Am. J. Potato Res. (2013) 90:158–170
DOI 10.1007/s12230-012-9280-1

Quantification of Field Resistance to *Verticillium dahliae* in Eight Russet-Skinned Potato Cultivars Using Real-Time PCR

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Published online: 14 December 2012

Abstract Changes in potato production over the past 10 to 20 years, have resulted in increased emphasis being placed on breeding for resistance to Verticillium wilt, caused by *Verticillium dahliae* Kleb. While many russet-skinned cultivars recently have been released with reported resistance to Verticillium wilt, information is lacking on the level of pathogen colonization, and therefore, the level of true genetic resistance is not known. Eight russet-skinned cultivars were grown in field trials with low and high levels of *V. dahliae* in the soil, and evaluated for wilt, stem colonization, yield, and tuber vascular discoloration. A recently developed QPCR assay was validated, with strong relationships to culture plating assays over three stem sampling dates. Additionally, stem colonization levels, as determined by QPCR, were related to wilt and tuber vascular discoloration. However, total yield did not exhibit a strong relationship to any other parameter evaluated in this study. Results from these studies indicate that varying levels of true resistance are present in the russet-skinned cultivars evaluated, and that the QPCR assay can be reliable in rapidly evaluating resistance to *V. dahliae* under field conditions. Based on pathogen quantification using stem colonization derived from traditional plating assays and QPCR, the resistance level of several cultivars is more clearly defined and discussed.

Resumen Los cambios en la producción de papa en los pasados 10 a 20 años, han resultado en un aumento en el énfasis ubicado en el mejoramiento para la resistencia al marchitamiento por *Verticillium*, causado por *Verticillium dahliae* Kleb. Mientras que muchos cultivares de piel tipo russet se han liberado recientemente con resistencia reportada al marchitamiento por *Verticillium*, la información carece del nivel de colonización del patógeno, y de aquí que no se conozca el nivel de resistencia genética verdadera. Se sembraron ocho cultivares con piel tipo russet en ensayos de campo con niveles bajo y alto de *V. dahliae* en el suelo, y evaluados para marchitez, colonización del tallo, rendimiento, y decoloración vascular del tubérculo. Se evaluó recientemente un ensayo QPCR, con relaciones fuertes a ensayos de cultivos en placas sobre tres fechas de muestreo del tallo. Adicionalmente, los niveles de colonización del tallo, como se determinaron por QPCR, se relacionaron al marchitamiento y a la decoloración vascular del tubérculo. No obstante, el rendimiento total no exhibió una relación fuerte a ningún otro parámetro evaluado en este estudio. Los resultados de estos estudios indican que están presentes diversos niveles de resistencia verdadera en las variedades de piel tipo russet evaluadas, y que el ensayo de QPCR puede ser confiable en la evaluación rápida de resistencia a *V. dahliae* bajo condiciones de campo. Con base en la cuantificación del patógeno mediante el uso de la colonización del tallo derivada de los ensayos tradicionales de placas y QPCR, el nivel de resistencia de varios cultivares está más claramente definido y discutido.

Keywords Solanum tuberosum · Verticillium dahliae · Disease resistance · Polymerase chain reaction · Verticillium wilt · Cultivar resistance

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Introduction

Verticillium wilt in United States potato (*Solanum tuberosum* L.) production 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 1993; 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 favorable for the build-up and survival of plant pathogens in the surface layers of the soil, 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, foliar chlorosis and necrosis. 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). 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. Additionally, vascular discoloration in tubers can result in browning of processed products and may render symptomatic potato tubers unmarketable (Rowe 1985).

While *V. dahliae* resistance mechanisms in potato 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).

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 2012). Of the top seven cultivars produced for seed-tubers in North America in 2000, cv. Russet Burbank was the most commonly grown comprising 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 based on acreage, Russet Norkotah, at 11 %, classified as very susceptible, and Ranger Russet at 4 %, classified as moderately resistant (Novy, et al. 2003). This represents a general lack of resistance to Verticillium wilt among russet-skinned cultivars (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, economic losses can 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 (Pasche et al. 2013). Quantification results from this QPCR assay correlated very well to traditional plating assays for pathogen quantification, and to wilt across eight russet-skinned potato cultivars grown under greenhouse conditions. Additionally, this assay can be performed in a fraction of the time required for traditional plating assays.

One objective of this research was to further validate the recently developed QPCR assay (Pasche et al. 2012) for the

quantification of *V. dahliae* in potato stem tissue grown under field conditions. Another objective was to separate true resistance from tolerance by evaluating pathogen colonization, symptom development and yield, for russet-skinned potato cultivars with varying levels of purported resistance to Verticillium wilt. 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 to quantify the host:pathogen interaction.

Materials and Methods

Cultivar Selection

Eight russet-skinned potato cultivars were selected for evaluation of colonization by *V. dahliae* (Table 1). Among these were very susceptible or susceptible control cv. Russet Norkotah and moderately resistant or resistant control cv. Ranger Russet, included for comparison purposes. Cultivar Russet Norkotah is known to be heavily colonized by *V. dahliae* under field conditions and commonly is used as a susceptible control (Bae et al. 2007; Frost et al. 2007; Hoyos et al. 1991; Jansky 2009). Cultivar Ranger Russet has been demonstrated to be resistant, although not completely, to colonization by *V. dahliae* 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 (AOND95249-1 Russ) also have been evaluated previously for colonization and were classified as susceptible to resistant, and very resistant 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 to very resistant, but stem colonization had not been evaluated. Previous claims of resistance were based solely on the presence or absence of wilt symptoms in cultivars Umatilla Russet (moderately susceptible/moderately resistant) (Mosley et al. 1999), Bannock Russet (very resistant) (Novy et al. 2002), Alturas (very resistant) (Novy et al. 2003) and Premier Russet (moderately resistant) (Novy et al. 2008) (Table 1).

Field Trial

A combination of four *V. dahliae* isolates from potato tissue produced in Minnesota was used to inoculate potato plants in field trials (Pasche et al. 2012). Two of these isolates were kindly provided by Dr. Jim Bradeen, Department of Plant

Table 1 Total yield, evaluated in 2009 and 2010, and potato tuber vacular discloration incidence and severity, evaluated in 2010, across eight russet-skinned potato cultivars with varying levels of reported susceptibility to Vertcillium wilt. Evaluations were performed on tubers grown in field soils infested at low and high *Verticillium dahliae* levels

Cultivar	Reported susceptibility	Total yield (mt/ha)	Tuber vascular discoloration[f]		
			Mild	Severe	Total
Russet Norkotah	very susceptible[a]	9.8 bc	31.7 a	4.1 ab	35.9 a
Ranger Russet	resistant[a]	10.1 abc	7.4 cde	1.2 cd	8.6 cd
Russet Burbank	moderately resistant/moderately susceptible[a]	9.9 bc	19.4 b	6.1 a	25.5 b
Umatilla Russet	moderately resistant/moderately susceptible[b]	11.0 a	11.8 c	2.0 bcd	13.8 c
Dakota Trailblazer	very resistant[a]	9.8 bc	4.3 e	1.4 cd	5.7 d
Bannock Russet	very resistant[c]	9.3 c	5.7 de	0.2 d	5.9 d
Alturas	very resistant[d]	10.4 ab	10.3 cd	3.1 bc	13.4 c
Premier Russet	moderately resistant[e]	9.3 c	6.6 cde	1.6 cd	8.2 cd
P value		<.0001	0.0002	<.0001	0.0023

Values within columns with the same letter are not statistically different based on Fisher's protected least significant difference (α=0.05)

[a] Jansky 2009

[b] Mosley et al. 2000

[c] Novy et al. 2002

[d] Novy et al. 2003

[e] Novy et al. 2008

[f] Mild tuber vascular discoloration: light discoloration not continuouse around the vascular ring. Severe tuber vascular discoloration: darker in color and continuous around the vascular ring, or nearly so

Pathology, University of Minnesota. The remaining two originated from potato tissue grown in central Minnesota, and were isolated and stored as described previously (Pasche et al. 2012). 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. Soil samples were sent to Pest Pros, Plainfield, WI for analysis of *V. dahliae* levels. Low infestation consisted of resident soil *V. dahliae* populations of 6.0 Verticillium propagules 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, to be used as additional inoculum incorporated during hilling operations. 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 during hilling operations 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). Symptom development was evaluated on a percentage wilt severity basis for each treatment/replicate combination in 2009 and 2010 and total yield was obtained at the end of each growing season. In 2010, tubers were evaluated for stem end incidence of mild, severe, and total vascular discoloration from 9 to 18 days post-harvest. Tubers were cut along the short axis just below the stolon attachment. 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 replicate.

Stem Colonization Analysis

V. dahliae colonization was quantified in potato stem samples using traditional culture plating assays, and by QPCR. Fresh stem sections from the basal region of all true stems (originating from the seed-tuber) of five plants in each cultivar/replicate were collected at 82 and 104 DAP in 2009 and 84 and 104 DAP in 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 (Pasche et al. 2012). At harvest, 139 and 132 DAP 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 (Pasche et al. 2012).

A duplex QPCR assay was 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). Pathogen quantification in potato stem tissue using the QPCR assay was compared to traditional plating methods described above (Pasche et al. 2012). Stem tissue used in PCR quantification was obtained directly adjacent to, and simultaneously with, that used in traditional culture 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 (Pasche et al.

2012). 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 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, cube-root transformed stem colonization, tuber yield and vascular discoloration were differentiated using Fisher's protected least significant difference test (α=0.05). Relationships among all parameters 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), or 110 (P=0.9981) DAP. However, a significant interaction was observed for cube root transformed CFU/g derived from plating assays at 82/84 (P=0.0312) and 104 (P=0.0064) DAP, as well as for *V. dahliae* mg/g of stem tissue (P=0.0135) derived from QPCR analyses of fresh stem tissue collected 104 DAP. The interaction between cultivar and infestation level derived from culture plating quantification on the first sampling date at 82/84 DAP was, in great part, due to a slight decrease in stem colonization from low to high soil infestation levels in cv. Bannock Russet, compared to an increase in all other cultivars. To demonstrate this, when the ANOVA was performed without cv. Bannock Russet, the interaction between main effects was no longer present (P=0.0866). At 104 DAP the situation was different. Stem tissue colonization increased across all cultivars between soil infestation levels, but differences in colonization for Russet Norkotah, the susceptible control cultivar, were greater than that observed in other cultivars. Therefore, when cv. Russet Norkotah was excluded from the analysis, no interactions between cultivar and infestation level were observed among the other seven cultivars for plating (P=0.3354) or QPCR (P=0.0753) assays at 104 DAP. No significant differences in colonization were observed between high and low soil infestation levels of *V. dahliae* for any cultivar with some level of resistance. No significant interaction of these main effects was observed in stems collected at 82/84 DAP and evaluated for *V. dahliae* colonization with QPCR (P=0.0886). Additionally, no significant interactions were observed among main effects of *V. dahliae* stem colonization collected at harvest and evaluated using traditional culture plating (P=0.2833) and QPCR (P=0.8754) assays, total yield (P=0.4521), and for mild (P=0.2910), severe (P=0.1253), or total (P=0.6296) vascular discoloration in tubers. 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 soil infestation level for each parameter.

Development of Verticillium Wilt Symptoms

Significant differences in wilt severity among cultivars were observed at all four data collections dates (Fig. 1). Wilt severity at the first data collection date, 83 DAP, was less than 10 % in all cultivars. Highly susceptible control cv. Russet Norkotah displayed a significantly higher level of wilt compared to cvs. Umatilla Russet, Bannock Russet, Alturas, Dakota Trailblazer, 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 substantially, especially 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, wilt in 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 was again 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.

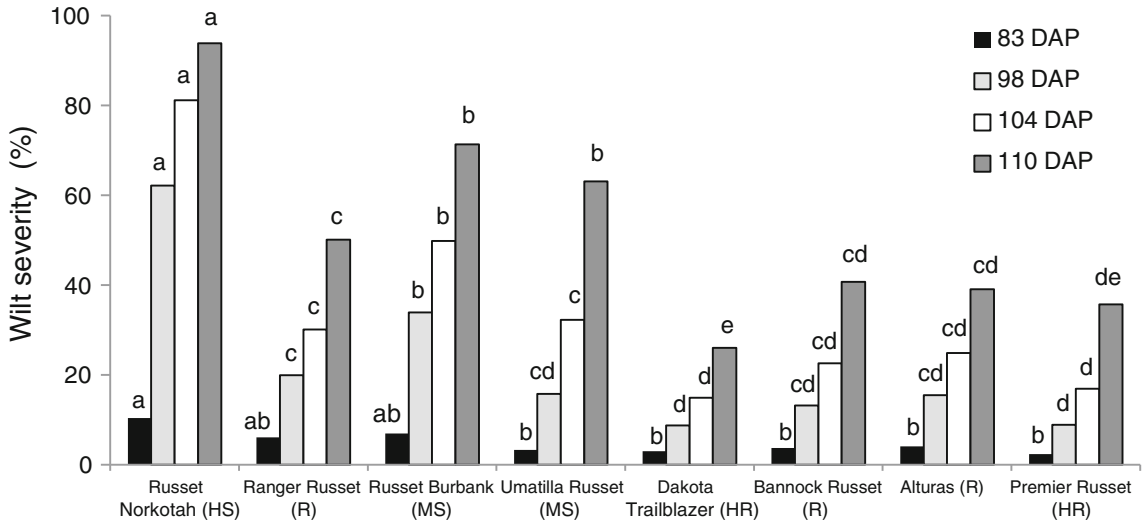

Fig. 1 Percentage Verticillium wilt severity evaluated at 83, 98, 104 and 110 days after planting (DAP) in 2009 and 2010. Evaluations made across eight russet-skinned potato cultivars reported as highly susceptible (HS), moderately susceptible (MS), resistant (R), or highly resistant (HR) to *V. dahliae* 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)

As observed previously, cvs. Dakota Trailblazer and Premier Russet were the least wilted among the cultivars evaluated, although not significantly so in every instance.

Quantification of *V. dahliae* in Fresh Stem Tissue

Significant differences among cultivars were observed in colonization levels in stem tissue collected 82/84 and104 DAP in 2009 and 2010 for both the traditional culture plating assays (P<0.0001; P<0.0001), as well as QPCR assays (P<0.0001; P<0.0001) (Fig. 2). At the first sampling date, results from culture plating assays indicated that susceptible control cv. Russet Norkotah and moderately susceptible cv. Russet Burbank were colonized at significantly higher levels than were all other cultivars (Fig. 2a). Moderately susceptible cv. Umatilla Russet was significantly less colonized than these two, but more so than the five other cultivars. Cultivars Alturas and Ranger Russet displayed intermediate levels of colonization, not significantly different from each other. While colonization in Alturas was significantly higher than for Dakota Trailblazer, colonization in cv. Ranger Russet was not. Cultivars Dakota Trailblazer, Bannock Russet and Premier Russet were least colonized, and no significant difference was observed among these three. Results from QPCR assays were similar to plating assays; however, colonization of cv. Russet Norkotah was determined to be significantly higher than all other cultivars (Fig. 2b). Moderately susceptible cvs. Russet Burbank and Umatilla Russet were significantly more colonized than all other cultivars except cv. Russet Norkotah. Again, cvs. Dakota Trailblazer, Bannock Russet and Premier Russet were least colonized. Significant differences were observed between infestation levels with both plating (P<0.0001) and QPCR(P=0.0012) assays 82/84 DAP (data not shown). At 104 DAP, susceptible control cv. Russet Norkotah had significantly higher levels of colonization than any other cultivar, when evaluated with either assay (Fig. 2a and b). 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. Results from plating assays indicated that colonization of cv. Alturas was not significantly lower than cvs. Russet Burbank and Umatilla Russet, but also was not significantly higher than cvs. Ranger Russet or Bannock Russet. The QPCR assay resulted in no significant differences among cvs. Ranger Russet, Bannock Russet, Alturas, Dakota Trailblazer and Premier Russet. However, results from both assays indicate that cvs. Dakota Trailblazer and Premier Russet are colonized at numerically lower levels than all other cultivars at this point in the growing season. Neither plating assays (P=0.2244), or QPCR (P=0.0706) assays detected a significant difference between infestation levels in these two cultivars at 104 DAP (data not shown).

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. 2). 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

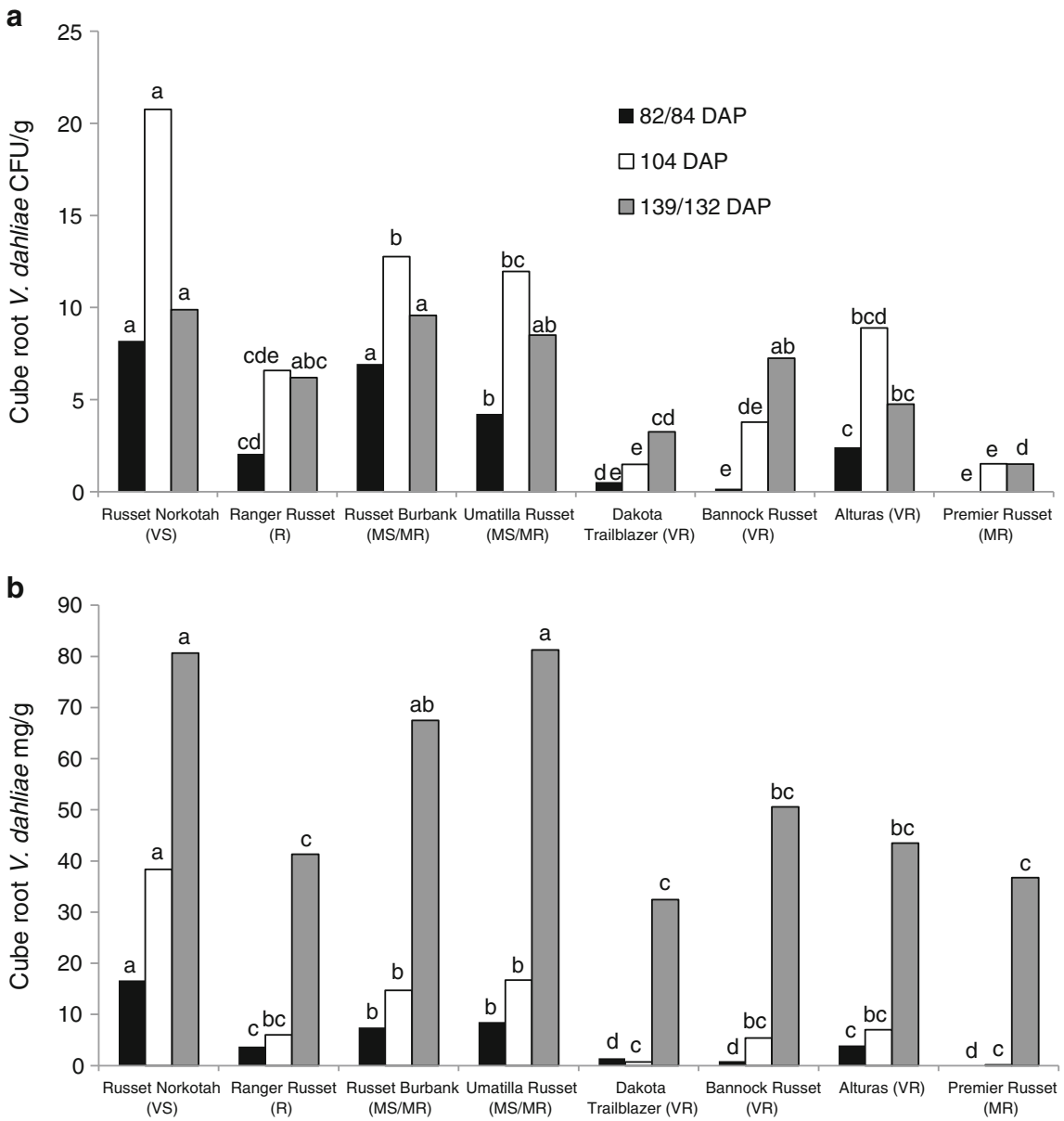

Fig. 2 Cube root transformed colony forming units of *Verticillium dahliae* per gram of potato stem tissue (*V. dahliae* CFU/g) generated using the traditional plating assay (**a**) and mg *V. dahliae* per gram of potato stem tissue (*V. dahliae* mg/g) generated using the duplex QPCR assay (**b**). Stem tissue was collected 82/84, 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 highly susceptible (HS), moderately susceptible (MS), resistant (R), or highly resistant (HR) to *V. dahliae*. *Bars* with the same letter are not statistically different based on Fisher's protected least significant difference (α=0.05)

among cvs. Russet Norkotah, Russet Burbank and Umatilla Russet. As determined by traditional plating assays, cvs. Bannock Russet and Ranger Russet also were not significantly less colonized than cv. Russet Norkotah (Fig. 2a). Cultivars Dakota Trailblazer and Premier Russet were colonized at the lowest levels, significantly less than all other cultivars in QPCR assays. However, no significant difference was observed between cvs. Dakota Trailblazer and Alturas in plating assays. Here, significant differences were observed between low and high soil infestation levels as determined by both plating (P=0.0448) and QPCR (P<0.0001) assays (data not shown).

Colonization of stem tissue increased from 82/84 DAP to 104 DAP in all cultivars, except Dakota Trailblazer, where the decrease was very small, and only when this cultivar was evaluated with QPCR (Fig. 2a and b). However, 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 (Fig. 2a). An increase in *V.*

dahliae colonization 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 (Fig. 2b). This increase is approximately proportional for all cultivars except susceptible control cultivar Russet Norkotah, in which colonization increased, but to a much lesser proportion 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 (Table 2). 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).

Table 2 Relationship between colonization of potato stems grown in field soils with low or high levels of *Verticillium dahliae* quantified using traditional plating assays and the duplex VTP1 QPCR assay, Verticllium wilt severity, total yield and tuber vascular discoloration determined by Pearson's correlation coefficient

Comparison parameters[a]	QPCR quantification		
	82/84 DAP	104 DAP	139/132 DAP
Traditional plating quantification			
82/84 DAP	r=0.92**[b]		
104 DAP		r=0.96**	
139/132 DAP			r=0.85**
Verticillium wilt severity			
82/84 DAP	r=0.83**		
104 DAP		r=0.94**	
139/132 DAP			r=0.88**
Total yield (mt/ha)	r=0.28^{ns}	r=0.21^{ns}	r=0.35^{ns}
Incidence of tuber vascular discoloration	r=0.91**	r=0.91**	r=0.75**

[a] Stem tissue was collected and processed fresh for traditional plating and QPCR assays at 82/84 and 104 days after planting (DAP) in 2009/2010. Stem tissue was collected and processed dried at 139/132 DAP

[b] Pearson correlation coefficients were significant (**) or not (ns) at the α=0.05 level, n=16

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 (Table 1). 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. 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=0.3012), severe (P=0.8569) or total (P=0.3902) tuber vascular discoloration.

Very strong and significant relationships existed in colonization between traditional plating and QPCR assays at all sampling dates as determined by Pearson's correlation (Table 2). Additionally, at all sampling dates, colonization as determined by QPCR, was closely related to wilt on the same date. Total tuber vascular discoloration also was closely related to colonization at all three sampling dates. However, total yield was not related to stem colonization as determined by QPCR at any date.

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 manage 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).

Traditional culture plating methods are accurate and reliable in quantifying pathogens such as *V. dahliae* in potato stems (Bae et al. 2008; Jansky 2009; Jansky and Miller 2010), however, because they are very labor intensive and time consuming, 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, and 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 (Pasche et al. 2012). The present research not only puts forth a rapid and reliable assay for use in screening cultivars and breeding selections for resistance to Verticillium wilt, it precisely quantifies the degree of genetic resistance present 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.

The difficulty in determining the true level of Verticillium wilt resistance in potato cultivars is compounded by the ambiguity in the classification of control cultivars used for comparative purposes (Table 1). For instance, cv. Russet Norkotah typically is included as the most susceptible control, but has been defined as both highly (very) susceptible and susceptible (Bae et al. 2007; Jansky 2009; Lynch et al. 1997; Mosley et al. 2000). Similarly, cv. Ranger Russet has been classified as moderately resistant or resistant (Bae et al. 2007, 2008; Jansky 2009; Novy et al. 2003, 2008) While these examples represent only slight differences in classification, more dramatic differences exist for cv. Russet Burbank, which has been classified as susceptible, moderately susceptible and moderately resistant to Verticillium wilt (Bae et al. 2008; Corsini et al. 1988; Hoyos et al. 1991; Lynch et al. 1997; Mosley et al. 2000; Novy et al. 2002, 2003, 2008). The authors believe the differences in response to *V. dahliae* are likely due to differences in environment under which the studies were conducted. Temperature and moisture can have a substantial impact on wilt development, therefore, these evaluations may differ from one growing season to the next and from region to region (Ben-Yephet and Szmulewich 1985; Cappeart et al. 1992; Nnodu and Harrison 1979; Powelson and Rowe 1993). In the trials reported here, total rainfall was slightly higher, 5.1 cm, in 2009 when compared to 2010. However, because these trials were grown under irrigation, this difference should not have had major implications on the results. Additionally, the average maximum temperature over the course of the growing season was 22.8 and 24.4 °C in 2009 and 2010, respectively. The average minimum temperature was 11.1 and 11.7 °C, respectively. Therefore, overall environmental conditions were very similar and most likely did not affect the results from the first year the field trials were performed to the second. It is apparent that situations exist where control cvs. Russet Norkotah and Russet Burbank both were classified as susceptible, and likewise, cvs. Russet Burbank and Ranger Russet are classified as resistant. These discrepancies can compromise new cultivar evaluations and classifications when used as the sole basis for comparison. The present research indicates that significant differences exist in colonization among these three cultivars, and supports the classification of cv. Russet Norkotah as highly (very) susceptible, cv. Russet Burbank as moderately susceptible and cv. Ranger Russet as resistant. However, as demonstrated by the research presented here, a narrow genetic window is represented when only these three cultivars are used for comparison. Until now, cv. Ranger Russet was considered the resistant standard among russet-skinned cultivars. The current, and other, research indicates that cultivars exist with higher levels of resistance than is present in cv. Ranger Russet, including Dakota Trailblazer and Premier Russet (Jansky 2009). Utilizing one of these cultivars for comparison purposes would allow breeders to more clearly define the classification of new cultivars compared to those currently available and that one of these should be utilized as controls in Verticillium wilt evaluations. Additionally, the use of cv. Russet Norkotah as the susceptible control should be replaced by cv. Russet Burbank. This is justified because cultivars with lower levels of resistance than cv. Russet Burbank do not represent a level of field resistance valuable to producers. Additionally, cv. Russet Norkotah is colonized at significantly greater levels than is observed in other cultivars, therefore, data transformation is necessary in order to meet normalization assumptions of the ANOVA. The use of cv. Russet Burbank would minimize the need for such transformations.

The research reported here further refines the Verticilllium wilt susceptibility classification from those proposed in cultivar releases and other previous research using rapid quantification of host colonization (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) (Table 1). The original cultivar release publications for Alturas, Bannock Russet, Premier Russet and Umatilla Russet were based solely on the development of wilt symptoms observed in the field. Cultivar Alturas originally was determined to be very resistant compared to cvs. Ranger Russet and Russet Burbank, defined as moderately resistant and susceptible, respectively, for the development of Verticillium wilt (Novy et al. 2003). Similarly, cv. Bannock Russet also was defined as very resistant, compared to moderately susceptible cv. Russet Burbank. However, based on stem colonization results from the current research, both cvs. Alturas and Bannock Russet should be classified as resistant, as both cultivars are colonized at a rate similar to resistant cultivar Ranger Russet (Table 1). Cultivar Premier Russet was rated previously 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 highly resistant, including Dakota Trailblazer. Therefore, the use of colonization assays provide a more refined analysis of the levels of resistance and the true nature of the host:parasite interaction.

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 moderately susceptible, 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, or lower, levels of resistance to *V. dahliae* as cv. Ranger Russet. These findings are paramount to the industry, redefining and precisely quantifying the host:pathogen interaction in currently available cultivars, at a time when management options are limited.

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, 1990; Davis et al. 1983; Hoyos et al. 1993; 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. Bannock Russet, Premier Russet and Alturas does reveal some interesting possibilities. For example, Bannock Russet and Alturas have the same male parent (A75188-3) and Premier Russet has Bannock Russet as a grandparent, and therefore A75188-3 as a great grandparent (R. Novy personal communication). 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 wilt resistant cultivars.

Attempts to evaluate trials conducted under field conditions for the development of symptoms of one disease often are confounded by infection of one or more non-target pathogens. The trials performed here are no exception to that, however, trends in Verticilium wilt severity among cultivars were consistent across all four dates, even at the earliest wilt evaluation date when severity was less than 10 %. 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 and cannot reliably distinguish cultivars that are resistant or highly resistant. Evaluating the absence of wilt symptoms 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, when using culture plating techniques (Jansky and Rouse 2003).

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 yield potential, and Verticillium wilt is but one factor that can affect tuber yield. While tuber vascular discoloration data only was collected in 1 year of the trial, results were very compelling. No data exist 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 has the potential to provide the industry with valuable information regarding tuber quality in Verticillium wilt resistant cultivars.

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 Russet 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 difference was 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 be stable under higher inoculum 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; 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; 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. The use of QPCR to quantify the host:pathogen interaction will provide a useful tool in more precisely defining the response of potato germplasm and cultivars to *V. dahliae*.

Acknowledgements We are grateful to Dean Peterson, Russell Benz and Roberta Sherman for technical assistance. We also acknowledge Curt Doetkott for assistance with statistical analyses. Funding for these studies was provided in part by the USDA-ARS-SCA (No. 58-3655-0-613) and the Minnesota Area II and Northern Plains Potato Growers Associations.

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