Mercado-Blanco et al., 2001; 2002; 2003; Nazar et al., 1991; Perez-Artez et al., 2000). The ITS regions of *V. dahliae* and *V. albo-atrum* were utilized for the development of endpoint PCR assays for quantifying *V. albo-atrum* in alfalfa and *V. dahliae* in sunflowers (Hu et al., 1993; Nazar et al., 1991). An additional endpoint PCR assay also using the ITS region initially, was developed and utilized to distinguish *Verticillium* spp. in soil, as well as field-grown potatoes (Mahuku et al., 1999), and subsequently to quantify *V. dahliae* in potato breeding clones and cultivars (Dan et al., 2001). RAPD polymorphisms were used to develop further primers to differentiate *V. dahliae* pathotypes of cotton and olive (Mercado-Blanco et al., 2001; 2002; 2003; Perez-Artez et al., 2000), as well as to quantify fungal biomass in potato in an endpoint PCR assay utilizing a competitive internal control (Li et al., 1999). The first real-time quantitative PCR assays were employed to quantify *V. dahliae* in olive and potato, utilizing SYBR-Green (Mercado-Blanco et al, 2003) and Plexor Q-PCR (Atallah et al., 2007) platforms, respectively. The primers developed to quantify *V. dahliae* in potato have been used also to quantify the pathogen in spinach seed (Duressa et al., 2012).

Each of these PCR assays improved upon detection of *V. dahliae* and advanced the knowledge of Verticillium wilt in numerous host:pathosystems. However, for quantification of fungal biomass, none has displayed sufficient utility to be adopted successfully by researchers as a replacement for time-consuming and laborious traditional plating assays for potato (Bae et al., 2008; Dun et al., 2012; Dung and Johnson, 2012; Jansky et al., 2009; Jansky and Miller, 2010). Therefore, the objectives of this research were to develop a real-time PCR assay, including a reference gene for normalizing the quantification of *V. dahliae in planta*. This duplexed real-time PCR assay will be compared to pathogen levels determined by traditional plating methods in potato stem sections produced under greenhouse conditions.

Materials and Methods

V. dahliae isolate preparation and maintenance. A combination of four isolates of *V. dahliae* isolated from potatoes produced in Minnesota, was used to develop, perform and validate QPCR assays. Two isolates were provided by Dr. Jim Bradeen, Department of Plant Pathology University of Minnesota, and were isolated from potato plants grown in research plots in central Minnesota. The remaining two were recovered from potato tissue grown in commercial potato fields in central Minnesota. Stem sections were washed free of soil and surface sterilized in a 10% household bleach solution for 10 min. Disks, approximately 3 to 4 mm wide, were plated onto solid medium containing 7% v/v ethyl alcohol and 1.5% w/v agar (EtOH agar). Plates were incubated in the dark for 5 to 10 days and fungal isolation was completed via hyphal tip methods. Isolates were preserved in long-term storage via transfer onto 10% clarified V8 juice agar medium (CV8) overlaid with two layers of sterilized Whatman #1 filter paper for approximately 3 to 4 weeks or until the filter paper was completely colonized. The top layer of filter paper was removed, cut into small pieces, placed into 2 ml Nalgene cryogenic vials

(Thermo Fisher Scientific, Rochester, NY) under sterile conditions, dried for 2 days and stored at -80°C for future use.

Extraction of total genomic DNA. Total genomic DNA was extracted from spores and mycelium, of the four previously described isolates of *V. dahliae* was grown in liquid 10% CV8 media for 7 to 10 days under constant shaking at 22±2°C. Media was removed via vacuum filtration, fungal material was freeze-dried and DNA was extracted using DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) following manufacturer's instructions. DNA quality and quantity was assessed for each of the four isolates individually using a NanoDrop 2000 (NanoDrop Technologies, LLC, Wilmington, DE). Total genomic DNA was extracted from stem segments excised from the basal region using the FastDNA Spin Kit (MPBiomedicals, Solon, OH) following manufacturer's instructions. Prior to DNA extraction, 150 mg of fresh stem tissue was pulverized for 45 s using an MP FastPrep-24. Serial dilutions of each fungal and stem DNA extract was performed in RNase and DNase free glass distilled water (Teknova, Hollister, CA) prior to use in PCR reactions.

V. dahliae target gene primer and probe development. Real-time PCR primers and probe were developed from the trypsin protease gene (*VTP1*) of *V. dahliae* (Dobinson et al., 2004) for quantification of fungal biomass *in planta*. A pair of forward and reverse primers was designed using Primer3 from NCBI GenBank accession AY354459. These oligonucleotide primers, designated VTP1-2F and VTP1-2R, amplify a 155 bp fragment of the *VTP1* gene (Table 2.1). The corresponding hybridization probe also was designed using Primer3 and designated VTP1-2P. This probe was labeled at 5'-terminal nucleotide with hexachlorofluorescein phosphoramidite (HEX) reported dye and at the 3'-terminal nucleotide with Black Hole Quencher (BHQ-1). Primer/probe specificity was evaluated via *in silico* analyses against

Verticillium spp., as well as numerous fungal and bacterial potato pathogens to ensure the amplicon was unique to *V. dahliae*. Sensitivity of the primer set was evaluated using conventional, end-point PCR reactions performed on serial dilutions of DNA extracted from fungal cultures and naturally infected stem tissue as described above and selected amplicons were sequenced. Specificity of the primer set was evaluated using conventional PCR reactions performed against numerous fungal and bacterial pathogens of potato (Table 2.2). DNA from bacterial spp. were grown in liquid nutrient broth yeast extract (NBY) medium inoculated with bacteria from -80C storage and grown for 1 to 3 days with shaking at 22±2C. Prior to conversion into a real-time PCR format, VTP1-2 primers were evaluated using the SYBR Green detection system to ensure primer dimers and non-specific binding did not affect target gene quantification. All assays were optimized for $MgCl_2$, dNTP and primer concentrations, as well as annealing temperature for use in a 25 µl reaction.

Table 2.1. Real-time PCR primers and probes designed for quantification of the trypsin protease gene (*VTP1*) of *Verticillium dahliae* and the reference actin gene in potato.

Primer/ probe	Target organism	Gene target	Sense	Sequence (5'-3')	Amplicon size
VTP1-2F	*Verticillium dahliae*	*VTP1* [b]	Forward	CTC GAT CGT CGT CAA CC	155
VTP1-2R			Reverse	TGG TGG TGA GAG TGT TG	
VTP1-2P			Probe	HEX/TAC GAC AAC GAC TTC GCC ATC/3BHQ_1	
PotAct-F[a]	*Solanum tuberosum*	*act* [c]	Forward	TGA ACA CGG AAT TGT CAG CA	123
PotAct-R[a]			Reverse	GGG GTT AAG SGG GGC TTC AG	
PotAct-P			Probe	Cy5/ACA ATG AGC TTC GTG TTG CC/3BHQ_2	

[a]PotAct primers originally described by Atallah and Stevenson, 2006.

[b]Genbank accession number AY354459.

[c]Genbank accession number X55751.

Table 2.2. Specificity of real-time polymerase chain reaction (PCR) assays used for the quantification of *Verticillium dahliae*.

Microorganism species	Disease	VTP1-2[a]	PotAct[a]
Alternaria alternata	brown spot	-	-
A. solani	early blight	-	-
Colletotrichum coccodes	black dot	-	-
C. lindemuthianum		-	-
Fusarium graminearum	Fusarium dry rot and wilt	-	-
F. oxysporum	Fusarium dry rot and wilt	-	-
F. sambucinum	Fusarium dry rot and wilt	-	-
F. solani	Fusarium dry rot and wilt	-	-
Pythium ultimum	leak	-	-
Phytophthora erythroseptica	pink rot	-	-
P. infestans	late blight	-	-
P. nicotianae	pink rot/late blight	-	-
Rhizoctonia solani	Rhizoctonia canker and black scurf	-	-
Sclerotinia sclerotiorum	white mold	-	-
Spongospora subteranea	pitted scab	-	-
Clavibacter michiganense subsp. *sepedonicum*	bacterial ring rot	-	-
Pectobacterium carotovorum subsp. *atrospetica*	blackleg	-	-
P. carotovorum subsp. *carotovorum*	soft rot	-	-
Serratia marcescens	a ubiquitous bacterium often found on potato	-	-
Streptomyces scabies	common scab	-	-
Verticillium dahliae	Verticillium wilt	+	-
V. albo-atrum	Verticillium wilt	-	-
Healthy potato		-	+
PCR grade water		-	-

[a]Positive or negative results denoted as +/-, respectively. Samples with quantification cycle (Cq) values greater than 35 are reported as negative.

Development and optimization of a duplex real-time PCR assay. The PCR assay developed for the target *VTP1* gene using VTP1-2F/R/P was duplexed with primers and probe for the potato actin gene (*act*) (Atallah and Stevenson, 2006). Primers previously were developed to amplify a 123 bp region of the *act* gene (Atallah and Stevenson, 2006). Because the original

assay using these primers was developed for the Plexor Q-PCR System, the hybridization probe PotAct-P was designed using Primer3 from the *act* gene sequence described by NCBI GenBank accession no. X55751 to enable multiplexing with the VTP1-2 assay (Table 2.1). This probe was labeled at 5'-terminal nucleotide with Cy5 reported dye and at the 3'-terminal nucleotide with Black Hole Quencher (BHQ-2). Cycling conditions for this duplex assay were optimized experimentally with 2 min at 95°C followed by 40 cycles of 30 s at 95°C, 60 s at 58°C and 30 s at 72°C, with data capture after the annealing step carried out in a Stratagene Mx3005P using polypropylene non-skirted QPCR 96-well tube plates (Agilent Technologies, Inc., Santa Clara, CA). Amplification and quantification of the *VTP1* gene was achieved using 0.5 µM VTP1-2 forward and VTP1-2 reverse primers, 0.4 µM VTP1-2 hybridization probe, 0.6 µM PotAct forward and PotAct reverse primers, 0.1 µM PotAct hybridization probe, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1× polymerase buffer, 1 unit GoTaq DNA polymerase and 2.0 µl template DNA in a 25 µl reaction. The host *act* gene was used for normalizing quantification of the *VTP1* gene in the duplex format. PCR efficiency was evaluated with each primer/probe combination individually and in combination to ensure that the addition of the reference gene would not affect target gene quantification.

Inoculation and growth of greenhouse derived plant material. Plants of eight russet-skinned potato cultivars, including two controls, were inoculated with *V. dahliae*, grown under greenhouse conditions and evaluated for Verticillium wilt resistance, as well as pathogen colonization (Table 2.3). The remaining six cultivars were reported to have resistance to Verticillium wilt (Jansky, 2009; Mosley et al., 2000; Novy et al., 2002, 2003, 2008; Thompson, unpublished). However, in most cases, cultivar resistance evaluations were based only on symptom development, not pathogen colonization. Isolates of *V. dahliae* were grown in liquid

media as described earlier. Fungal material was filtered through two layers of sterile cheesecloth to remove mycelium and the conidial concentrations were standardized to 1.0×10^5 conidia/ml in sterile water with the aid of a hemacytometer. Spore suspensions were combined at equal volumes of each isolate to produce the inoculum suspension.

Table 2.3. Russet-skinned culitvars evaluated for resistance to *Verticillium dahliae* in three greenhouse trials, and previously reported level of genetic resistance.

Cultivar	Resistance rating	Source
Russet Norkotah	Susceptible control	Jansky, 2009
Ranger Russet	Resistant control	Jansky, 2009
Russet Burbank	Moderately susceptible/ moderately resistant	Jansky, 2009
Umatilla Russet	Moderately susceptible/ moderately resistant	Mosley, et al., 2000
Dakota Trailblazer	Very resistant	Thompson, unpublished; Jansky, 2009
Bannock Russet	Very resistant	Novy et al., 2002
Alturas	Very resistant	Novy et al., 2003
Premier Russet	Moderately resistant	Novy et al., 2008

Ten plants per replicates of each cultivar were inoculated, while ten were used as non-inoculated controls. Portions of certified seed tubers 2 cm in diameter containing one eye were excised and suberized at 55°C in 80 to 85% relative humidity for 48h and planted into 8 cm pots containing Sunshine potting mixture of peat, vermiculite, and perlite (1:1:1, v/v/v) (Sun-Gro Horticulture Canada, LTD; Seba Beach, Alberta). Plants were arranged in a randomized complete block design (RCBD) with four replicates and grown with 16h light for five weeks before carefully being removed from these pots. Roots were washed free of soil under running tap water and soaked in the inoculum suspension for 10 min. Non-inoculated plants were washed in the same manner as inoculated plates and soaked in sterile water. All plants were transplanted immediately into 20 cm pots containing a 1:1 mixture of Sunshine Mix and Scotts Premium Topsoil (The Scotts Miracle-Gro Company, Maryville, OH). Percent Verticillium wilt severity

was recorded on a weekly basis beginning five weeks after inoculation and continuing for 5 to 8 weeks. The area under the wilt progress curve (AUWPC) was calculated from weekly Verticillium wilt severity data (Shaner and Finney, 1977):

$$AUWPC = \sum_{i=1}^{n} [(W_{i+1} + W_i) / 2] [t_{i+1} - t_i]$$

where n = total number of observations, W_i = percentage wilt severity at the *ith* observation, and ti = time in days at the *ith* observation. To permit comparisons across trials, AUWPC values were standardized by dividing the AUWPC values by the total area of the graph for each treatment/replicate/trial combination, resulting in the relative area under the wilt progress curve (RAUWPC). Additionally, RNA was extracted and foliage from each cultivar in all three greenhouse trials was tested for the presence of *Tomato Spotted Wilt Virus* (TSWV) using reverse-transcription (RT)-PCR primers TSWV 1 and 2 (Navarre et al., 2009), as well as forward (S1983) and reverse (S2767) primers (Tsompana et al., 2005), also specific for TSWV.

Duplex QPCR assay validation. Validation of target gene amplification was achieved using total genomic DNA extracted from mycelia and spores of four isolates of *V. dahliae* as described previously. Extracts from each of the *V. dahliae* isolates were diluted to 25 ng/µl, equal volumes of DNA from each were combined, and the resulting solution was diluted serially 10-fold in PCR grade water over 6 orders of magnitude. DNA from the host for validation of the duplex assay was obtained from tissue culture derived, greenhouse-reared plants. Additionally, serial dilutions of DNA extracted from non-inoculated and inoculated plants grown from certified seed-tubers under greenhouse conditions as described previously were used in assay validation. The final validation step involved comparing the quantification of the target pathogen derived from the duplex QPCR assay using primers VTP1-2F/R and probe VTP1-2P in conjunction with normalizing primers PotActF/R and probe PotActP to traditional plating

methods in potato stem tissue generated in the greenhouse. A standard curve for both the pathogen *VTP1* and the host *act* normalizing gene was developed to allow quantification of the *VTP1* gene, as well as account for differences in DNA extraction and PCR efficiency. Dilution series of both the fungal and tissue culture derived stem DNA were included in duplicate on each PCR plate as internal positive controls, as well as quantification standards.

Fungal quantification via traditional plating assays. A portion of a single stem per plant extending from approximately 2.5 cm below and 18 cm above the soil line was harvested from each of five plants of each cultivar, inoculation type, and replicate (320 total) at 8 and 11 weeks after inoculation (Jansky, 2009). Stem segments were surface sterilized in a 10% household bleach solution and air dried. A longitudinal cross-section of stem tissue weighing approximately one gram was excised from the basal region of each stem segment, placed into a plastic bag and sterile distilled water was added at a 1:1 weight to volume ratio. Stem segments then were crushed and 50 µl were spread onto solid Sorenson's NP-10 semi-selective media (Farley, 1972). Plates were incubated in the dark for 4 to 5 weeks, read directly under 60× magnification with the aid a stereo-microscope, and the number of *V. dahliae* colony forming units per gram (CFU/g) of potato stem tissue was calculated.

Statistical analyses. Slopes and intercepts of linear regressions resulting from serial 10-fold dilutions were compared across *V. dahliae* isolates, to the corresponding overall regression line using random coefficients models. The Bonferroni adjustment was utilized to control the overall Family-wise Type I Error ($\alpha = 0.05$) for comparisons across all parameters (slopes and intercepts) (Westfall et al., 1999). An analysis of covariance (ANCOVA) was performed to compare linear regressions developed from VTP1-2F/R/P in simplex and duplex QPCR assays. All QPCR reactions performed to validate the VTP1-2 assay were run in triplicate. Colonization

data from three greenhouse trials were transformed using cube-root transformations to meet normality assumptions for the Analyses of variance (ANOVA). Data then were combined based on variance homogeneity and the interaction of trial with the treatment effect. ANOVA were performed for percentage wilt severity, cube root transformed *V. dahliae* CFU/g stem tissue, and *V. dahliae* mg/g *S. tuberosum* stem tissue. Means were separated using Fisher's protected least significant differences test (LSD) ($\alpha = 0.05$). Pearson's correlation analyses were performed to show the relationship between *V. dahliae* mg/g *S. tuberosum* DNA and *V. dahliae* CFU/g stem tissue results from traditional plating assays. All QPCR reactions performed on greenhouse derived stems were run in duplicate.

Results

PCR primer development. *In silico* analyses with primer pairs VTP1-2F and VTP1-2R showed no homology to GenBank sequences. Following optimization, conventional PCR assays with primers VTP1-2F/R and PotActF/R were sensitive to 25 pg fungal DNA (Fig. 2.1A) and total genomic potato stem DNA, respectively (Fig. 2.1B). Additionally, amplification was not observed from VTP1-2F/R and PotActF/R assays conducted on common potato pathogens (Table 2.2). No amplification of a product resulted from tissue culture derived greenhouse reared plantlets evaluated within the VTP1-2 assay or *V. dahliae* culture DNA with the PotAct assay. The evaluation of VTP1-2F/R using the SYBR Green format resulted in detection of *V. dahliae* at 0.25 pg at a single fluorescence peak indicating this assay is both sensitive and specific (Fig. 2.2).

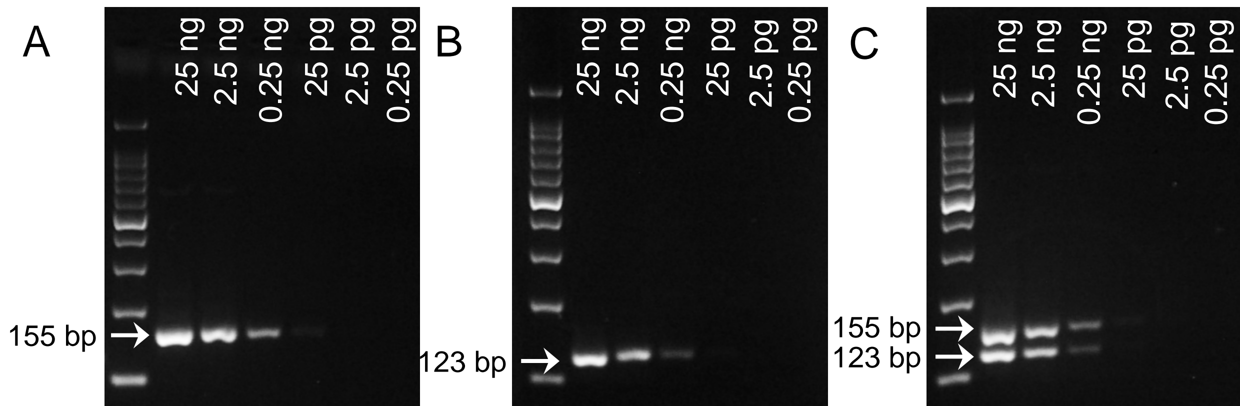


Figure 2.1. Conventional endpoint simplex PCR assays using primers VTP1-2F/R (A), PotActF/R (B) and the duplex assay with the same primers (C) used to detect serial dilutions of *Verticillium dahliae* DNA from pure culture and host DNA from greenhouse reared tissue culture derived plantlets across six orders of magnitude.

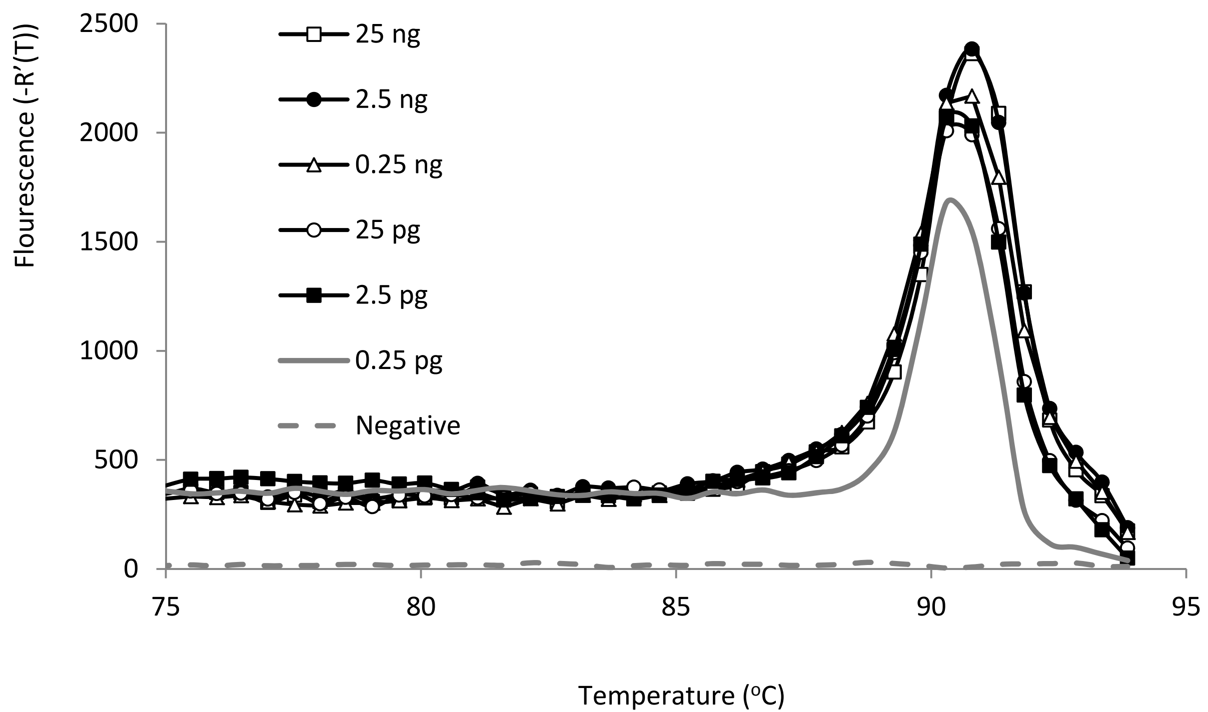
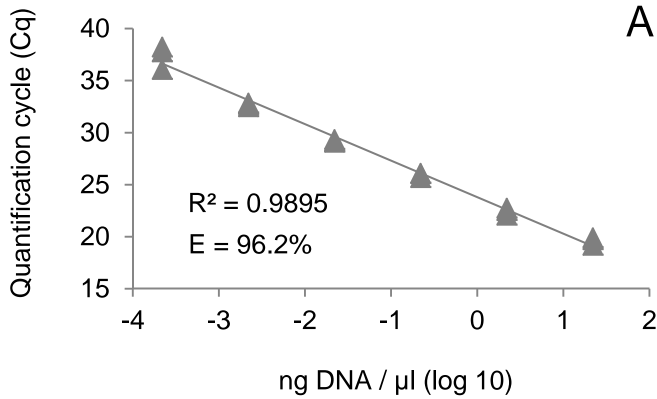
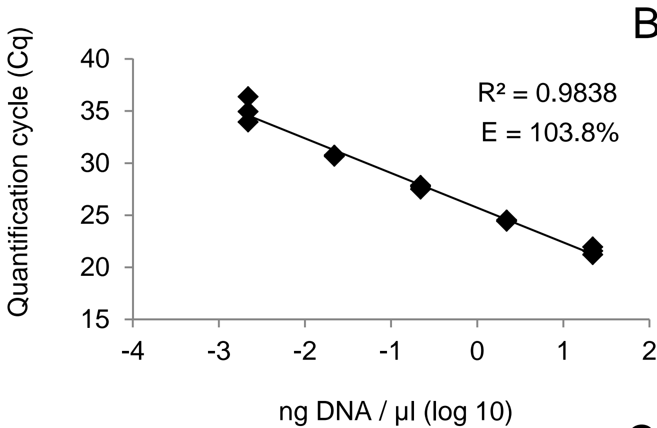


Figure 2.2. SYBR green assay using PCR primers VTP1-2F/R to detect the *VTP1* gene of *Verticillium dahliae* DNA from pure culture across six orders of magnitude.

Development, optimization and validation of a real-time PCR assay. Following optimization of the VTP1-2 QPCR assay, utilizing the VTP1-2 probe, target DNA was detected in a linear range over 6 orders of magnitude to a limit of detection (LOD) of 250 fg (Fig. 2.3A). Regression lines developed for each of four *V. dahliae* isolates grown from pure culture indicated no significant difference among the slopes and intercepts when the Bonferroni adjustment was applied (Table 2.4). Results from preliminary conventional duplex PCR assays indicated that the two primer sets were amenable to duplexing when compared to individual assays (Figs. 2.1A, B and C). Subsequently, all primers and probes were combined to enable the quantification of the target *VTP1* gene in a duplex assay normalized via the amplification of the host *act* gene. When comparing simplex QPCR VTP1-2 to the duplex assay, efficiencies were not compromised when amplifying DNA from *V. dahliae* cultures and tissue culture derived plantlets. Therefore, no significant difference was observed in the slope of the simplex VTP1-2 assay when compared to the duplex assay ($P = 0.3020$). Differences were observed in the intercepts of the regression lines when comparing the simplex to duplex assay ($P < 0.0001$). Amplification of the target and normalizing genes were achieved across 5 orders of magnitude. Similar results were achieved with DNA from plants inoculated and grown under greenhouse conditions. Preliminary results from infected tissue indicated that PCR efficiency and regression coefficients were not compromised when compared to the combination of *V. dahliae* from culture and tissue culture plants (Figs. 2.4A and B).





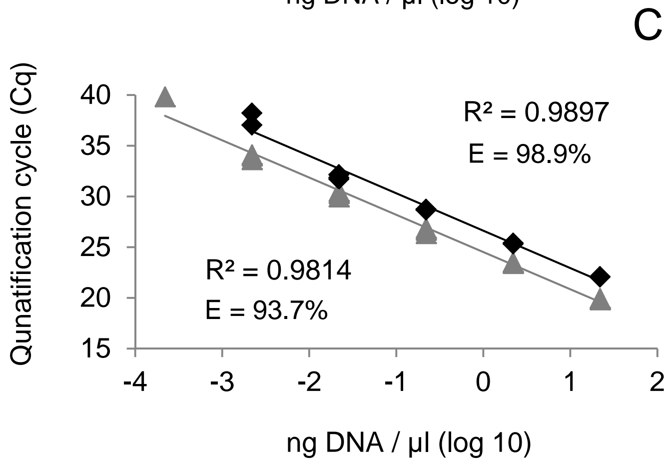


Figure 2.3. Standard curve of *Verticillium dahliae* DNA from pure culture, and DNA extracted from greenhouse-reared, root-inoculated, plants of cv. Russet Norkotah. Quantification cycle (Cq) is plotted against log DNA concentration for hybridization probe based simplex QPCR assays VTP1-2F/R/P to detect the *VTP1* gene represented by the gray triangle (A) and PotActF/R/P to detect the *act* gene represented by the black diamond (B) and the duplex assay (C). DNA was diluted serially across six orders of magnitude and the amplification efficiency was calculated using the formula $E = 10^{(-1/slope)} - 1$.

Table 2.4. Linear Regression equations obtained using the random coefficents model generated from 10-fold serial dilutions over 6 orders of magnitude of total genomic DNA extracted from cultures of *Verticillium dahliae* .

Isolate	Intercept	*P* value[b]	Slope	*P* value	E	R^2
VD05MNH5	25.42	0.1242	-3.175	0.2502	106.52	0.998
VD05MN1-3	25.50	0.2395	-3.301	0.4760	100.88	0.985
VD05UM1	26.27	0.0340	-3.345	0.1869	99.05	0.997
VD05UM2	25.90	0.5674	-3.192	0.3607	105.72	0.997

[a]Amplification efficiency (E) was calculated from the slope of each regression line using the equation $E = 10^{(-1/slope)} - 1$.

[b]Bonferroni adjustment apllied to control the overall Family-wise Type I Error for comparisons across parameters ($\alpha = 0.006$).

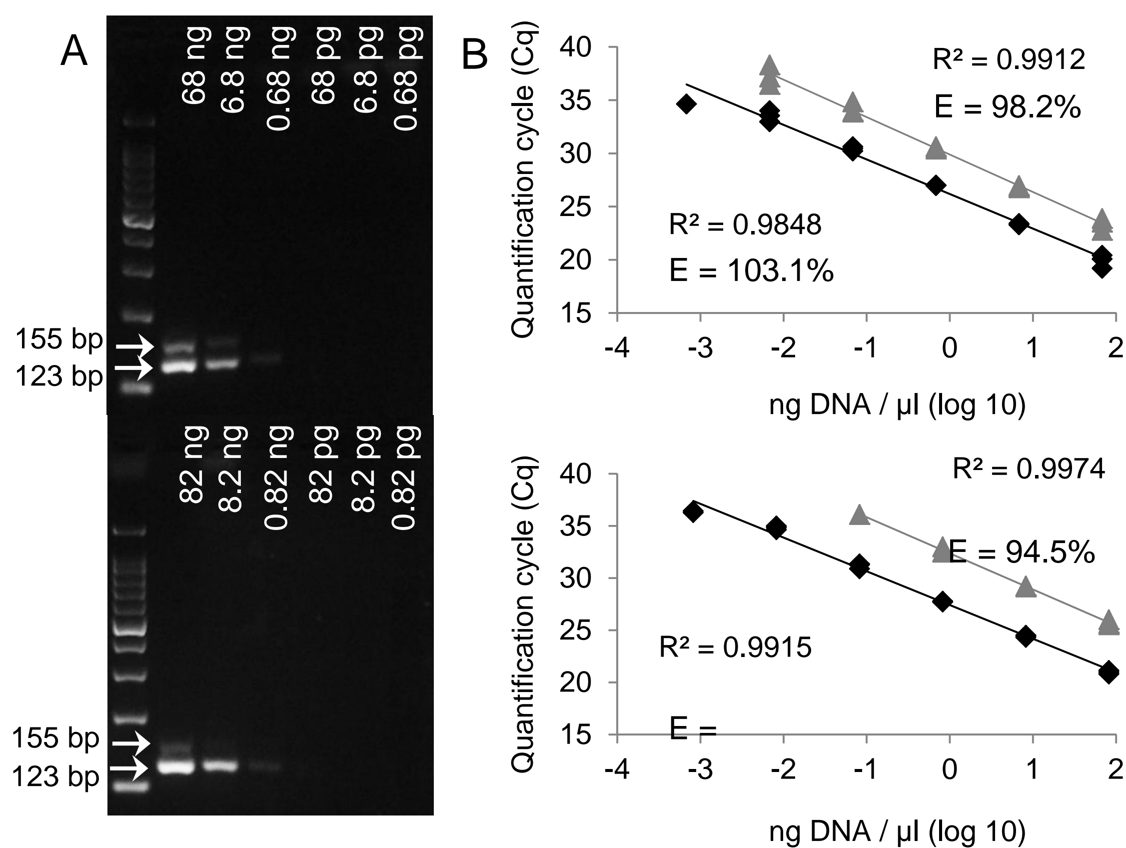


Figure 2.4. Conventional duplex VTP1-2F/R (A) and real-time duplex VTP1-2F/R/P QPCR (B) assays to detect the *VTP1* gene from *Verticillium dahliae* (153 bp), represented by the gray triangle, and the *act* gene from potato (123 bp), represented by the black diamond, in two greenhouse-reared, root-inoculated, plants of cv. Russet Norkotah. DNA was serially diluted across six orders of magnitude and the amplification efficiency was calculated using the formula E = 10(-1/slope) – 1. Quantification cycle (Cq) is plotted against log DNA concentration.

Comparison of traditional plating to PCR quantification of *V. dahliae in planta*. Quantification of *V. dahliae* using traditional plating, as well as QPCR methods resulted in significant interactions between the main effects of inoculum and cultivar at the first (P = 0.0008; P < 0.0001) and second (P < 0.0001; P < 0.0001) sampling dates. Additionally, at both the first and second sampling dates, significant differences were observed between inoculated and non-inoculated plants (P = 0.0187; P = 0.0033), as well as among cultivars (P = 0.0292; P = 0.0101). The interactions between main effects likely were due to seed-borne inoculum which resulted in low, but varying, levels of infection in non-inoculated plants (data not shown); therefore, further analysis includes comparisons among cultivars for inoculated treatments only.

At the first sampling date 8 weeks after inoculation, significant differences were observed in cube root transformed *V. dahliae* CFU/g among inoculated cultivars (P = 0.0146). Susceptible control cv. Russet Norkotah was most heavily colonized by *V. dahliae*, but not significantly more so than moderately susceptible cv. Russet Burbank and very resistant cv. Alturas (Fig. 2.5A). Resistant control cv. Ranger Russet and cvs. Umatilla Russet, Premier Russet and Dakota Trailblazer had moderate levels of *V. dahliae* colonization, significantly less than cv. Russet Norkotah and significantly more than cv. Bannock Russet. At the second sampling date 11 weeks after inoculation, increases in colonization by *V. dahliae* were observed in most cultivars. Cultivars Ranger Russet, Umatilla Russet and Dakota Trailblazer displayed decreases in colonization of 8%, 16% and 6%, respectively. Interestingly, Bannock Russet, which had the lowest colonization in the first sampling date, displayed the largest relative colonization increase of nearly 74% by the second sampling date 3 weeks later. As expected, *V. dahliae* colonization in the susceptible control cv. Russet Norkotah increased substantially (30%). Cvs. Russet Burbank, Alturas, and Premier Russet displayed colonization increases of 24%, 3% and 15%,

respectively. Overall, this resulted in cv. Russet Norkotah having significantly higher levels of *V. dahliae* colonization than all cultivars except cv. Russet Burbank. Fungal colonization in cv. Bannock Russet was significantly lower than that observed in all other cultivars at both sampling dates.

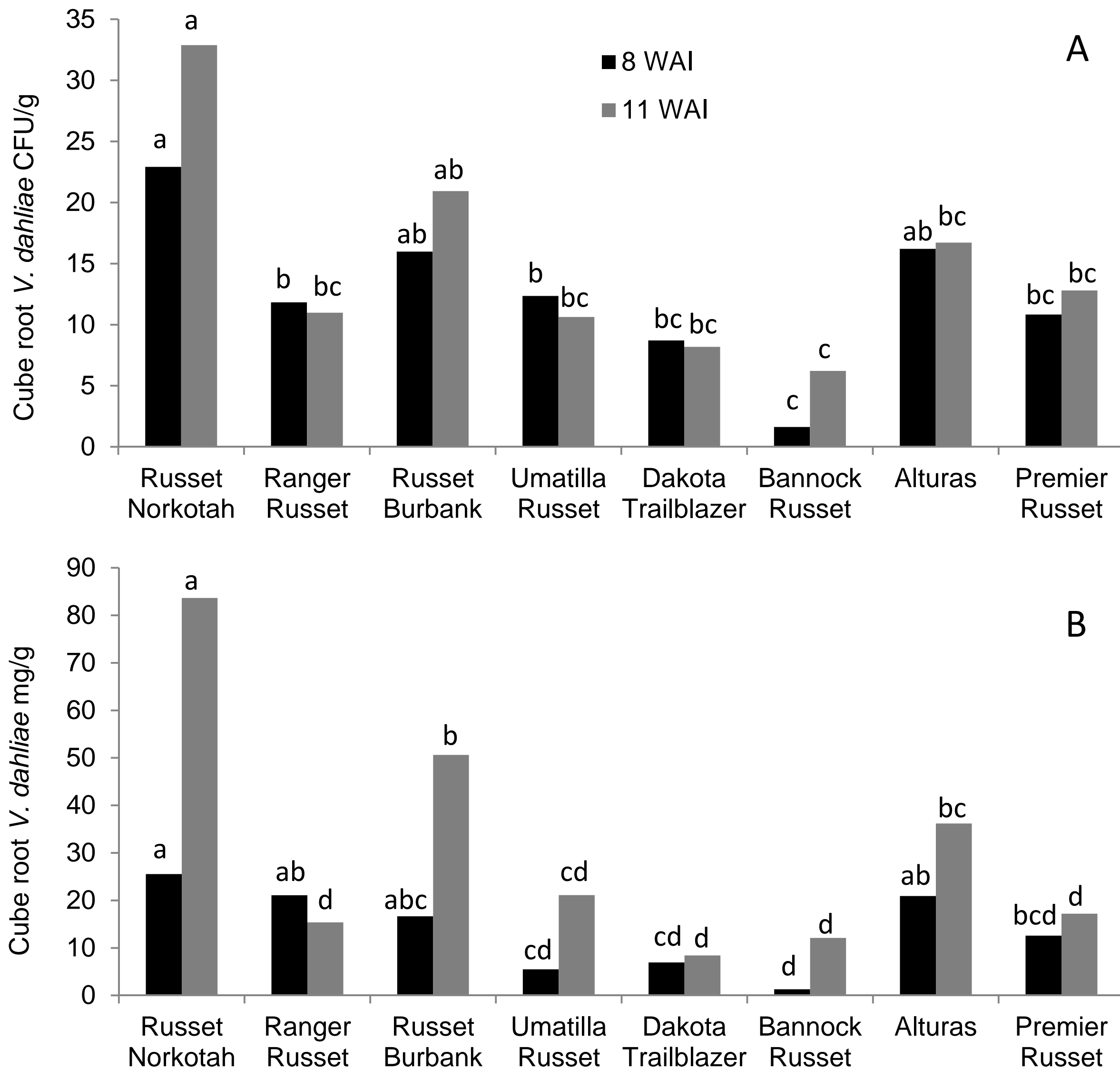


Figure 2.5. Cube root transformed colony forming units of *Verticillium dahliae* per gram of potato stem tissue (*V. dahliae* CFU/g) generated using the traditional plating assay (A) and mg *V. dahliae* per gram of potato stem tissue (*V. dahliae* mg/g) generated using the duplex QPCR assay (B). Evaluations performed 8 and 11 weeks after inoculation (WAI) across eight russet potato cultivars with varying level of resistance to *V. dahliae*.

QPCR quantification of *V. dahliae*. Employing QPCR methods to the quantification of *V. dahliae in planta* resulted in similar results as observed with traditional plating methods at the first sampling date. Again, susceptible control cv. Russet Norkotah was colonized by *V. dahliae* at the highest levels, but not significantly more so than resistant control cvs. Ranger Russet, or cvs. Russet Burbank and Alturas (Fig 2.5B). *V. dahliae* DNA quantified from cv. Bannock Russet was significantly lower than all cultivars. However, while trends were similar at the second sampling date, the level of increase in *V. dahliae* detected by QPCR methods was much greater than that observed by traditional plating methods, the largest of which was observed with cvs. Bannock Russet (89%), Umatilla Russet (73%), Russet Nokotah (69%) and Russet Burbank (67%). Cultivars Alturas, and Premier Russet displayed moderate increases of 42% and 27%, respectively, while colonization in cv. Dakota Trailblazer increased by only 17%. As was observed with traditional plating assays, colonization decreased in cv. Ranger Russet. With the large relative levels of increases in colonization by *V. dahliae*, significant differences were observed more commonly among cultivars. Significantly higher colonization was observed in cv. Russet Norkotah, compared to all other cultivars, followed by Russet Burbank and Alturas. Cultivars Ranger Russet, Dakota Trailblazer, Bannock Russet and Premier Russet were colonized at significantly lower levels than all other cultivars. Pearson correlation coefficients indicate that a strong and significant relationship exists between cube root transformed data for fungal quantification between traditional and QPCR methods at both the first ($n = 16$; $r = 0.94$; $P < 0.0001$) and second ($n = 16$; $r = 0.96$; $P < 0.0001$) sampling dates (Figs. 2.6A and B).

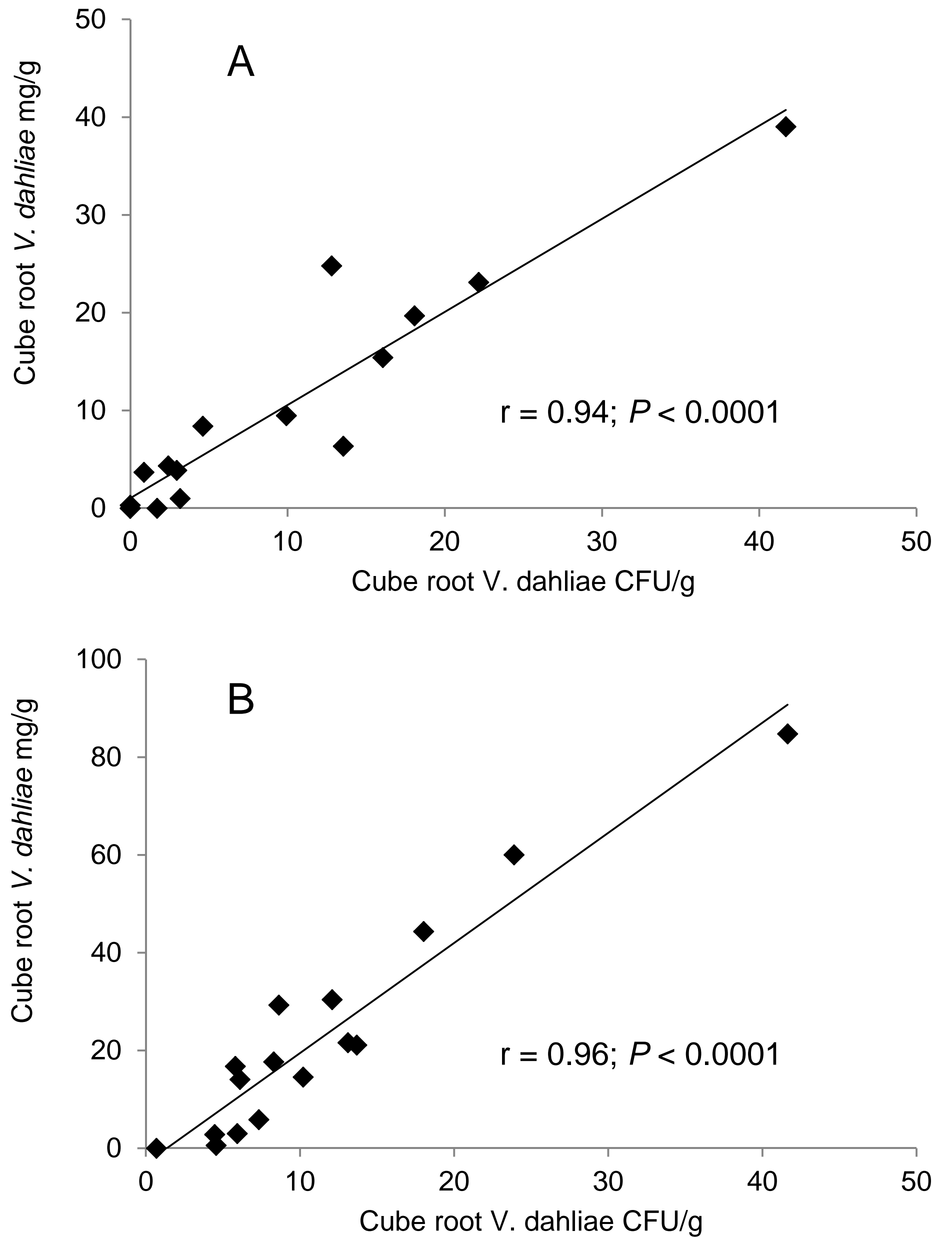


Figure 2.6. Relationship between cube root transformed *Verticillium dahliae* colony forming units per gram of potato stem tissue (*V. dahliae* CFU/g) generated using the traditional plating assay mg *V. dahliae* per gram of potato stem tissue (*V. dahliae* mg/g) generated using the duplex QPCR assay. Colonization evaluated 8 (A) and 11(B) weeks after inoculation across eight russet-skinned potato cultivars with varying levels of resistance to *V. dahliae*.

Verticillium wilt development. Because foliage from all cultivars was confirmed via molecular detection methods to be infected with TSWV in all greenhouse trials, Verticillium wilt was difficult to evaluate accurately. Subsequently, few differences were observed among cultivars, as well as between *V. dahliae* inoculated and non-inoculated plants (Table 2.5). Susceptible control cv. Russet Norkotah consistently displayed significantly higher levels of Verticillium wilt in inoculated plants than in non-inoculated plants in all three greenhouse trials, and moderately susceptible cv. Russet Burbank displayed higher RAUWPC values in inoculated, compared to non-inoculated, plants in two of three trials. However, no significant differences in RAUWPC were observed among the other cultivars with higher levels of resistance to Verticillium wilt.

Table 2.5. Relative area under the wilt progress curve (RAUWPC) generated from greenhouse reared plants of eight russet-skinned potato cultivars inoculated or not inoculated with *Verticillium dahliae* .

Cultivar	Inoculation	Trial 1	Trial 2	Trial 3
Russet Norkotah	None	0.14 c	0.27 de	0.43 b
Russet Norkotah	Yes	0.30 a	0.53 a	0.53 a
Ranger Russet	None	0.31 a	0.43 b	0.56 a
Ranger Russet	Yes	0.22 b	0.30 d	0.41 b
Russet Burbank	None	0.08 ef	0.21 ef	0.18 d
Russet Burbank	Yes	0.09 def	0.29 d	0.33 c
Umatilla Russet	None	0.07 ef	0.10 g	0.07 f
Umatilla Russet	Yes	0.05 f	0.11 g	0.10 ef
Dakota Trailblazer	None	0.08 ef	0.16 fg	0.16 de
Dakota Trailblazer	Yes	0.08 ef	0.16 fg	0.14 de
Bannock Russet	None	0.09 ef	0.17 fg	0.10 ef
Bannock Russet	Yes	0.11 cde	0.19 f	0.10 ef
Alturas	None	0.09 def	0.39 bc	0.13 def
Alturas	Yes	0.13 cd	0.34 cd	0.11 ef
Premier Russet	None	0.11 cde	0.15 fg	0.10 ef
Premier Russet	Yes	0.11 cde	0.15 fg	0.13 def
P value[a]		<0.0001	<0.0001	<0.0001

[a]Values in a column followed by the same letter are not statistically different based on Fisher's protected least significant difference (α = 0.05). *P* value represents the probability of observing a greater value in the F test.

Discussion

The importance of Verticillium wilt in potato production is increasing due to several factors, not limited to tuber quality and increased restrictions on metam sodium use for disease control (MacRae and Noling, 2010; Rowe and Powelson, 2002). Because of the lack of viable alternatives to metam sodium for controlling the disease, the need for resistance in cultivars with acceptable agronomic and end-use consumer qualities is greater than ever (Powelson et al., 1993; Rowe and Powelson, 2002). The quantification of *V. dahliae* has taken many forms, from enumerating microsclerotia with photographs or sieving from ground tissue, to plating either fresh or dried stem tissue onto semi-selective media (Davis et al., 1983; Evans et al., 1966; Hoyos et al., 1991; Isaac et al., 1971; Tsai and Erwin, 1975). The evaluation of genetic resistance in breeding selections and cultivars has been based on these methods in many instances (Bae et al., 2008; Concibido et al., 1994; Corsini et al., 1985; 1990; Davis et al., 1983; Hoyos et al., 1993; Jansky, 2009; Jansky and Miller, 2010; Jansky and Rouse, 2000; 2003; Jansky et al., 2004; Mohan et al., 1990). More recently, the validity of plating methods to accurately determine the level of colonization in potato cultivars and breeding selections was confirmed (Jansky, 2009; Jansky and Miller, 2010). However, the fact remains that these methods require much greater efforts than can be reasonably justified to screen hundreds of breeding selections on a regular basis (Bae et al., 2008). Additionally, it can be difficult to differentiate *V. dahliae* and *V. albo-atrum*, both of which infect potato, as well as in some instances microsclerotial producing *C. coccodes,* which also is isolated commonly from potato stem tissue, especially when high densities exist on an agar plate. Over the past 10-20 years, several russet-skinned cultivars have been released with purported resistance to *V. dahliae*, but the majority of these cultivars have not been evaluated for pathogen colonization (Johansen et al., 1994; Love et al., 2002, 2005, 2006;

Mosely et al., 2000a, 2000b; Novy et al., 2002, 2003, 2006, 2008, 2010; Stark et al., 2009). One reason for this may be that traditional methods available to evaluate colonization are extremely time and labor intensive. Therefore, a reliable and accurate quantitative real-time PCR assay would enhance greatly efforts towards breeding for genetic resistance to Verticillium wilt.

Numerous PCR primers and assays have been developed for the detection and quantification of *V. dahliae*, but these have not been utilized for quantification on a large scale. Instead, researchers continue to utilize traditional plating methods, over more rapid PCR methods currently available (Bae et al., 2008; Janksy, 2009; Jansky and Miller, 2010). The current research was undertaken to improve quantification of *V. dahliae in planta*, by developing a method that could replace plating assays for large-scale evaluations of cultivars and breeding material. *VTP1* was chosen as the target amplicon for this assay because it was determined to be a single copy, highly conserved gene in *V. dahliae* isolates from several host plants (Dobinson et al., 2004). A duplex assay utilizing an internal normalizing gene from the host eliminates the need for evaluating DNA quantity and quality, as well as controls for DNA extraction and PCR efficiency. Additionally, it controls for the presence of PCR inhibitors, as a product should always be produced with the host detection system. Of major importance when developing an assay intended to replace an industry standard is the ability to demonstrate that the novel technique can meet or surpass the performance of existing methods. This QPCR for detection of the *VTP1* gene assay was able to detect *V. dahliae* when the plating assay was not. The QPCR described here was 100× more sensitive when compared to the conventional end-point PCR assay using the same primers and PCR efficiency and sensitivity remained high in the duplex format, even when evaluating potato stem tissue infected at low levels. Additionally, the use of

an amplicon specific probe may enhance specificity of the assay over non-probe based techniques (Bustin and Nolan, 2004).

The level of increase in colonization in potato stem tissue from the first to the second sampling dates is an important aspect of screening for cultivar resistance. A cultivar which is not colonized rapidly in the first part of the growing season, may have higher rates of colonization as the season progresses, resulting in higher colonization levels than other cultivars later in the season as was determined for cv. Bannock Russet. This reinforces the need for multiple, or at least timely, evaluations of colonization to accurately assess the level of resistance in a particular cultivar, as well as to estimate the amount of pathogen returned to the soil by a given cultivar. Evaluating cultivars too early in the growing season may result in overestimating the level of resistance in that cultivar. Additionally, evaluations performed too late may provide as assessment of inoculum returned to the soil, but may not accurately evaluate the effect the pathogen has on plants during the critical stage of tuberization. Here, the second sampling date 11 weeks after inoculation resulted in the best timing to differentiate host resistance. Some differences observed at 11 weeks after inoculation were not as clear at 8 weeks after inoculation, and therefore, care should be taken to ensure that colonization is evaluated at the proper timing. For example, results from QPCR assays performed at 8 WAI indicate no significant difference between the susceptible control cultivar Russet Norkotah and moderately resistant cultivar Russet Burbank, while at 11 WAI a significant differences in colonization is observed. The optimal timing of evaluation may fluctuate under varying field conditions and production systems and certainly should be evaluated. Additionally, cultivar maturity has been reported to significantly affect wilt, and in some instances, stem colonization in *V. dahliae* infested field trials (Bae et al., 2008; Busch and Edgington, 1967; Jansky and Miller, 2010). Strains of cv.

Russet Norkotah selected from somatic variants, display later maturity and lower severity of Verticillium wilt when compared to standard cv. Russet Norkotah (Jansky and Miller, 2010). Greenhouse trials conducted with cv. Kennebec resulted in a correlation between tuber formation and Verticillium wilt symptoms (Busch and Edgington, 1967). Plants which were grown under long photo-periods to prevent tuberization, also did not display symptoms of Verticillium wilt, however, stem colonization was not evaluated in these trials. When evaluating early generation breeding selections, stem colonization was significantly correlated with maturity and Verticillium wilt severity in the first year of the field trial, but not the second (Bae et al., 2008). The results reported here demonstrate that colonization can change substantially within a three week period under controlled greenhouse conditions, it therefore would be expected that even greater differences also would be observed under field conditions.

It is the belief of the author that the larger differences observed between the first and second sampling dates in cultivars with higher colonization levels using QPCR vs. plating, including cvs. Russet Norkotah, Russet Burbank and Alturas, was due to an underestimation of colonization using traditional plating methods. In these cultivars, the number of colonies growing on agar media was difficult to differentiate and accurately enumerate. While colonization in some cultivars were not significantly different than Russet Norkotah, this most likely is an artifact of the plating assay, in particular at the second sampling date when this cultivar was significantly more colonized by *V. dahliae* than all other cultivars evaluated. Data from colonization trials were not normally distributed and therefore required transformation prior to analysis to meet the assumptions of the ANOVA. Very high colonization in susceptible check cv. Russet Norkotah was the main reason for the need for transformation. This especially was true for data generated via QPCR. Additionally, the variability in colonization levels in individual

stems within and among replicates was somewhat high, also contributing to the need for data transformation (Bae et al., 2008). This may be attributed to inoculation method and further investigations of methods which may result in more uniform infection levels currently are underway. The refinement of the *in planta* assay, in addition to the increased accuracy of the QPCR assay reported here, will result in further improvements in the ability to differentiate levels of resistance to *V. dahliae* in breeding material and cultivars.

Systemic infection of non-inoculated plants occurred in greenhouse assays presented here, even though certified seed-tubers were used. This was attributed to seed-borne infection, as great care was taken to eliminate contamination during the inoculation procedure. Transmission of *V. dahliae* infected seed tubers is not a major contributor to overall disease pressure when soil-borne inoculum is present, however, seed-borne inoculum has been reported to cause Verticllium wilt and systemic fungal colonization (Dung and Johnson, 2012; Dung et al., 2012; Hoyman, 1974; Robinson and Ayers, 1961). Additionally, it has been shown previously that inoculum on the surface of seed tubers resulted in more wilted plants than did tubers with vascular *V. dahliae* infections (Robinson and Ayers, 1961). Seed-tubers were not washed prior to use in greenhouse assays. Washing may have reduced the amount of infection observed in non-inoculated treatments and should be considered in future research of this type. Also the frequency of vascular colonization in seed-tubers was not assessed, therefore the impact of these two sources of inoculation cannot be evaluated here.

The development and validation of the QPCR assay described here is paramount in the efforts towards breeding for resistance to *V. dahliae*. This assay proved to be sensitive and specific when evaluating greenhouse produced plants. Also, this duplex assay includes the use of a normalizing gene from the host which replaces the need for determining DNA quantity and

quality, prior to quantification of the target gene. The target gene of the pathogen was amplified in all cultivars evaluated in this study, even when plating techniques failed to detect the presence of *V. dahliae*. Subsequently, this QPCR assay reduces the time, labor, and expense of quantifying the host:pathogen interaction. Plating assays are limited in that colonies can be difficult to discern in the presence of closely related fungi, as well as in instances when colonization levels are high, possibly resulting in under- and over-estimation of fungal quantification. QPCR methods do not have that limitation. Therefore, it serves as a valuable tool for breeders and other researchers in the identification of genetic resistance as well as other control measures for Verticillium wilt.

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CHAPTER THREE. QUANTIFICATION OF FIELD RESISTANCE TO *VERTICILLIUM DAHLIAE* IN EIGHT RUSSET-SKINNED POTATO CULTIVARS USING REAL-TIME PCR

Abstract

Numerous aspects of potato production have evolved over the past 10-20 years, including soil tillage practices, a reduction in years between potato crops and cultivars grown, as well as an increased importance placed on tuber size and quality. Many of these changes have had a profound effect on the importance of management of Verticillium wilt, caused by *Verticillium dahliae* Kleb. Subsequently, increased emphasis has been placed on breeding for resistance, particularly in russet-skinned cultivars for the frozen processing industry. While many russet-skinned cultivars recently have been released with resistance to the wilt phase of Verticillium wilt, information is lacking on the level of pathogen colonization for these cultivars, and therefore, the level of true genetic resistance is not known. The differentiation of resistance to colonization and tolerance to disease symptoms is crucial in sustainable potato production. Tolerant cultivars support high levels of colonization with little or no disease symptoms and will return large amounts of inoculum to the soil for infection of subsequent crops. Eight russet-skinned cultivars were grown in field trials with low and high levels of *V. dahliae* in the soil. Each treatment was evaluated for wilt, stem colonization (comparing QPCR to traditional plating methods) and yield over two growing seasons and tuber vascular discoloration in one growing season. The QPCR assay was validated with strong relationships to plating assays over two stem sampling dates, as well as to wilt across growing seasons. Additionally, stem colonization levels were related to wilt and tuber vascular discoloration. Total yield did not exhibit a strong

relationship to any other parameter evaluated in this study. Results from these studies indicate that true resistance is present, to varying degrees, in the russet-skinned cultivars evaluated.

Introduction

Verticillium Wilt in United States potato production (*Solanum tuberosum* L.) is caused by *Verticillium dahliae* Kleb. and *V. albo-atrum* Reinke and Berthold. *V. dahliae* is often the most common and devastating of these two pathogens, mainly because microsclerotia persist in the soil for as many as 14 years (Powelson and Rowe, 1994; Wilhelm, 1955). Due to the ease of introduction of *V. dahliae* into non-infested fields, the wide host range, and the longevity of microsclerotial survival in the soil, most agricultural soils are infested with the pathogen to some extent (Rowe, 1985; Powelson et al., 1993). Effects on yield can be more substantial when susceptible crops are grown in short rotations. Additionally, the soil micro-environment has become increasingly more favorable for the build-up and survival of plant pathogens, including *V. dahliae,* with the implementation of conservation tillage and cropping practices (Bockus and Shroyer, 1998; Taylor et al., 2005).

The symptoms of successful infection by *V. dahliae* include overall wilting of the host plant, premature vine death, and foliar chlorosis and necrosis (Perry and Evert, 1983a; 1983b). Infected vascular bundles appear light brown and plants remain erect after senescence, which distinguishes symptoms of Verticillium wilt from symptoms of other wilt diseases caused by pathogens including *Colletotrichum coccodes* (Wallr.) Hughes and *Fusarium* spp. (Rich, 1983; Rowe, 1985). Symptoms of Verticillium wilt may not be evident until plants reach maturity and may not be distinguishable from natural senescence at any growth stage (Davis, 1985; Isaac and Harrison, 1968). Therefore, wilt symptoms should not be relied upon solely for evaluating disease management strategies including soil fumigants, cultural practices or genetic resistance.

While wilting eventually leads to premature death of the host, it may not be a direct indication of yield loss (Rowe et al., 1987). Additionally, vascular discoloration in tubers can result in browning of processed products and may render symptomatic potato tubers unmarketable (Rowe, 1985).

While resistance mechanisms in potato to *V. dahliae* are not well understood, host resistance and the level of pathogen in the soil often are considered the main parameters affecting the development of Verticillium wilt. In moderately susceptible cv. Russet Burbank, stem colonization increased with increasing *V. dahliae* soil infestation levels (Nicot and Rouse, 1987). However, many factors including temperature, soil-water content, soil type, organic matter content, and the availability of nutrients, also affect the level of Verticillium wilt in a potato crop (Ben-Yephet and Szmulewich, 1985; Cappeart et al., 1992; Davis et al., 2001; Nnodu and Harrison, 1979; Powelson and Rowe, 1993). The control of these factors, when possible, can aid in lowering disease severity by improving plant health and limiting the infectivity of inoculum and spread of the pathogen in the plant. However, management of Verticillium wilt has proven extremely difficult to achieve without the use of soil fumigants (Powelson et al., 1993; Rowe and Powelson, 2002). Soil fumigation with metam sodium can result in an up to 80% reduction in soil infestation levels and corresponding reductions in disease (Taylor et al., 2005). Unfortunately, this method of control is expensive and has detrimental effects on the environment (Davis, 1985; Powelson et al., 1993). Additionally, regulations outlined by the EPA will make the application of this chemical much more limited in the future (MacRae and Noling, 2010). Ultimately, because cultivars with resistance to Verticillium wilt are not grown widely, soil fumigation will continue to be relied upon heavily for disease management until a suitable

replacement is found or registration is suspended (Powelson et al., 1993; Rowe and Powelson, 2002).

Russet-skinned potato cultivars for use in frozen processing, as well as table stock comprise greater than 70% of all potato acreage in the United States (National Potato Council, 2010). Of the top seven cultivars produced for seed-tubers in North America in 2000, cv. Russet Burbank was the most commonly grown overall with 30% of total acres, and was categorized as moderately susceptible to Verticillium wilt (Rowe and Powelson, 2002). Two other russet-skinned cultivars ranked in the top seven, Russet Norkotah, 11%, classified as very susceptible, and Ranger Russet, 4%, classified as moderately resistant. This represents a general lack of resistance to Verticillium wilt among russet-skinned cultivars grown for the frozen processing industry (Rowe and Powelson, 2002). However, over the past two decades, numerous russet-skinned cultivars have been released with reported resistance to Verticillium wilt (Johansen et al., 1994; Love et al., 2002; 2005; 2006; Mosley et al., 1999; 2000; 2001; Novy et al., 2002; 2003; 2006; 2008; 2010; Stark et al., 2009). The difficulty in recommending the utilization of many of these cultivars lies in the fact that many have not been evaluated for true resistance, but merely have been evaluated for symptoms of wilt. In the latter case, growing these cultivars can result in the build-up of high levels of inoculum without losses in the current season (Rowe, 1985). Unfortunately, drastic losses will result when a susceptible cultivar is planted in that soil. Additionally, when soil inoculum reaches high levels it may require several rotations away from a susceptible host, even if control measures such as soil fumigation are used.

Recent research to quantify *V. dahliae* in potato was performed using traditional plant tissue plating assays (Bae et al., 2008; Jansky 2009; Jansky and Miller , 2010). While results from this research provided valuable information concerning the evaluation of Verticillium wilt

resistance in potato, studies of this type are difficult to perform due to the time and labor intensive nature of plating assays. A duplex QPCR method recently was developed utilizing a host normalizing gene for absolute quantification of the potato:*V. dahliae* interaction (chapter 2). Quantification results from this assay correlated very well to traditional plating assays for pathogen quantification and to wilt across eight russet-skinned potato cultivars grown under greenhouse conditions. However, the QPCR assay can be performed in a fraction of the time required for traditional plating assays.

The objectives of this research include the further validation the utility of the recently developed QPCR assay for the quantification of *V. dahliae* from potato stem tissue grown under field conditions (chapter 2). Additionally, colonization by *V. dahliae* was quantified for russet-skinned potato cultivars with varying levels of purported resistance to Verticillium wilt. The goal was to separate true resistance from tolerance by evaluating pathogen colonization, symptom development and yield. The objectives were accomplished by growing these russet-skinned potato cultivars along with control cultivars with established resistance levels in *V. dahliae* infested field research plots. Real-time QPCR methods were compared to traditional plating methods to determine the amount of colonization in stem tissue.

Materials and Methods

Isolate preparation and maintenance. A combination of four *V. dahliae* isolates originally isolated from potato tissue produced in Minnesota was used to inoculate potato plants in field trials (chapter 2). Two of these isolates were kindly provided by Dr. Jim Bradeen, Department of Plant Pathology, University of Minnesota. The remaining two were recovered from potato tissue grown in central Minnesota by first surface sterilizing infected stem tissue in a 10% household bleach solution for ten minutes. Disks, approximately 3 to 4 mm wide, were plated onto solid

medium containing 7% v/v ethyl alcohol and 1.5% w/v agar (EtOH agar). Plates were incubated in the dark for 5 to 10 days and fungal isolation was completed via hyphal tip methods. Isolates were maintained by growing them on 10% clarified V8 Agar medium (CV8) over two layers of sterilized Whatman #1 filter paper for approximately 3 to 4 weeks or until the filter paper was completely colonized. The top layer of filter paper was removed aseptically, cut into small pieces, placed into 2 ml Nalgene cryovials (Thermo Fisher Scientific, Rochester, NY), dried for 2 days and stored at -80°C for future use.

Cultivar selection. Eight russet skin-type potato cultivars were selected for evaluation of colonization by *V. dahliae*. Among these were very susceptible (VS) control cv. Russet Norkotah and resistant (R) control cv. Ranger Russet, included for comparison purposes. Cultivars Russet Norkotah and Ranger Russet have been reported to be the most susceptible and resistant standard cultivars, respectively (Jansky, 2009). Additionally, cv. Russet Norkotah is known to be heavily colonized by *V. dahliae* under field conditions and commonly is used as a susceptible control cultivar (Bae et al., 2007; Frost et al., 2007; Hoyos et al., 1991; Jansky, 2009). Cultivar Ranger Russet has been demonstrated to be resistant to colonization by *V. dahliae*, although not completely, under field conditions and has been included in previous studies as a resistant control cultivar (Bae et al., 2007, 2008; Jansky, 2009). Cultivars Russet Burbank and Dakota Trailblazer also have been evaluated previously for colonization and were classified as susceptible (S) to resistant (R), and very resistant (VR) to pathogen colonization, respectively (Hoyos et al., 1991; Jansky, 2009). The other cultivars evaluated had been reported previously to range from moderately susceptible/moderately resistant (MS/MR) to VR, but stem colonization had not been evaluated and claims of resistance are based solely on the presence or absence of wilt symptoms. These cultivars include, Umatilla Russet (MS/MR) (Mosley et al., 1999),

Bannock Russet (VR) (Novy et al., 2002), Alturas (VR) (Novy et al., 2003) and Premier Russet (MR) (Novy, et al., 2008).

Field trial. All four isolates of *V. dahliae* were sent to Northwest Mycological Consultants, Corvallis, OR to be used to infest grain. Briefly, sterilized grain seed was infested with each of the four isolates in individual bags each containing approximately 3.2 kg of fully hydrated grain. These mushroom spawn bags, with number seven vents to allow gas exchange, were subsequently incubated in the dark at 22°C for 3 weeks. Grain was sent to North Dakota State University where it was dried on greenhouse benches for 7 to 14 days before milling in 2009. In 2010, grain remained in the spawn bags until soil infestation. Certified seed tubers of each cultivar were cut into seed pieces weighing approximately 70 g and suberized at 13°C for approximately 48 h prior to planting. Sixteen treatments, consisting of eight cultivars by two infestation levels, were planted in a randomized complete block design (RCBD) with four replicates. Each experimental unit contained two rows of ten plants per replicate. Low infestation consisted of resident soil *V. dahliae* populations of 6 Verticillium prapagules per gram of soil (Vppg) in 2009 and 4.5 Vppg in 2010. High infestation was achieved by adding approximately 19 kg of dried and ground *V. dahliae*-infested grain applied in-furrow at planting, at a rate of 96.7 g/row m in 2009. In 2010, 33 kg of hydrated grain was applied at 178.6 g/row m. The increased weight of grain was added to account for the difference between dry and hydrated grain weight. The same four *V. dahliae* isolates were plated to solid CV8 agar and grown in the dark at 25 ± 2°C for 3 weeks. Contents of the culture plates (agar and fungus) were mixed with distilled water at a rate of 25 ml/plate, pureed with an electric blender and adjusted to a final concentration of 1×10^{4} microsclerotia/ml using a hemocytometer. The agar and fungal slurry was applied to the soil of each high infestation plot at a rate of 30 ml/row m with hand held CO_2

pressure operated sprayer and incorporated using a typical hilling operation on June 11, 2009 and June 2, 2010. Trials were maintained following typical commercial growing practices for northeastern North Dakota, including overhead irrigation and cultivation, as well as fungicide, insecticide and herbicide applications.

Disease evaluation. Visual assessments for signs of Verticillium Wilt were performed four times from 83 to 110 days after planting (DAP). Symptoms of Verticillium wilt were scored on a percentage wilt severity basis for each treatment/replication combination. The area under the wilt progress curve (AUWPC) was calculated from wilt severity ratings and these values were standardized across years by dividing by the number of days within the rating period, resulting in the relative area under the wilt progress curve (RAUWPC) (Shaner and Finney, 1977). Total yield was obtained and stem sections again were collected at harvest, 139 and 132 DAP in 2009 and 2010, respectively. In 2010, tubers were evaluated for stem end incidence of mild, severe, and total vascular discoloration from 9 to 18 days post-harvest. Mild discoloration was defined as a light discoloration which did not follow the entire vascular ring, while severe discoloration was darker in color and extended around the vascular ring, or nearly so. Soil samples were obtained at harvest to evaluate the pathogen infestation level in each field replication.

Stem colonization analysis. Fresh stem sections from the basal region of all true stems (originating from the seed-tuber) of five plants in each cultivar/replication were collected at 104 DAP in 2009 and 2010. Samples were processed following previously described protocols with some modifications (Jansky, 2009). Stem sections were surface sterilized and a disk weighing approximately 1 g was excised from each stem and placed into a sealable plastic bag and sterile distilled water was added at a 1:1 weight to volume ratio. Sections were crushed and 50 µl

spread onto solid NP media. At harvest, 139 and 132 DP in 2009 and 2010, respectively, when plants were senescent or nearly so, stem sections were dried, ground in a Wiley Mill using a 40 mesh screen and 50 mg were plated onto solid NP media. Plates from both crushed fresh and ground dried stem sections were incubated in the dark for 4 to 5 weeks. Plates from fresh stems were read directly, while stem debris from dried stems was washed from plates under running tap water and plates were dried overnight before examination under 60× magnification using a stereomicroscope. The number of colony forming units (CFU)/g stem tissue then was calculated.

A duplex QPCR assay performed using primers and probe developed from the Trypsin Protease gene (*VTP1*) of *V. dahliae* (Dobinson et al., 2004) and internal control actin gene (*act*) of *Solanum tuberosum* (Atallah and Stevenson, 2006) was compared to traditional plating methods described above for pathogen quantification in potato stem tissue, as described previously (Chapter 2). Stem tissue used in PCR quantification was obtained directly adjacent to, and simultaneously with, that used in traditional plating methods. Total genomic DNA was extracted from fresh and dried stems using the FastDNA Spin Kit (MPBiomedicals, Solon, OH) following manufacturer's instructions. Prior to DNA extraction, tissue was pulverized for 45 s using an MP FastPrep-24. In fresh and dried stems, 150 and 50 mg tissue was used for DNA extraction, respectively. This PCR assay was optimized for use in quantification of DNA extracted from stem tissue, as described previously (Chapter 2). Cycling conditions were initiated with 2 min at 95°C followed by 40 cycles of 30 s at 95°C, 60 s at 58°C and 30 s at 72°C with data capture after the annealing step performed in a Stratagene Mx3005P using polypropylene QPCR 96-well tube plates, non-skirted (Agilent Technologies, Inc., Santa Clara, CA). Amplification and quantification of the *VTP1* gene was achieved using 0.5 µM VTP1-2 forward and VTP1-2 reverse primers, 0.4 µM VTP1-2 Taqman probe, 0.6 µM PotAct forward

and PotAct reverse primers, 0.1 μM PotAct Taqman probe 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1× polymerase buffer, 1 unit GoTaq DNA polymerase and 2.0 μl template DNA in a 25 μl reaction. All QPCR reactions performed on stems collected from field trials were run in duplicate.

Statistical analyses. Colonization data from field trials conducted in 2009 and 2010 were transformed using cube-root transformations to meet normality assumptions for the analysis of variance (ANOVA). Data then were combined based on the interaction of trial year with the main effects of cultivar and infestation level for wilt, colonization, yield and tuber vascular discoloration. Two-way analyses of variance (ANOVA) were conducted on combined data from the 2009 and 2010 field trials using cultivar and soil infestation level as main affects in PROC GLM of SAS. Mean wilt severity, RAUWPC, cube-root transformed stem colonization, tuber yield and vascular discoloration were differentiated using Fisher's protected least significant difference test ($\alpha = 0.05$). Relationships among wilt symptoms, cube-root transformed CFU *V. dahliae*/g stem tissue from traditional plating assays, cube root transformed colonization as determined by QPCR, yield and tuber vascular discoloration were evaluated using Pearson's correlation.

Results

No significant interaction was observed between the main effects of cultivar and infestation level for wilt severity at 83 ($P = 0.5855$), 98 ($P = 0.0549$), 104 ($P = 0.7263$), 110 ($P = 0.9981$) DAP or RAUWPC ($P = 0.2774$). However, a significant interaction was observed for cube root transformed CFU/g ($P = 0.0064$) derived from plating assays, and for *V. dahliae* mg/g of stem tissue ($P = 0.0135$) derived from QPCR analyses of fresh stem tissue collected 104 DAP. Here, significant interactions were due to the differences in colonization between infestation levels for Russet Norkotah, the susceptible control cultivar. To demonstrate this, no interactions

between cultivar and infestation level were observed among the other seven cultivars in either plating (P = 0.3354) or QPCR (P = 0.0753) assays. No significant differences in colonization were observed between high and low infestation levels for any cultivar with some level of resistance to *V. dahliae*. No significant interaction of these main effects was observed in stems collected at harvest (P = 0.2833; P = 0.8754) total yield (P = 0.4521), mild (P = 0.2910), severe (P = 0.1253), or total (P = 0.6296) vascular discoloration. Because interactions of the main effects were not significant in the majority of parameters, all data are presented within the main effects of cultivar and infestation for each parameter.

Development of Verticillium wilt symptoms. Significant differences in wilt severity among cultivars were observed at all four data collections dates (Fig. 3.1). Wilt severity at the first data collection date 83 DAP was less than 10% in all cultivars. Susceptible control cv. Russet Norkotah displayed a significantly higher level of wilt compared to cvs. Umatilla Russet, Bannock Russet, Alturas and Premier Russet. Wilt in cvs. Ranger Russet and Russet Burbank was not different than any other cultivar. At the second wilt evaluation 15 days later, wilt severity increased approximately 3- to 6-fold, depending on cultivar. Increases were more substantial in cultivars with little, or moderate, resistance to the pathogen. Wilt at 98 DAP in cv. Russet Norkotah was significantly higher than all other cultivars, cv. Russet Burbank was higher than all the remaining cultivars, and cvs. Dakota Trailblazer and Premier Russet were significantly less wilted compared to cvs. Russet Norkotah, Russet Burbank and Ranger Russet. Wilt symptoms continued to increase throughout the remainder of the growing season, but not as dramatically as was observed between the first and second evaluations. At 104 DAP, wilt in cv. Russet Norkotah again was significantly higher than other cultivars, followed by cv. Russet Burbank. Verticillium wilt was least severe in cvs. Dakota Trailblazer and Premier Russet, but

not significantly lower than that observed in Alturas and Bannock Russet. At the final data collection date 110 DAP, wilt in susceptible control cv. Russet Norkotah reached over 90%, and remained significantly higher than all other cultivars. This again was followed by cv. Russet Burbank, as well as Umatilla Russet. As observed previously, cvs. Dakota Trailblazer and Premier Russet were the least wilted among the cultivars evaluated, although not significantly so in every instance. The trends observed over all evaluation dates were reflected in the RAUWPC (Fig. 3.2). Wilt development across the growing season was significantly higher in cv. Russet Norkotah, followed by cv. Russet Burbank. No significant difference was observed among the remaining six cultivars. The effect of infestation level was significant only at 98 DAP and with the RAUWPC.

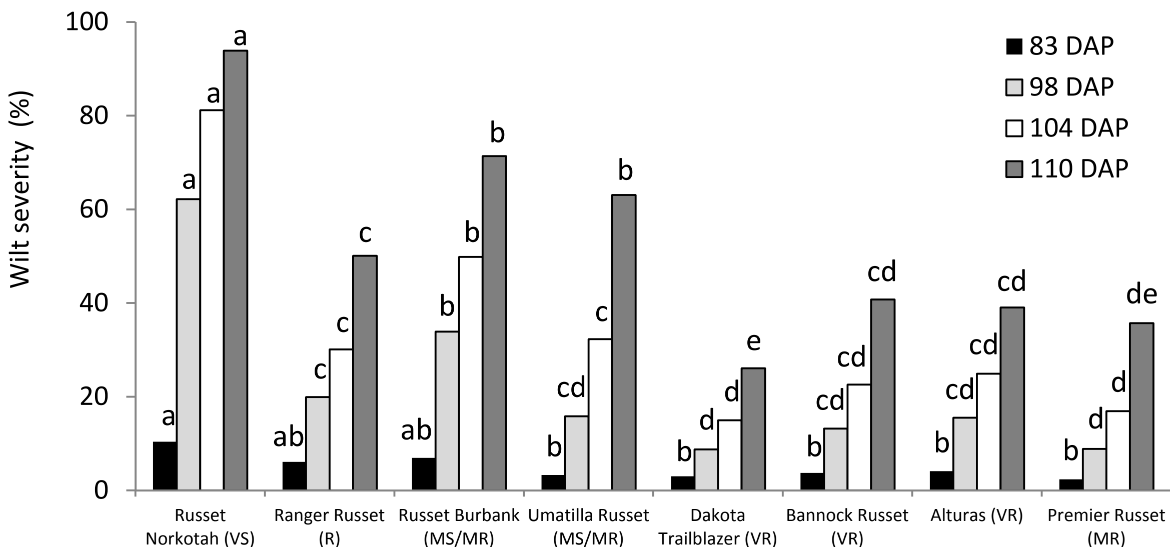

Figure 3.1. Percentage Verticillium wilt severity evaluated at 83, 98, 104 and 110 days after planting (DAP) in 2009 and 2010. Evaluations made across eight russet-skinned potato cultivars reported as very susceptible (VS), moderately susceptible/moderately resistant (MS/MR), moderately resistant (MR), resistant (R), or very resistant (VR) to *V. dahliae* from soils infested at low and high pathogen levels. Bars within evaluation date with the same letter are not statistically different based on Fisher's protected least significant difference (α = 0.05).

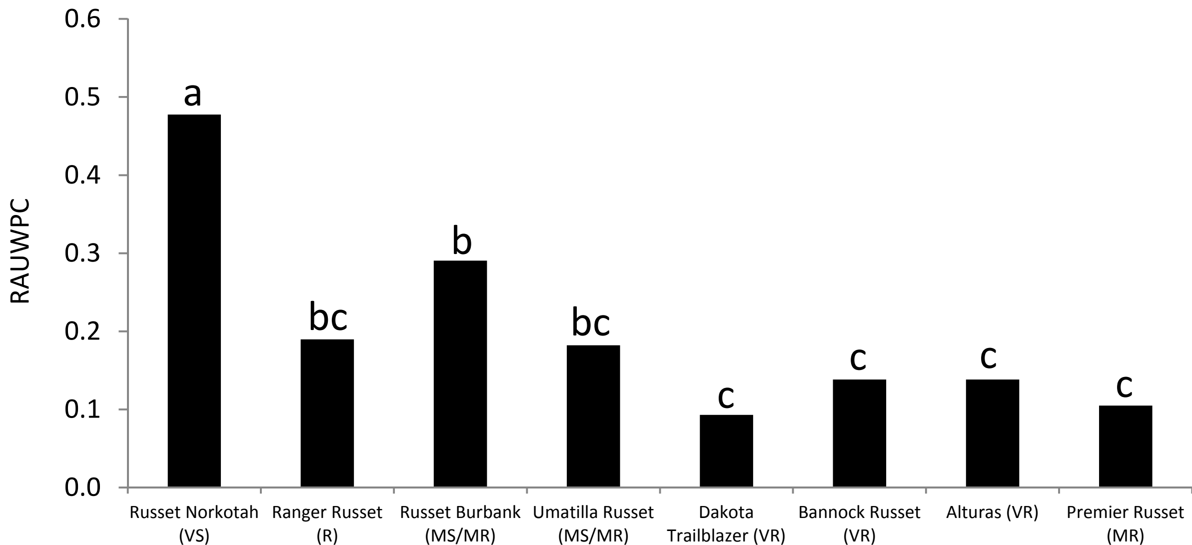

Figure 3.2. Relative area under the wilt progress curve (RAUWPC) evaluated in 2009 and 2010 across eight russet-skinned potato cultivars reported as very susceptible (VS), moderately susceptible/moderately resistant (MS/MR), moderately resistant (MR), resistant (R), or very resistant (VR) to *V. dahliae* from soils infested at low and high pathogen levels. Bars with the same letter are not statistically different based on Fisher's protected least significant difference ($\alpha = 0.05$).

Quantification of *V. dahliae* in fresh stem tissue. Significant differences were observed in colonization levels in stem tissue collected 104 DAP in 2009 and 2010 among cultivars for both the traditional plating assays ($P < 0.0001$), as well as QPCR assays ($P < 0.0001$). Susceptible control cv. Russet Norkotah had significantly higher levels of colonization than any other cultivar (Fig. 3.3). Also, in both assays, cvs. Russet Burbank and Umatilla Russet, were colonized significantly less than cv. Russet Norkotah, but significantly more than cvs. Premier Russet and Dakota Trailblazer. Differences in colonization among these moderately resistant cultivars and those with reportedly higher levels of resistance, were significant in some instances, depending on the assay. Results from both assays indicate that cvs. Dakota Trailblazer and Premier Russet are colonized at extremely low levels at this point in the growing season. Neither plating assays ($P = 0.2244$), or QPCR assays ($P = 0.0706$) assays detected a significant difference between infestation levels.

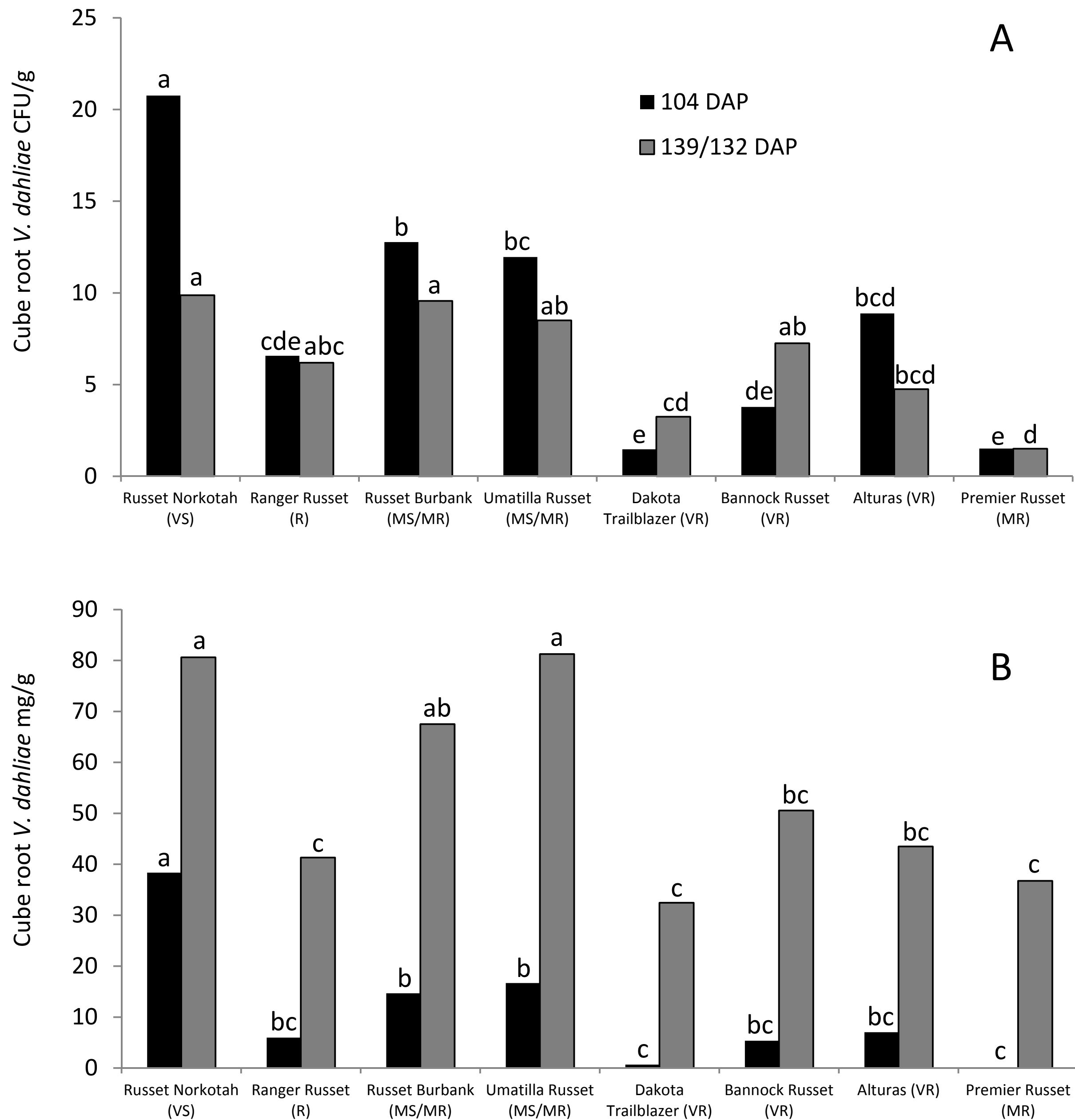

Figure 3.3. Cube root transformed colony forming units of *Verticillium dahliae* per gram of potato stem tissue (*V. dahliae* CFU/g) generated using the traditional plating assay (A) and mg *V. dahliae* per gram of potato stem tissue (*V. dahliae* mg/g) generated using the duplex QPCR assay (B). Stem tissue was collected 104 and 139/132 days after planting in 2009 and 2010 from soils infested at low and high levels with *V. dahliae*. Evaluations made across eight russet-skinned potato cultivars reported as very susceptible (VS), moderately susceptible/moderately resistant (MS/MR), moderately resistant (MR), resistant (R), or very resistant (VR) to *V. dahliae*. Bars with the same letter are not statistically different based on Fisher's protected least significant difference ($\alpha = 0.05$).

Quantification of *V. dahliae* in dried stem tissue. Trends observed from the colonization of stem tissue collected at harvest, 139 and 132 DAP in 2009 and 2010, respectively were similar to those observed with the colonization of fresh stem data (Fig. 3.3). Significant differences were observed among cultivars using both plating assays ($P < 0.0001$), as well as QPCR methods ($P < 0.0001$). In both assays, the difference in colonization level was not significant among cvs. Russet Norkotah, Russet Burbank and Umatilla Russet. As determined by traditional plating assays, cv. Bannock Russet also was not significantly less colonized than cv. Russet Norkotah. As was observed earlier in the growing season, cvs. Dakota Trailblazer and Premier Russet were colonization at the lowest levels, significantly less than cvs. Russet Norkotah, Russet Burbank and Umatilla Russet in both assays. Here, significant differences were observed between low and high infestation levels as determined by both plating ($P = 0.0448$) and QPCR ($P < 0.0001$) assays.

Colonization levels of *V. dahliae* as determined by traditional plating assays, decreased from fresh stem samples collected 104 DAP, to stems collected at harvest, and dried before plating, in five of eight russet cultivars evaluated (Figs. 3.3A). An increase was observed in cvs. Bannock Russet and Dakota Trailblazer, while no change in colonization was observed in cv. Premier Russet. Results differed considerably using the QPCR assay. Colonization levels, expressed as mg of *V. dahliae* DNA/g potato tissue, increased dramatically in all eight cultivars from 104 DAP to harvest (Figs. 3.3B). This increase is approximately proportional for all cultivars except susceptible control cultivar Russet Norkotah, in which colonization increased, but to a much lesser extent than observed in other cultivars. The relative increase in colonization at harvest was most substantial in cvs. Dakota Trailblazer and Premier Russet, which had the lowest colonization levels of the cultivars evaluated at 104 DAP.

Tuber yield and quality evaluations. Significant differences were observed in total yield among cultivars ($P = 0.0023$), however, these differences were not related to levels of reported resistance (Fig. 3.4). Cultivar Umatilla Russet yielded significantly higher than cvs. Russet Burbank, Dakota Trailblazer, Russet Norkotah, Premier Russet, and Bannock Russet. Cultivars Premier Russet and Bannock Russet yielded the lowest, but only significantly so when compared to cvs. Umatilla Russet and Alturas. Additionally, no significant differences in total yield were observed between low and high infestation levels ($P = 0.3335$).

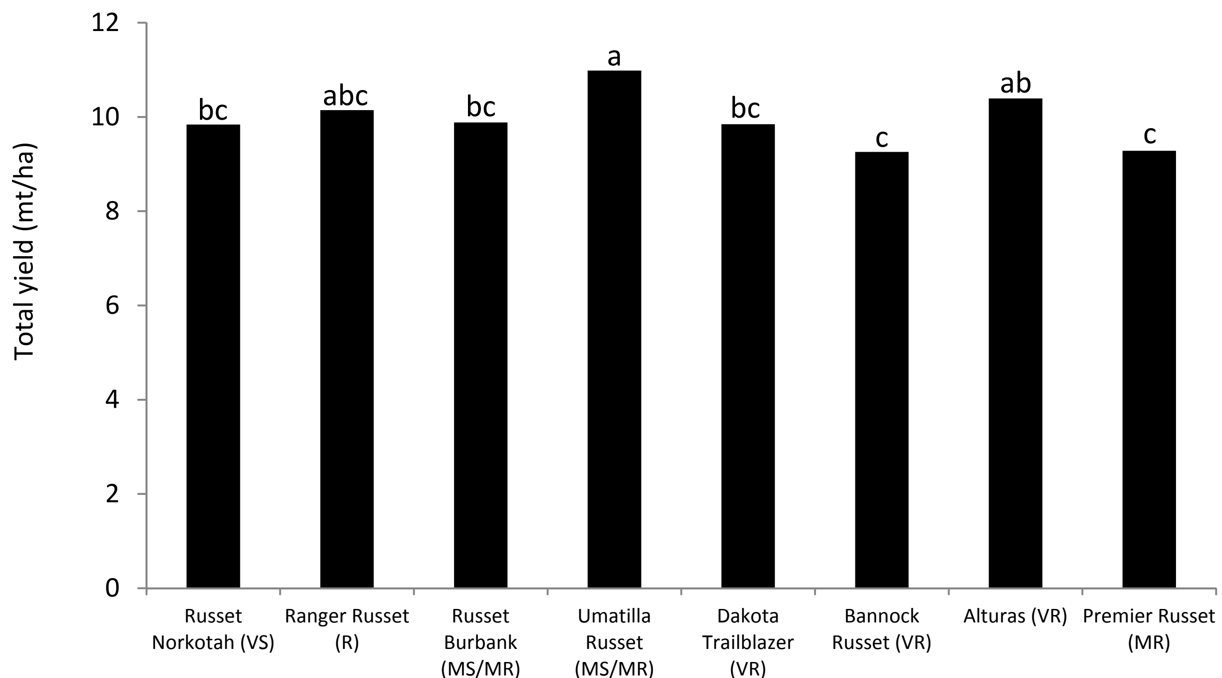

Figure 3.4. Total yield (mt/ha) evaluated in 2009 and 2010 across eight russet-skinned potato cultivars reported as very susceptible (VS), moderately susceptible/moderately resistant (MS/MR), moderately resistant (MR), resistant (R), or very resistant (VR) to *V. dahliae* from soils infested at low and high pathogen levels. Bars with the same letter are not statistically different based on Fisher's protected least significant difference ($\alpha = 0.05$).

Significant differences also were observed among cultivars for incidence of mild ($P < 0.0001$), severe ($P = 0.0002$) and total ($P < 0.0001$) vascular discoloration (Fig. 3.5). Unlike total yield, discoloration in tuber vascular tissue followed similar trends as were observed with

colonization and wilt. Susceptible control cv. Russet Norkotah displayed significantly higher incidences of mild discoloration than all other cultivars, while cv. Russet Burbank followed. Cultivars Ranger Russet, Premier Russet, Bannock Russet and Dakota Trailblazer all had significantly lower incidences of discoloration than the other cultivars evaluated. Trends in levels of severe discoloration were similar to that observed with mild discoloration. However, cv. Russet Burbank had higher incidence of severe discoloration than cv. Russet Norkotah, but not significantly so. Total incidence of vascular discoloration indicated that those cultivars with some level of resistance had significantly less discoloration than both cvs. Russet Norkotah and Russet Burbank. No significant differences were observed between infestation levels for incidence of mild (P = 0.301), severe (P = 0.857) or total (P = 0.390) tuber vascular discoloration.

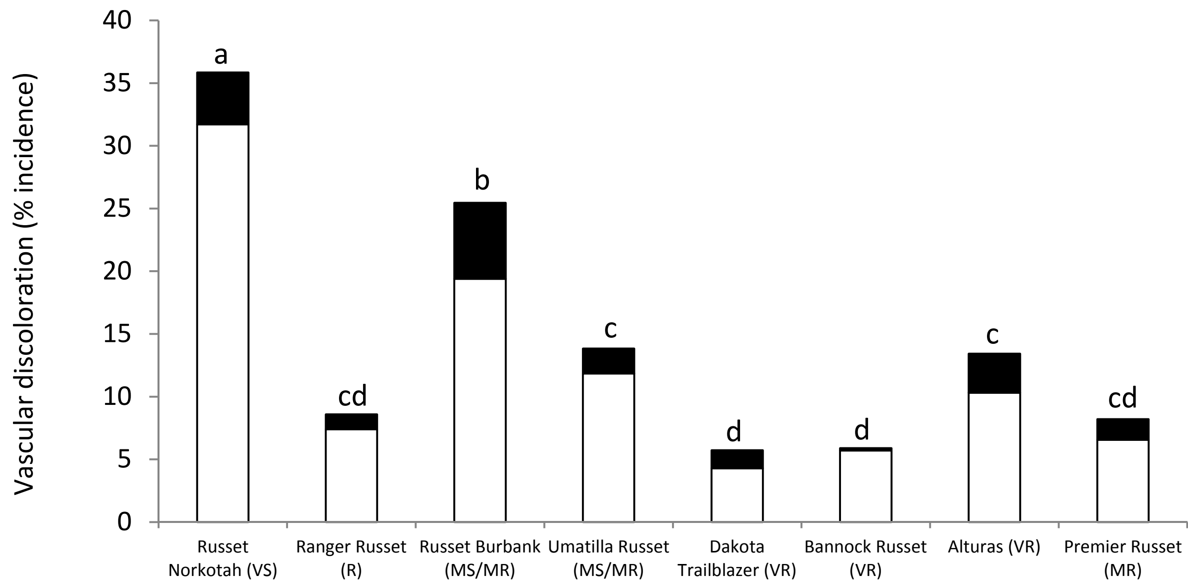

Figure 3.5. Percentage incidence of mild (white bars), severe (black bars) and total (white + black bars) tuber vascular discoloration evaluated in 2010 across eight russet-skinned potato cultivars reported as very susceptible (VS), moderately susceptible/moderately resistant (MS/MR), moderately resistant (MR), resistant (R), or very resistant (VR) to *V. dahliae* from soils infested at low and high pathogen levels. Bars with the same letter are not statistically different based on Fisher's protected least significant difference (α = 0.05).

Percentage Verticillium wilt severity at 104 DAP was closely related to stem colonization by *V. dahliae* on the same date as determined by both the plating ($n = 16$; $r = 0.92$; $P < 0.0001$) and QPCR assays ($n = 16$; $r = 0.94$; $P < 0.0001$) using Pearson's correlation (Figs. 3.6A and B). Similarly, colonization of stems collected at harvest, 139/132 DAP, as determined by plating ($n = 16$; $r = 0.82$; $P = 0.0001$) and QPCR ($n = 16$; $r = 0.88$; $P < 0.0001$) were related to wilt evaluations at 110 DAP (Figs. 3.6C and D). Trends in colonization levels determined by both assays were similar, and Pearson's correlation revealed a strong relationship ($n = 16$; $r = 0.96$; $P < 0.0001$) between the traditional plating and QPCR assays at 104 DAP (Fig. 3.7A). A highly significant and robust correlation was observed also for colonization of stem tissue collected at harvest as measured by plating and QPCR assays ($n = 16$; $r = 0.85$; $P < 0.0001$) (Fig. 3.7B). However, total yield was not related to either stem colonization at 104 DAP as determined by QPCR ($n = 16$; $r = 0.21$; $P = 0.438$) or RAUWPC ($n = 16$; $r = 0.03$; $P = 0.900$). Total tuber vascular discoloration also was related to colonization at 104 DAP as determined by QPCR ($n = 16$; $r = 0.91$; $P < 0.0001$) (Fig. 3.8).

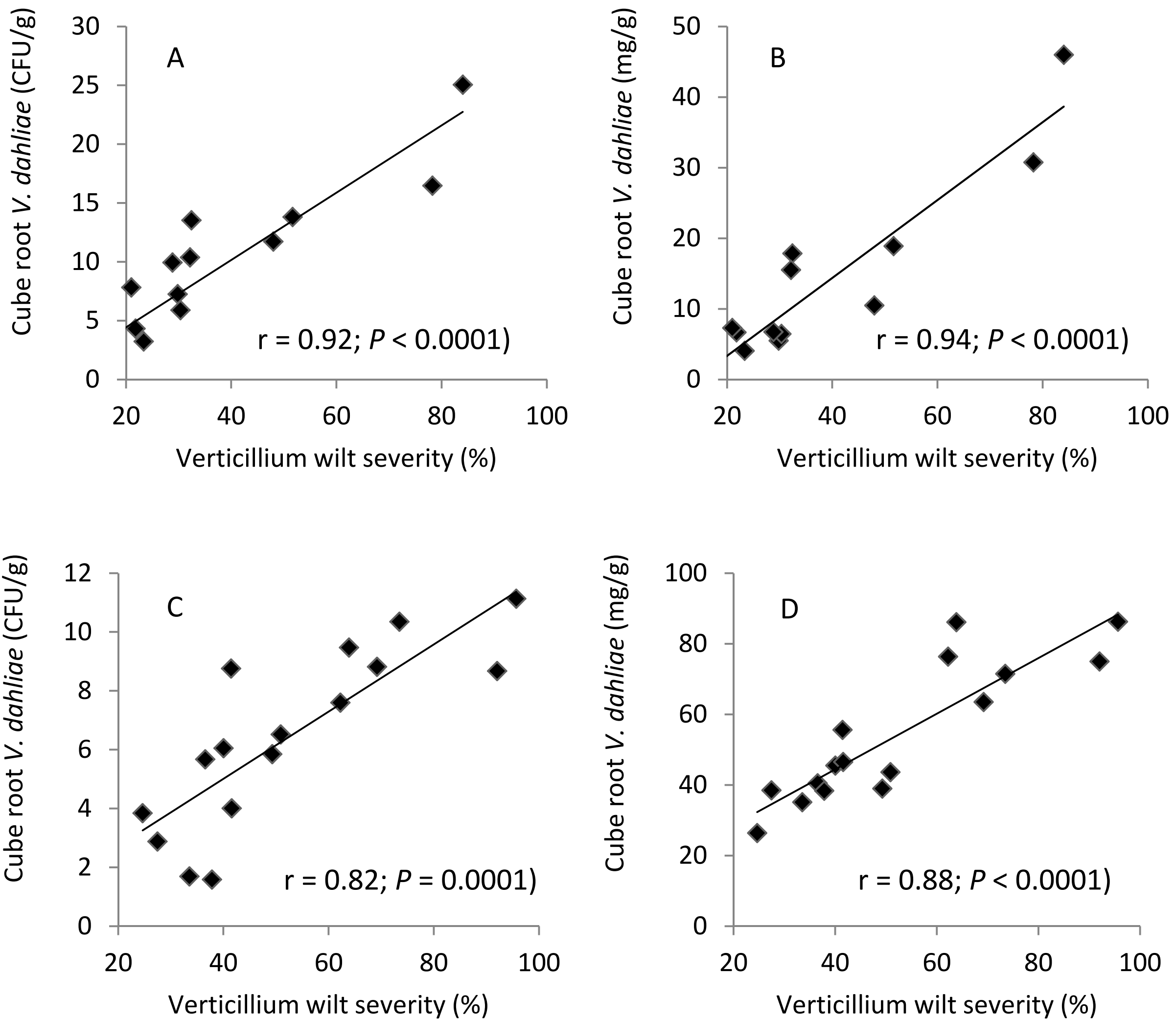

Figure 3.6. Relationships between cube root transformed *Verticillium dahliae* colony forming units per gram of potato stem tissue (*V. dahliae* CFU/g) generated using the traditional plating assay and Verticillium wilt at 104 DAP (A) and 110 DAP (C) and mg *V. dahliae* per gram of potato stem tissue (*V. dahliae* mg/g) generated using the duplex QPCR assay and Verticillium wilt at 104 DAP (B) and 110 DAP (D) as determined using Pearson's correlation. Stem tissue collected and wilt evaluated in 2009 and 2010 from eight russet-skinned potato cultivars with varying levels of resistance to *V. dahliae* grown in soils infested at low and high pathogen levels.

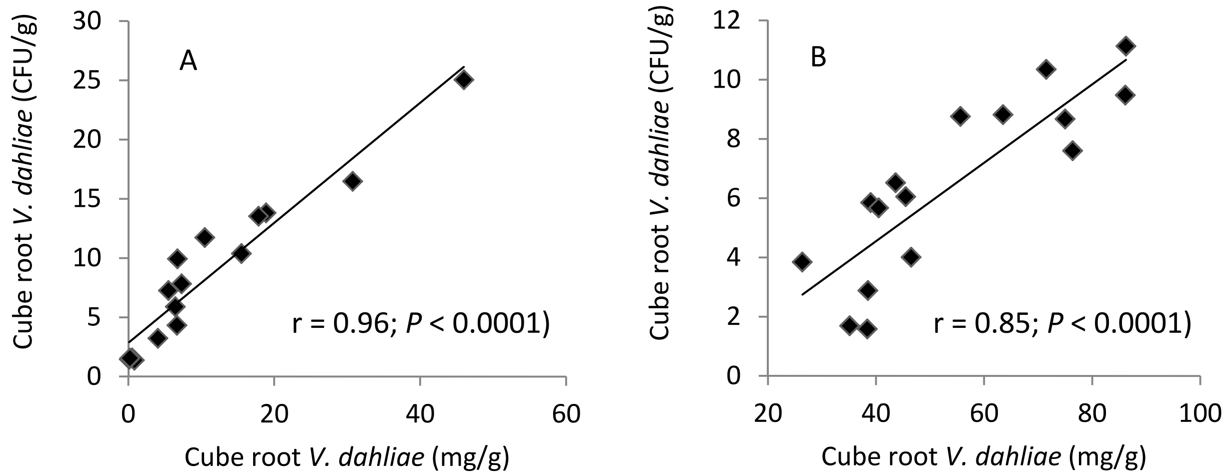

Figure 3.7. Relationship between cube root transformed *Verticillium dahliae* colony forming units per gram of potato stem tissue (*V. dahliae* CFU/g) generated using the traditional plating assay and mg *V. dahliae* per gram of potato stem tissue (*V. dahliae* mg/g) generated using the duplex QPCR assay as determined using Pearson's correlation. Stem tissue collected 104 (A) and 139/132 (B) days after planting in 2009 and 2010 from eight russet-skinned potato cultivars with varying levels of resistance to *V. dahliae* grown in soils infested at low and high pathogen levels.

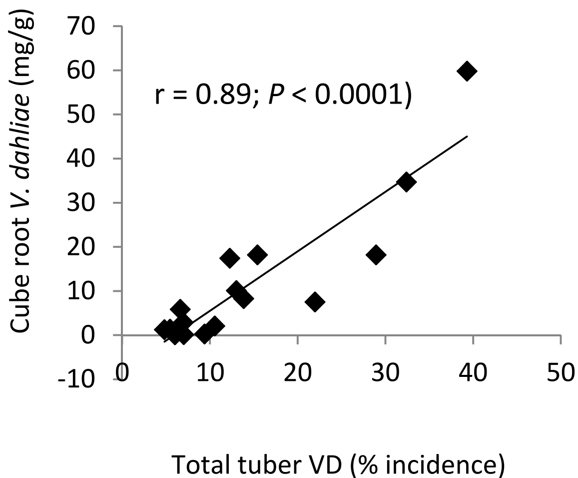

Figure 3.8. Relationships between cube root transformed *Verticillium dahliae* mg per gram of potato stem tissue (*V. dahliae* mg/g) generated using the duplex QPCR assay at 104 DAP and total tuber vascular discoloration (VD) as determined using Pearson's correlation. Stem tissue colonization was compared to VD in 2010 only, from eight russet-skinned potato cultivars with varying levels of resistance to *V. dahliae* grown in soils infested at low and high pathogen levels.

Discussion

Verticillium wilt is extremely damaging to potato growers not only because of loss of tuber yield, size and quality, but also because of the extraordinary expense in controlling the disease via the use of the soil fumigant metam sodium (Rowe and Powelson, 2002). In the past, the general lack of cultivars with market acceptance and resistance to Verticillium wilt left growers with few alternative options to control the disease (Powelson et al., 1993; Rowe and Powelson, 2002). Recently, several russet-skinned cultivars were released with purported to Verticillium wilt, however, for many of these no attempts were made to quantify the host:pathogen interaction at the time of their release to the industry (Johansen et al., 1994; Love et al., 2002; 2005; 2006; Mosley et al., 1999; 2000; 2001; Novy et al., 2002; 2003; 2006; 2008; 2010; Stark et al., 2009).

While plating methods are accurate and reliable, they are labor intensive, and therefore, many breeders do not perform these evaluations. This in turn represents an increase in the need for accurate and rapid assays to detect the pathogen in host tissue. This study was developed, and performed, to quantify *V. dahliae* colonization in eight russet-skinned potato cultivars to determine if the resistance level reported was truly resistance, or merely tolerance, to symptom development. Additionally, stems collected from field trials were used to further validate a previously developed duplex QPCR assay under field conditions to replace standard plating techniques (Chapter 2). The present research not only puts forth a rapid and reliable assay for use in screening cultivars and potentially breeding selections for resistance to Verticillium wilt, it demonstrates that resistance is present to varying degrees among the russet-skinned cultivars evaluated. This is evident not only in the levels of colonization present in each cultivar, but also

in the relationship between colonization and symptoms of Verticillium wilt, including foliar wilt and tuber vascular discoloration.

Verticillium wilt evaluations preformed here generally were consistent with variety releases and other previous research with the exception of cv. Premier Russet (Bae et al., 2007, 2008; Frost et al., 2007; Hoyos et al., 1991; Jansky, 2009; Mosley et al., 1999; Novy et al., 2002; 2003; 2008). Cultivar Premier Russet was rated as moderately resistant, or similar to cv. Ranger Russet. In this study, depending on the evaluation date, it displayed wilt symptoms significantly lower than cv. Ranger Russet and statistically similar to that of cultivars rated as very resistant including cvs. Dakota Trailblazer, Bannock Russet and Alturas. However, temperature and moisture can have a substantial impact on wilt development, and therefore these evaluations may differ from one season or growing region to the next (Ben-Yephet and Szmulewich 1985; Cappeart et al., 1992; Nnodu and Harrison, 1979; Powelson and Rowe, 1993).

Stem colonization by *V. dahliae* also was similar to previously reported data for the control cultivars Russet Norkotah, Ranger Russet as well as cvs. Russet Burbank and Dakota Trailblazer (Bae et al., 2007, 2008; Frost et al., 2007; Hoyos et al., 1991; Jansky, 2009). This provides a level of confidence that plating and QPCR techniques employed in this research were reliable. Susceptible control cultivar Russet Norkotah consistently had the highest levels of wilt and colonization. As expected, cultivars rated as MS/MR, Russet Burbank and Umatilla Russet, had wilt and colonization levels intermediate to that of the susceptible and resistant control cultivars. Interestingly, the four remaining cultivars, Dakota Trailblazer, Bannock Russet, Alturas and Premier Russet all had statistically similar, while generally numerically lower, levels of resistance to *V. dahliae* as cv. Ranger Russet. These findings are paramount to the industry,

providing additional sources of resistance to Verticillium wilt at a time when management options are limited.

Significant differences in total yield were observed among the cultivars evaluated, however, the trend did not follow other parameters evaluated in this study. This is not too surprising as inconsistencies in yield reactions have been observed previously in Verticillium wilt evaluations (Frost et al., 2007; Mohan et al., 1990). Cultivars differ genetically in their yielding ability and Verticillium wilt is but one factor that can affect tuber yield. While tuber vascular discoloration data only was collected in one year of the trial, results were very compelling. No data exists prior to this study on the relationship between wilt or stem colonization to the level of tuber vascular discoloration. While this research should be confirmed in future trials, it could provide the industry with valuable information regarding tuber quality in Verticillium resistant cultivars.

Several wild *Solanum* spp. have been identified as sources of resistance to *V. dahliae*, and in crosses made with one resistant and one susceptible parent, this resistance appears to be simply inherited and stable (Corsini et al., 1985; 1988; 1990; Davis, 2009; Davis et al., 1983; Hoyos et al., 1993; Hunter et al., 1968; Jansky and Rouse, 2000; 2003). The sources of resistance in the cultivars evaluated in this study are not identified and potentially are not known (Mosley et al., 1999; Novy et al., 2002; 2003; 2008). However, comparing pedigrees among cvs. Umatilla Russet, Bannock Russet, Premier Russet and Alturas does reveal some interesting possibilities. For example, Bannock Russet and Alturas have the same male parent and Premier Russet has Bannock Russet in its female lineage. Other similarities in the pedigrees of these cultivars exist. The development of this rapid, reliable and accurate QPCR technique facilitates screening the lineages of resistant cultivars with good agronomic characteristics, therefore providing a better

understanding of the sources of resistance. This subsequently will accelerate the development of new Verticillium resistant cultivars.

Previous research has indicated that Verticillium wilt evaluated on a single date was correlated to the AUWPC (Jansky, 2009). This observation is confirmed in this study, even at the earliest wilt evaluation date when severity was less than 10%. Trends among cultivars were consistent across all four dates and with RAUWPC. As was proposed previously, this may allow breeders to assess wilt symptoms early in the growing season and subsequently have time to collect stems from those selections which show promise for resistance (Jansky, 2009; Jansky and Rouse, 2000). The three-tiered method developed in previous research was successful in identifying resistance among breeding clones using a combination of wilt symptoms, plating fresh stem sap and plating dried stem tissue (Jansky, 2009). This approach allows for accurate screening and identification of true resistance while systematically eliminating clones with no resistance early in the screening process, and therefore, reducing the labor required for stem colonization assays. The research reported here not only confirms the use of these three parameters for the identification of resistance, but successfully uses a QPCR assay to further reduce the time and labor required to quantify stem colonization by *V. dahliae*.

Reports of the correlation between stem colonization and symptom expression have been inconsistent (Davis et al., 1983; Frost et al., 2007; Jansky, 2009; Jansky and Rouse, 2000; Lynch et al., 1997; Mohan et al., 1990). The research reported here indicates that symptom expression is well correlated with colonization on the same date, and others have made similar observations (Davis et al., 1983; Mohan et al., 1990). However, some researchers report that no correlation between wilt and colonization exists (Jansky, 2009; Jansky and Rouse, 2000; Lynch et al., 1997). The disparity in these results indicates that tolerant cultivars are present in some populations

evaluated, and not others (Lynch et al., 1997). In the current study, wilt was well correlated with colonization, indicating that true resistance is present in those cultivars displaying low levels of wilt symptoms and concurrent low levels of pathogen colonization. However, the use of visual symptoms alone cannot separate resistance from tolerance. Evaluating the absence of wilt symptoms recorded alone leaves the possibility that cultivars could be harboring high pathogen populations. This inoculum would be returned to the soil to infect subsequent crops of potato, perhaps planted using susceptible cultivars. Therefore, visual assessments alone should not be relied upon to screen cultivars and germplasm for resistance to colonization by *V. dahliae*. It also has been reported that stem colonization quantified during the growing season did not correlate well with colonization of dried stems at the end of the season (Jansky et al., 2004; Jansky and Rouse, 2003). The current research, and others, disagrees with this observation (Davis et al., 1983). Stem colonization of fresh stems 104 DAP as determined by QPCR methods was well correlated with colonization of dried stem tissue collected 139/132 DAP ($n = 16$; $r = 0.83$; $P < 0.0001$). However, results from the current study do agree with previous observation that populations recovered from fresh stems were higher than that recovered from dried stems (Jansky and Rouse, 2003). The relationship between yield and severity of Verticillium wilt also has been inconsistent (Frost et al., 2007; Mohan et al., 1990). Here, neither wilt severity nor colonization was correlated to total yield.

The root lesion nematode *P. penetrans* interacts synergistically with *V. dahliae*, resulting in larger yield reductions than either pathogen alone (Martin et al., 1982; Rowe et al., 1985). The mechanisms of this interaction are not well understood, and therefore the effects of the presence of *P. penetrans* on resistance to *V. dahliae* observed in the cultivars evaluated in these studies need further examination. Additionally, previous research indicates that very few propagules of

V. dahliae are required to cause high levels of disease and that the level of inoculum can be related to the level of stem colonization in cv. Russet Burbank and yield in cvs. Norgold and Norchip (Ben-Yephet and Szmulewich, 1985; Davis, 1985; Nicot and Rouse, 1987; Nnodu and Harrison, 1979). While two infestation levels were evaluated here, little differences were observed between them in any of the eight cultivars. Therefore, further studies are warranted to determine whether or not the resistance observed in these cultivars will break-down under higher disease pressure.

The quantification of *V. dahliae* for the purpose of evaluating resistance is an important tool, and has been used extensively in potato breeding (Bae et al., 2008; Concibido et al., 1994; Corsini et al., 1985; 1990; Davis et al., 1983; Frost et al., 2007; Hoyos et al., 1993; Jansky, 2009; Jansky and Miller, 2010; Jansky and Rouse, 2000; 2003; Jansky et al., 2004; Mohan et al., 1990). Additionally, traditional plating methods have been utilized successfully to evaluate Verticillium wilt control measures (Nicot and Rouse, 1987). However, these studies were performed using labor-intensive plating techniques, even after more rapid PCR techniques had been developed (Atallah et al., 2007; Dan et al., 2001; Hu et al., 1993; Li et al., 1999; Mahuku et al., 1999; Mercado-Blanco et al., 2001; 2002; 2003; Nazar et al., 1991; Pérez-Artés et al., 2000). One study utilized a QPCR assay for the quantification of *V. dahliae* in cvs. Russet Norkotah and Ranger Russet at several points over the growing season (Bae et al., 2007). However, these methods were not used in subsequent colonization studies performed by similar authors (Bae et al., 2008). There may be several reasons for the lack of widespread adoption of PCR assays in *V. dahliae* quantification, including the difficulty of successfully incorporating of new methods into standard protocols or the reliability of the available PCR assays. The studies reported here

demonstrate the use of a QPCR assay to quantify *V. dahliae* in potato stems produced under field conditions with great accuracy and reliability.

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APPENDIX A. SUMMARY OF STATSTICAL ANALYSES FOR BLACK DOT SEVERITY

Table A.1. Analysis of variance of relative area under the *Colletotrichum coccodes* colonization progress curve (RAUCPC) for inoculation/infestation sites and potato tissues sampled. The trial was conducted in North Dakota in 2003.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Rep	3	0.02116	4.98	0.0020
Inoculation/infestation	7	0.06601	15.52	<.0001
Tissue	3	0.52824	124.23	<.0001
Inoculation/infestation × Tissue	21	0.00645	1.52	0.0653

Table A.2. Analysis of variance of relative area under the *Colletotrichum coccodes* colonization progress curve (RAUCPC) for inoculation/infestation sites and potato tissues sampled. The trial was conducted in North Dakota in 2004.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Rep	3	0.0164	3.54	0.0145
Inoculation/infestation	7	0.0892	19.27	<.0001
Tissue	3	2.09759	453.11	<.0001
Inoculation/infestation × Tissue	21	0.01647	3.56	<.0001

Table A.3. Analysis of variance of relative area under the *Colletotrichum coccodes* colonization progress curve (RAUCPC) for inoculation/infestation sites and potato tissues sampled. The trial was conducted in Minnesota in 2003.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Rep	3	0.03701	5.47	0.0010
Inoculation/infestation	7	0.03432	5.07	<.0001
Tissue	3	3.22467	476.51	<.0001
Inoculation/infestation × Tissue	21	0.00527	0.78	0.7483

Table A.4. Analysis of variance of relative area under the *Colletotrichum coccodes* colonization progress curve (RAUCPC) for inoculation/infestation sites and potato tissues sampled. The trial was conducted in Minnesota in 2004.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Rep	3	0.07481	5.32	<.0001
Inoculation/infestation	7	0.11925	8.48	<.0001
Tissue	3	0.00475	0.34	0.9978
Inoculation/infestation × Tissue	21	0.00219	0.16	0.9261

APPENDIX B. SUMMARY OF STATSTICAL ANALYSES FOR DEVELOPMENT AND VALIDATION OF THE DUPLEX QPCR ASSAY

Table B.1. Random coefficients analysis of regression lines generated from 10-fold serial dilutions over six orders of magnitude of DNA from *Verticillium dahliae* cultures and tissue culture derived plantlets using single and duplex QPCR assays.

Source of variation	Isolate	Degrees of freedom	Std Err Pred	t Value	Pr > |t|
Log DNA concentration	1	61	0.2283	-1.56	0.1242
Intercept	1	61	0.06738	1.16	0.2502
Log DNA concentration	2	61	0.2282	-1.19	0.2395
Intercept	2	61	0.06643	-0.72	0.4760
Log DNA concentration	3	61	0.2285	2.17	0.0340
Intercept	3	61	0.06873	-1.33	0.1869
Log DNA concentration	4	61	0.2282	0.58	0.5674
Intercept	4	61	0.06643	0.92	0.3607

APPENDIX C. SUMMARY OF STATSTICAL ANALYSES FOR QUANTIFICATION OF *VERTICILLIUM DAHLIAE* COLONIZATION IN THREE GREENHOUSE TRIALS

Table C.1. Combined analysis of variance of pathogen colonization as determined by traditional plating assays at eight weeks after inoculation for eight russet-skinned potato cultivars across three greenhouse trials.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Trial	2	393.894	4.39	0.0164
Rep (Trial)	9	51.9179	0.58	0.8097
Cultivar	7	463.241	5.16	0.0001
Cultivar × Trial	14	119.004	1.33	0.2180

Table C.2. Combined analysis of variance of pathogen colonization as determined by QPCR assays at eight weeks after inoculation for eight russet-skinned potato cultivars across three greenhouse trials.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Trial	2	367.786	5.41	0.0068
Rep (Trial)	9	31.0486	0.46	0.8977
Cultivar	7	898.19	13.22	<.0001
Cultivar × Trial	14	190.859	2.81	0.0026

Table C.3. Combined analysis of variance of pathogen colonization as determined by traditional plating assays at 11 weeks after inoculation for eight russet-skinned potato cultivars across three greenhouse trials.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Trial	2	219.702	7.23	0.0015
Rep (Trial)	9	30.129	0.99	0.4561
Cultivar	7	733.679	24.15	<.0001
Cultivar × Trial	14	243.026	8.00	<.0001

Table C.4. Combined analysis of variance of pathogen colonization as determined by QPCR assays at 11 weeks after inoculation for eight russet-skinned potato cultivars across three greenhouse trials.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Trial	2	1727.04	11.88	0.0001
Rep (Trial)	9	309.427	2.13	0.0406
Cultivar	7	5553.83	2.00	<.0001
Cultivar × Trial	14	295.082	2.03	0.0332

APPENDIX D. SUMMARY OF STATSTICAL ANALYSES FOR VERTICILLIUM WILT SEVERITY IN THREE GREENHOUSE TRIALS

Table D.1. Analysis of variance of percent Verticillium wilt severity for eight russet-skinned potato cultivars and two *Verticillium dahliae* infestation levels across the growing season as represented by Relative Area Under the Wilt Progress Curve (RAUWPC) in greenhouse trial 1.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Rep	3	0.1164	22.49	<.0001
Inoculum	1	0.01794	3.47	0.0637
Cultivar	7	0.19006	36.72	<.0001
Cultivar × Inoculum	7	0.04413	8.53	<.0001

Table D.2. Analysis of variance of percent Verticillium wilt severity for eight russet-skinned potato cultivars and two *Verticillium dahliae* infestation levels across the growing season as represented by Relative Area Under the Wilt Progress Curve (RAUWPC) in greenhouse trial 2.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Rep	3	0.05471	4.29	0.0056
Inoculum	1	0.03991	3.13	0.0781
Cultivar	7	0.47526	37.23	<.0001
Cultivar × Inoculum	7	0.12021	9.42	<.0001

Table D.3. Analysis of variance of percent Verticillium wilt severity for eight russet-skinned potato cultivars and two *Verticillium dahliae* infestation levels across the growing season as represented by Relative Area Under the Wilt Progress Curve (RAUWPC) in greenhouse trial 3.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Rep	3	0.05533	5.05	0.0020
Inoculum	1	0.01393	1.27	0.2601
Cultivar	7	1.10564	101.00	<.0001
Cultivar × Inoculum	7	0.07734	7.07	<.0001

APPENDIX E. SUMMARY OF STATSTICAL ANALYSES FOR VERTICILLIUM WILT SEVERITY IN FIELD TRIALS CONDUCTED IN 2009 AND 2010

Table E.1. Combined analysis of variance of percent Verticillium wilt severity for eight russet-skinned potato cultivars and two *Verticillium dahliae* infestation levels at 83 days after planting in 2009 and 2010.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Year	1	1969.41	222.40	<.0001
Rep (Year)	6	4.85683	0.55	0.7700
Cultivar	7	114.879	12.97	<.0001
Infestation	1	12.7892	1.44	0.2326
Cultivar × Infestation	7	7.12673	0.80	0.5855
Year × Cultivar	7	42.3855	4.79	0.0001
Year × Infestation	1	1.53563	0.17	0.6781
Year × Cultivar × Infestation	7	3.3558	0.38	0.9124

Table E.2. Combined analysis of variance of percent Verticillium wilt severity for eight russet-skinned potato cultivars and two *Verticillium dahliae* infestation levels at 98 days after planting in 2009 and 2010.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Year	1	0.29549	0.01	0.9371
Rep (Year)	6	22.5845	0.48	0.8225
Cultivar	7	5174.34	109.70	<.0001
Infestation	1	282.893	6.00	0.0163
Cultivar × Infestation	7	97.6032	2.07	0.0549
Year × Cultivar	7	103.588	2.20	0.0418
Year × Infestation	1	3.13438	0.07	0.7972
Year × Cultivar × Infestation	7	26.6398	0.56	0.7826

Table E.3. Combined analysis of variance of percent Verticillium wilt severity for eight russet-skinned potato cultivars and two *Verticillium dahliae* infestation levels at 104 days after planting in 2009 and 2010.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Year	1	86.4284	1.37	0.2452
Rep (Year)	6	112.395	1.78	0.1122
Cultivar	7	7677.35	121.55	<.0001
Infestation	1	167.537	2.65	0.1069
Cultivar × Infestation	7	40.0664	0.63	0.7263
Year × Cultivar	7	228.039	3.61	0.0018
Year × Infestation	1	18.7119	0.30	0.5876
Year × Cultivar × Infestation	7	59.1171	0.94	0.4830

Table E.4. Combined analysis of variance of percent Verticillium wilt severity for eight russet-skinned potato cultivars and two *Verticillium dahliae* infestation levels at 110 days after planting in 2009 and 2010.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Year	1	366.765	4.65	0.0338
Rep (Year)	6	257.005	3.26	0.0061
Cultivar	7	7961.21	100.88	<.0001
Infestation	1	304.459	3.86	0.0526
Cultivar × Infestation	7	8.00179	0.10	0.9981
Year × Cultivar	7	213.549	2.71	0.0136
Year × Infestation	1	21.8213	0.28	0.6003
Year × Cultivar × Infestation	7	56.1098	0.71	0.6628

Table E.5. Combined analysis of variance of percent Verticillium wilt severity for eight russet-skinned potato cultivars and two *Verticillium dahliae* infestation levels across the growing season as represented by Relative Area Under the Wilt Progress Curve (RAUWPC) in 2009 and 2010.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Year	1	0.41093	235.68	<.0001
Rep (Year)	6	0.00159	0.91	0.4895
Cultivar	7	0.25984	149.03	<.0001
Infestation	1	0.01086	6.23	0.0144
Cultivar × Infestation	7	0.0022	1.26	0.2774
Year × Cultivar	7	0.01741	9.98	<.0001
Year × Infestation	1	0.00069	0.39	0.5320
Year × Cultivar × Infestation	7	0.00152	0.87	0.5318

APPENDIX F. SUMMARY OF STATSTICAL ANALYSES FOR COLONIZATION BY *VERTICILLIUM DAHLIAE* IN FIELD TRIALS CONDUCTED IN 2009 AND 2010

Table F.1. Combined analysis of variance of pathogen colonization as determined by traditional plating assays at 104 days after planting for eight russet-skinned potato cultivars and two *Verticillium dahliae* infestation levels in 2009 and 2010.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Year	1	238.547	14.25	0.0003
Rep (Year)	6	50.0231	2.99	0.0104
Cultivar	7	696.539	41.62	<.0001
Infestation	1	25.0458	1.50	0.2244
Cultivar × Infestation	7	50.9299	3.04	0.0064
Year × Cultivar	7	48.8146	2.92	0.0085
Year × Infestation	1	7.77179	0.46	0.4973
Year × Cultivar × Infestation	7	15.3432	0.92	0.4974

Table F.2. Combined analysis of variance of pathogen colonization as determined by QPCR assays at 104 days after planting for eight russet-skinned potato cultivars and two *Verticillium dahliae* infestation levels in 2009 and 2010.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Year	1	48.462	0.85	0.3578
Rep (Year)	6	140.092	2.47	0.0294
Cultivar	7	2494.43	43.98	<.0001
Infestation	1	189.93	3.35	0.0706
Cultivar × Infestation	7	153.665	2.71	0.0135
Year × Cultivar	7	228.993	4.04	0.0007
Year × Infestation	1	321.755	5.67	0.0193
Year × Cultivar × Infestation	7	36.8195	0.65	0.7141

Table F.3. Combined analysis of variance of pathogen colonization as determined by traditional plating assays at 139/132 days after planting for eight russet-skinned potato cultivars and two *Verticillium dahliae* infestation levels in 2009 and 2010, respectively.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Year	1	7.03125	1.19	0.2787
Rep (Year)	6	8.46875	1.43	0.2116
Cultivar	7	146.049	24.68	<.0001
Infestation	1	24.5	4.14	0.0448
Cultivar × Infestation	7	7.41071	1.25	0.2833
Year × Cultivar	7	20.7634	3.51	0.0023
Year × Infestation	1	40.5	6.84	0.0104
Year × Cultivar × Infestation	7	14.625	2.47	0.0229

Table F.4. Combined analysis of variance of pathogen colonization as determined by QPCR assays at 139/132 days after planting for eight russet-skinned potato cultivars and two *Verticillium dahliae* infestation levels in 2009 and 2010, respectively.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Year	1	69238.5	728.59	<.0001
Rep (Year)	6	182.237	1.92	0.0864
Cultivar	7	6122.92	64.43	<.0001
Infestation	1	2120.63	22.32	<.0001
Cultivar × Infestation	7	41.6864	0.44	0.8754
Year × Cultivar	7	831.704	8.75	<.0001
Year × Infestation	1	114.383	1.20	0.2755
Year × Cultivar × Infestation	7	226.794	2.39	0.0276

APPENDIX G. SUMMARY OF STATSTICAL ANALYSIS FOR TUBER YIELD IN FIELD TRIALS CONDUCTED IN 2009 AND 2010

Table G.1. Combined analysis of variance of total yield (mt/ha) for eight russet-skinned potato cultivars and two *Verticillium dahliae* infestation levels in 2009 and 2010.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Year	1	197151	21.28	<.0001
Rep (Year)	6	18698.5	2.02	0.0645
Cultivar	7	30607.4	3.30	0.0023
Infestation	1	8705.87	0.94	0.3335
Cultivar × Infestation	7	9012.65	0.97	0.4521
Year × Cultivar	7	9680.58	1.04	0.4008
Year × Infestation	1	2092.6	0.23	0.6351
Year × Cultivar × Infestation	7	12994.3	1.40	0.2056

APPENDIX H. SUMMARY OF STATSTICAL ANALYSES FOR TUBER QUALITY IN FIELD TRIALS CONDUCTED IN 2010

Table H.1. Combined analysis of variance of mild tuber vascular discoloration for eight russet-skinned potato cultivars and two *Verticillium dahliae* infestation levels.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Rep	3	110.0018	1.55	0.2060
Cultivar	7	1362.003	19.18	<.0001
Infestation	1	76.63816	1.08	0.3012
Cultivar × Infestation	7	87.57873	1.23	0.2910

Table H.2. Combined analysis of variance of severe tuber vascular discoloration for eight russet-skinned potato cultivars and two *Verticillium dahliae* infestation levels.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Rep	3	3.564371	0.28	0.8369
Cultivar	7	56.98656	4.54	0.0002
Infestation	1	0.410	0.03	0.8569
Cultivar × Infestation	7	20.89267	1.66	0.1253

Table H.3. Combined analysis of variance of total tuber vascular discoloration for eight russet-skinned potato cultivars and two *Verticillium dahliae* infestation levels.

Source of variation	Degrees of freedom	Mean squares	F value	*P* value
Rep	3	77.71116	0.88	0.4546
Cultivar	7	1835.764	20.76	<.0001
Infestation	1	65.83769	0.74	0.3902
Cultivar × Infestation	7	66.40354	0.75	0.6296