Colonization Dynamics and Spatial Progression of *Verticillium dahliae* in Individual Stems of Two Potato Cultivars with Differing Responses to Potato Early Dying

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**ABSTRACT**


Potato early dying (PED), caused by *Verticillium dahliae*, is a chronic yield-limiting disease of potato (*Solanum tuberosum*). In this study, we describe the colonization dynamics of *V. dahliae* in two potato cultivars with varying responses to PED. We utilized a quantitative real-time polymerase chain reaction (Q-PCR) assay to assess the colonization and spatial progression of *V. dahliae* in cvs. Ranger Russet (moderately resistant) and Russet Norkotah (highly susceptible). Ninety plants per cultivar were inoculated with a conidial suspension in the greenhouse. Every 2 weeks until week 10, we collected basal samples from 15 plants, and repeatedly sampled the growing apices of another 15 plants. The mean infection coefficient (IC) values in the basal and apical samples were significantly lower in cv. Ranger Russet at all five sampling dates. The pathogen was detected in basal samples of both cultivars by week 2, and in apical samples of cv. Russet Norkotah at week 4 and of cv. Ranger Russet at week 6. Colonization of cv. Russet Norkotah consistently increased in apical and basal samples during the 10 weeks, while it plateaued after week 6 in cv. Ranger Russet. Differences in response to PED appear associated with the speed of colonization and the establishment of a higher population density by *V. dahliae* in the plant.

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nadia/ml suspension of *V. dahliae* isolate V18 (race 4A). This isolate is highly aggressive on potato and was isolated from a severely affected potato plant in Wisconsin. Additionally, 10 plants of each cultivar served as controls and were treated similarly to the inoculated plants, except that the roots were dipped in sterile deionized water rather than a suspension of *V. dahliae* conidia. Furthermore, five additional plants of each cultivar were inoculated with *V. dahliae* isolate V18 and treated similarly, but were raised to maturity, along with noninoculated checks, to confirm expression of typical PED symptoms. Inoculations were performed on 7 March and 3 May for trial 1 and trial 2, respectively.

After dipping in the conidial suspension or water, all plants were transferred to 15-cm pots filled with the potting mix described above. All 200 plants in each of the two trials were fertilized at the time of transfer to the commercial potting mix using 20-20-20 commercial fertilizer (J. R. Peters, Allentown, PA). Growing conditions were 25 ± 2°C during the day and 18 ± 2°C at night, with a 16-h photoperiod and water as needed. Plants were grown in 15-cm plastic pots, which were placed in plastic flats holding 12 plants of one cultivar each. The position of individual flats was arbitrarily assigned in the greenhouse. The two trials were conducted using the same seed and following the same procedures described above.

**Sample collection.** Fifteen plants of each cultivar were destructively sampled biweekly during the 10 weeks of the trial, starting 2 weeks after inoculation (total 75 inoculated plants). Samples were collected at weeks 2 and 10 after inoculation, thus providing 30 basal stem cuttings (15 per cultivar) every 2 weeks. Samples 5 cm long were collected from the base of the stem and cut into small pieces (0.2 cm) divided into two groups. All basal stem cuttings were surface-disinfested in 20% bleach for 5 min before processing. One group was squeezed to release 150 µl of sap, which was used for the CFU quantification assay, by spreading onto petri dishes filled with NPX semi-selective medium. Petri dishes were incubated at room temperature in the dark for 2 weeks. The second group was ground to a powder in liquid nitrogen, using a mortar and pestle, and two 50-µg aliquots were used for DNA extraction.

Apical cuttings from the remaining 15 inoculated plants of each cultivar were taken every 2 weeks starting 2 weeks after inoculation on the same dates the basal stems were sampled. A 1-cm-long portion (~100 mg) of the growing tip of each plant was excised with a sterile blade. The axillary bud just below the apex was then left to grow and the sampling was repeated after 2 weeks. Fifteen apical samples were collected from each cultivar every 2 weeks, and DNA extractions were performed on two aliquots of each apical sample, as described above. Only Q-PCR quantification was performed on apical samples because of insufficient plant material to conduct the plating assay described above. Compared with nonsampled growing plants of the same age, the latter plants were about 20% smaller and more branched, because of the removal of dominant apical meristem.

**CFU quantification.** Two 100-µl aliquots of squeezed sap were spread onto one petri plate filled with NPX medium. Colonies were counted using a dissecting microscope after a 2-week incubation in the dark at room temperature.

**DNA extraction.** All DNA samples were extracted using the FastDNA kit (MP Biomedicals, Irvine, CA) following the manufacturer’s recommendations. Subsequently, DNA extracts were subjected to a cleanup using AMPure magnetic beads to eliminate remaining potential PCR-inhibitors. Total DNA amounts were estimated by gel electrophoresis (1% agarose), by comparison to a standardized quantification DNA ladder (High DNA Mass ladder, Invitrogen, Carlsbad, CA). Samples were stored at 4°C until used.

**Q-PCR amplification.** Q-PCR amplifications were conducted in triplicate, in a Bio-Rad iCycler qPCR real-time PCR system using the iQ Supermix SYBR-Green (Bio-Rad) with ~1 ng of DNA and 200 nM of each primer in 20 µl total volume. The following amplification protocol was used: initial denaturation of 3 min at 95°C, then 40 cycles of 95°C for 10 s and 63°C for 35 s. Melt curve analysis was used to distinguish potential primer dimers and nonspecific amplification products. *V. dahliae* DNA was amplified using the previously described primers VertBt-F (AAC AAC AGT CCG GAT GAT TC) and VertBt-R (TGA CCG GGC TCG AGA TCG) (2). The absence of PCR inhibitors in DNA samples was assessed by spiking each DNA sample with 10 pg of *Phytophthora infestans* DNA (isolate US-8 940480) and computing the ΔCt (ΔCt = Ct sample – Ctcontrol) (9). In all amplifications, DNA from pure cultures of each *V. dahliae* was used as the positive control and a no-template water sample as the negative control. The infection coefficient (IC), which is the ratio of Cхаst/Сpathogen (28), was computed for each sample tested. Potato DNA amounts were quantified using previously described primers PotAct-F (TGA ACA CCG AAT TCG CAG CA) and PotAct-R (GGG GGT TGT AAG GGC GTC AG) (3).

**Data analysis.** The IC and CFU data generated from the quantification of *V. dahliae* in basal samples were analyzed in a two-way ANOVA, using Proc GLM in SAS (SAS Institute, Cary, NC). The impact of the date of sampling (weeks after inoculation), cultivar, and the interaction of these two variables were tested. Least square means separation was performed on the significant cultivar*×*week interactions. No significant differences were observed between the two trials.

Data from the quantification of *V. dahliae* in apical samples were analyzed in a repeated time measures framework, using Proc Mixed in SAS (17). One of the essential criteria for the ANOVA is the independence of variables, which in this situation was not met, as the same plants were sampled every 2 weeks for 10 weeks. Hence, we opted to use a mixed linear model approach, where sampling dates were random factors and cultivars were fixed factors. Data analysis was performed under three different covariance structure models: compound symmetry, unstructured, and autoregressive 1. The model that produced the lowest Akaike information criterion (AIC) value is considered the model with the best fit. Least squares mean separations were performed in the model with the best fit. No significant differences were observed between the two trials.

**RESULTS**

*V. dahliae* was detected in stem bases of inoculated plants 2 weeks after inoculation (Fig. 1A), and in the apices after 4 weeks (Fig. 2). Conversely, no evidence of the presence of the fungus was found in noninoculated control plants, throughout the 10 weeks of each experimental trial. As early as 2 weeks after inoculation, cv. Russet Norkotah showed a significantly higher incidence of basal infection with *V. dahliae* (93 and 86.7% in trials 1 and 2, respectively) compared with cv. Ranger Russet (0 and 26.7% in trials 1 and 2, respectively). Carbohydrate symptoms were not observed on cv. Ranger Russet throughout the study, while symptoms were observed on cv. Russet Norkotah plants as early as week 4. These symptoms included wilting and chlorosis of foliage, in addition to a marked reduction in canopy growth compared with controls. Subsequently, inoculated plants of cv. Russet Norkotah died 2 to 3 weeks earlier than uninoculated controls.

Two weeks after inoculation, the incidence of basal *V. dahliae* infections was significantly higher (*P* = 0.012) in cv. Russet Norkotah compared with cv. Ranger Russet (Fig. 1A). Pathogen incidence in basal samples of cv. Russet Norkotah reached 86.7% 2 weeks after inoculation, and it reached 100% at 4 weeks after inoculation (Fig. 3). Conversely, incidence was 26.7% in cv. Ranger Russet at week 2, and gradually increased to above 90% after week 8 (Fig. 3).

Colonization of basal areas of cvs. Russet Norkotah and Ranger Russet differed significantly over time, with cv. Russet Norkotah exhibiting higher IC and CFU values across all 10 weeks (Fig. 1A). At week 2, the mean IC for cv. Russet Norko-
V. dahliae was capable of infecting and colonizing both cultivars. Russet Norkotah plants consistently exhibited higher colonization rates of both basal and apical regions compared with cv. Ranger Russet (Figs. 1 and 2). PED is particularly severe on cv. Russet Norkotah, leading to a premature senescence of potato vines, as well
as a significant reduction in yield and number of U.S. No. 1 tubers (10,26). In comparison, cv. Ranger Russet is described as moderately resistant to the disease (26) with adequate tolerance to moisture stress and limited pathogen colonization (1). We found that *V. dahliae* colonized the basal areas of both cultivars as early as 2 weeks after inoculation. But, while cv. Russet Norkotah reached a mean IC close to 0.60, the mean IC for cv. Ranger Russet was significantly lower at less than 0.20 (Fig. 1A). During the 10 weeks of the study, IC values in cv. Russet Norkotah increased to reach almost 1.0, where the DNA amount of the pathogen and host were even. Conversely, IC values for cv. Ranger Russet increased after week 4, but reached a plateau between weeks 6 and 10, with the highest mean IC reaching 0.60 (Fig. 1A). At every sampling date, mean IC values were significantly different from each other. Such a finding is in agreement with the described response of each cultivar to potato early dying. Additionally, we found high positive correlations between the date of sampling and IC values, with coefficient correlations in excess of 95%

Davis et al. (7) found a comparable correlation (R² = 87.6%) between CFU counts and the date of sampling in naturally infested plots, starting 2 months after planting. In this study, we sampled inoculated plants starting 25 days after planting (2 weeks after inoculation), before any disease symptoms are normally apparent.

*V. dahliae* was detected in the apical portions of cv. Russet Norkotah at week 4, where the mean IC value exceeded 0.60, while the fungus was detected in cv. Ranger Russet at week 6 and the mean IC value exceeded 0.60 (Fig. 2). The colonization of apices increased with time in cv. Russet Norkotah where the mean IC value reached 0.97. Significant differences were observed among most sampling dates in cv. Russet Norkotah, with the exception of weeks 6 and 8, after which IC values increased significantly again (Table 1). In cv. Ranger Russet, colonization ceased to increase significantly after the detection of the pathogen in week 6 (Table 1 and Fig. 2). The significant decrease in IC values between weeks 6 and 8 in cv. Ranger Russet (P = 0.0108) may be attributed to a lag phase in the growth of the inoculum in newly colonized tissues, rather than a decrease in general colonization of the plant. This seems to be substantiated by the lack of significant differences between weeks 6 and 10 (Table 1). The two cultivars were significantly different from each other at all sampling dates except week 2, where no *V. dahliae* DNA was detected. In a previous study (2), we detected 148 fg of *V. dahliae* DNA, which is the equivalent of five nuclei. Such a level of sensitivity indicates an absence of the pathogen in the apical tissues sampled in week 2 in both cultivars and week 4 in cv. Ranger Russet, rather than a failure to detect the pathogen.

A previous study (8) showed that no less than 525 CFU/g of tissue were needed for a significant correlation with disease incidence. In this study, we found that IC values around 0.80 in the basal samples, and above 0.60 in apical samples, were correlated with symptom appearance in cv. Russet Norkotah. Meanwhile, in cv. Ranger Russet, IC values never exceeded 0.60 in basal samples, and 0.70 in apical samples, and no symptoms of early dying could be observed. Additionally, mean CFU counts never exceeded 100 CFU/g in basal samples of cv. Ranger Russet, while they were more than 700 CFU/g in cv. Russet Norkotah (Fig. 1B).

Over the five sampling dates, we found a 97% correlation between basal and apical IC values in cv. Russet Norkotah, and 85% in cv. Ranger Russet. These high correlations indicate that the colonization of plant tissues in cv. Ranger Russet goes largely unhampered, while symptom expression does not occur as readily. Nevertheless, the mechanisms leading to the differential expression of early dying symptoms remain unclear, but are perhaps associated with the speed of colonization of apical tissues after the basal areas, and the level of colonization attained. Moreover, *V. dahliae* incidence in cv. Russet Norkotah was higher than 85% at week 2, compared with 25% in cv. Ranger Russet (Fig. 3). Incidence reached 100% in cv. Russet Norkotah by week 4, while it only reached 85% by week 10 in cv. Ranger Russet. Hence, it is probable that cv. Russet Norkotah is more susceptible than cv. Ranger Russet because of how fast *V. dahliae* colonizes and establishes a higher population density in potato plants. From the data presented here, we suggest that breeders examine plant material 6 weeks after inoculation with *V. dahliae* conidia (67 days after planting), when the apices become colonized.

In this study, we investigated, using real-time quantitative PCR, the colonization

![Figure 3](image)

Fig. 3. Comparison of the fortnightly infection incidence over 10 weeks after inoculation by Verticillium dahliae, between the moderately resistant cv. Ranger Russet (white bars) and the highly susceptible cv. Russet Norkotah (black bars), based on the detection of *V. dahliae* DNA in basal or apical plant tissues using quantitative real-time polymerase chain reaction (Q-PCR). Plantlets of both cultivars were inoculated by root immersion in an 8 \times 10^6 conidia/ml suspension on the same date in the greenhouse.

Table 1. Least square mean comparisons of infection coefficient (IC) values from apical samples between the five sampling dates for each cultivar

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Weeks compared</th>
<th>Estimate**</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Russet Norkotah</td>
<td>2 and 4</td>
<td>–0.30 ***</td>
<td>0.106</td>
</tr>
<tr>
<td></td>
<td>2 and 6</td>
<td>–0.76 ***</td>
<td>0.106</td>
</tr>
<tr>
<td></td>
<td>2 and 8</td>
<td>–0.64 ***</td>
<td>0.112</td>
</tr>
<tr>
<td></td>
<td>2 and 10</td>
<td>–0.33 **</td>
<td>0.103</td>
</tr>
<tr>
<td></td>
<td>4 and 6</td>
<td>–0.46 ***</td>
<td>0.106</td>
</tr>
<tr>
<td></td>
<td>4 and 8</td>
<td>–0.34 **</td>
<td>0.112</td>
</tr>
<tr>
<td></td>
<td>4 and 10</td>
<td>–0.33 ***</td>
<td>0.080</td>
</tr>
<tr>
<td></td>
<td>6 and 8</td>
<td>0.12 **</td>
<td>0.112</td>
</tr>
<tr>
<td></td>
<td>6 and 10</td>
<td>0.43 **</td>
<td>0.103</td>
</tr>
<tr>
<td></td>
<td>8 and 10</td>
<td>0.32 **</td>
<td>0.108</td>
</tr>
<tr>
<td>Ranger Russet</td>
<td>2 and 4</td>
<td>0.00</td>
<td>0.106</td>
</tr>
<tr>
<td></td>
<td>2 and 6</td>
<td>–0.40 **</td>
<td>0.106</td>
</tr>
<tr>
<td></td>
<td>2 and 8</td>
<td>–0.61 ***</td>
<td>0.175</td>
</tr>
<tr>
<td></td>
<td>2 and 10</td>
<td>–0.57 ***</td>
<td>0.103</td>
</tr>
<tr>
<td></td>
<td>4 and 6</td>
<td>–0.40 ***</td>
<td>0.106</td>
</tr>
<tr>
<td></td>
<td>4 and 8</td>
<td>–0.61 ***</td>
<td>0.175</td>
</tr>
<tr>
<td></td>
<td>4 and 10</td>
<td>–0.57 ***</td>
<td>0.103</td>
</tr>
<tr>
<td></td>
<td>6 and 8</td>
<td>0.28 *</td>
<td>0.106</td>
</tr>
<tr>
<td></td>
<td>6 and 10</td>
<td>–0.17</td>
<td>0.103</td>
</tr>
<tr>
<td></td>
<td>8 and 10</td>
<td>–0.45 ***</td>
<td>0.103</td>
</tr>
</tbody>
</table>

**Negative values indicate an increase in mean IC values, and positive values indicate a decrease in mean IC values (* significant at α = 0.05, ** significant at α = 0.01, *** significant at α = 0.001).**
and spatial progression by *V. dahliae* of two potato cultivars with varying responses to PED. The pathogen was detected in basal samples of cvs. Russet Norkotah (highly susceptible) and Ranger Russet (moderately resistant) 2 weeks after inoculation of plantlets in the greenhouse with conidia of *V. dahliae* isolate V18. Four weeks after inoculation, *V. dahliae* was detected in apical samples of cv. Russet Norkotah, while it was not detected in cv. Ranger Russet until week 6. Colonization of cv. Russet Norkotah consistently increased in both apical and basal samples during the 10 weeks of the experiment, while it reached a plateau after week 6 in cv. Ranger Russet. This study is an attempt at understanding the population dynamics of *V. dahliae* in potato cultivars to explain the process of resistance to PED. This research provides breeders with much needed clarifications regarding the nature of resistance to *V. dahliae* in cvs. Ranger Russet and Russet Norkotah and describes a new time-saving approach to utilize in the selection of breeding lines with enhanced resistance to PED.

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**LITERATURE CITED**
