Effect of Co-inoculations of *Pectobacterium* spp. and *Verticillium dahliae* in Early Dying and Aerial Stem Rot of Potato

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Potato early dying (PED) is a disease complex found in many potato-producing regions. Fields affected by PED exhibit wilting, chlorosis, necrosis, and premature vine death. Several pathogens can be associated with the potato early dying complex including fungi, bacteria, and nematodes (6,7). *Verticillium dahliae*, the causal agent of Verticillium wilt of potato, is considered to be the primary pathogen involved in the early dying complex of potato in most locations where potato is grown (9). Bacteria in the genus *Pectobacterium*, formerly known as *Erwinia*, cause several diseases of potato including tuber soft rot, aerial stem rot, and early dying symptoms similar to those caused by *V. dahliae* (3,7,9).

When multiple pathogens infect potato, interactions may occur resulting in additive or synergistic (greater than additive) increases in disease incidence and severity or decreases in yield (2). Interactions among pathogens can also lead to an earlier onset of disease or reduced inoculum thresholds required for disease development (9). Individually, *V. dahliae* and *Pectobacterium* spp. can have significant detrimental effects on potato production, but it is not entirely clear if additive or synergistic interactions occur when both are present on potato (3,4,8). A preliminary survey was carried out during the 2008 growing season to determine the co-occurrence of pectolytic bacteria and *V. dahliae* in fields located in the Columbia Basin of Washington State. The survey was conducted in fields exhibiting severe early dying and aerial stem rot symptoms. Pectolytic bacteria were isolated from 86% of *V. dahliae*-infected Russet Burbank plants (*unpublished data*), suggesting a high potential for interactions between these two pathogens in commercial potato fields.

The objective of this study was to determine if additive or synergistic interactions exist between *V. dahliae* and *Pectobacterium* spp. in the development of early dying or aerial stem rot of potato. Two isolates of *Pectobacterium* were tested: *P. carotovorum* subsp. *carotovorum* isolate Ec101, which causes early dying of potato and *P. wasabiae* isolate PwO405, which causes aerial stem rot symptoms. A non-invasive procedure was used to inoculate *Pectobacterium* to aerial stems which attempted to mimic artificial wounding caused by insects, windblown sand, or wind damage. Potato early dying and aerial stem rot severity were measured in greenhouse assays to determine if additive or synergistic (greater than additive) increases in disease severity occurred following co-inoculations with *V. dahliae* and *Pectobacterium* spp. compared to inoculations with either pathogen alone. In addition, pathogen DNA levels were measured using quantitative real-time quantitative PCR (q-RTPCR) in order to determine if co-inoculations with *V. dahliae* and *Pectobacterium* spp. resulted in increased pathogen colonization in potato stems.

MATERIALS AND METHODS

The interaction between *V. dahliae* and *Pectobacterium* spp. in the development and severity of early dying symptoms and aerial stem rot of potato was investigated in replicated greenhouse trials. Potato plants (cv. 'Russet Burbank') were inoculated by applying a soil drench of *V. dahliae* isolate 653 (VCG 4A from potato) or a water control. Approximately four weeks after *V. dahliae* inoculations, plants were wound-inoculated (10^4 CFU) using *P. carotovorum* subsp. *carotovorum* isolate Ec101, *P. wasabiae* isolate PwO405, or a sterile water control. Plants were exposed to a 24 to 48 hr period of leaf wetness and lesions were measured to assess aerial stem rot severity. Wilt incidence, total chlorosis, and total necrosis were recorded at one, two- and three weeks post-bacterial inoculation to assess PED severity. Chlorosis and necrosis were converted to areas under chlorosis progress curves (AUCPC) and areas under necrosis progress curves (AUNPC), respectively. Chlorosis and necrosis data were also combined into areas under senescence progress curves (AUSPC). Both *Pectobacterium* isolates were also tested for the ability to cause tuber soft rot by inoculating 5×10^6 CFU onto 'Russet Burbank' potato slices. All data were analyzed using analysis of variance (ANOVA).

Verticillium dahliae and *Pectobacterium* spp. were quantified in plant tissue using a SYBR-Green (BioRad, Hercules, CA) q-RTPCR protocol which detects the amplification of target DNA during each PCR cycle using a fluorescent dye which binds to double-stranded DNA. The amount of fluorescence in a sample is compared to a standard curve comprised of known amounts of target DNA, and the starting amount of target DNA in the sample is determined based on the cycle threshold (Ct), which is the amplification cycle at which fluorescence is detected above the threshold of normal background fluorescence.

Plants from the greenhouse assays described above were sampled for q-RTPCR analysis. Plant tissue samples were taken from the site of bacterial inoculation as well as from basal (below) and apical (above) portions of the plant to quantify the movement of the pathogens in aboveground stems. Amplifications were conducted essentially as described by Atallah and Stevenson (1). Detection limits ranged between 5^{-4} to 5 ng of *Pectobacterium* DNA and 1.5^{-4} to 15 ng of *V. dahliae* DNA, or roughly 9 to 900,000 *Pectobacterium* cells and 5 to 500,000 *V. dahliae* nuclei. The amount of potato DNA in each sample was also quantified to obtain a relative amount of pathogen DNA to host DNA and calculate infection coefficients (IC) for each pathogen ($Ct_{host}/Ct_{pathogen}$). Melt curve analysis was performed to distinguish nonspecific amplification products by differences in melting temperature. Data were analyzed using ANOVA.

RESULTS AND DISCUSSION

Significant interactions were not detected between *V. dahliae* and *Pectobacterium* spp. on potato in greenhouse assays ($P > 0.05$) and disease symptoms were not significantly different among plants inoculated with either *Pectobacterium* isolate alone compared to plants co-infected with *V. dahliae* (Table 1). These results suggest that co-infections of potato by *Pectobacterium* spp. and *V. dahliae* do not result in additive or synergistic increases in disease severity. PED

symptoms caused by Ec101 or *V. dahliae* alone were nearly indistinguishable (Fig. 1). The similarity in early dying symptoms caused by *Pectobacterium carotovorum* subsp. *carotovorum*, a bacterium and *V. dahliae*, a fungus, demonstrates the importance of correct disease diagnosis prior to the application of chemical or other control measures. Isolate PwO405 caused typical aerial stem rot symptoms in the presence and absence of *V. dahliae.* However, both Ec101 and PwO405 were able to cause tuber soft rot despite obvious differences in pathogenicity on stems (Table 2).

Significant interactions were not observed in infection coefficient (IC) values or relative levels of pathogen DNA using q-RTPCR ($P > 0.05$). *Pectobacterium* and *V. dahliae* IC values were also not significantly different in plants infected with one pathogen compared to plants co-infected with both pathogens (Figs. 2 and 3). These results indicate that co-infections by *Pectobacterium* spp. and *V. dahliae* do not result in significant synergistic increases in pathogen populations in potato stems.

These results indicate that co-infections of potato by *Pectobacterium* spp. and *V. dahliae* do not result in additive or synergistic increases in disease severity. Even though *V. dahliae* is often considered to be the primary causal agent of potato early dying, pectolytic bacteria may be responsible for early dying symptoms in fields from which *V. dahliae* cannot be detected or in fields not previously cropped to potato (3,5) and care must be taken when diagnosing the causal agent(s) of PED. In addition, a high potential exists for progeny tubers to be infested by inoculum of *Pectobacterium* originating from decaying plant debris, particularly in fields exhibiting aerial stem rot.

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Table 1. Lesion size (log-transformed), area under chlorosis progress curves (AUCPC), area under necrosis progress curves (AUNPC), area under senescence progress curves (AUSPC), and incidence of wilt in 'Russet Burbank' potato plants inoculated with *Pectobacterium carotovorum* subsp. *carotovorum* isolate Ec101and *P. wasabiae* isolate PwO405 in the presence and absence of *Verticillium dahliae*[z]

***Pectobacterium* treatment**	***V. dahliae* treatment**	**log(lesion)**	**Wilt incidence**	**AUCPC**	**AUNPC**	**AUSPC**
Water control	**Water control**	0.01 a	0.00 a	286 a	129 a	415 a
	Isolate 653	0.00 a	0.91 c	507 b	392 a	900 b
***P. carotovorum* subsp. *carotovorum* isolate Ec101**	**Water control**	0.65 b	0.88 c	509 b	306 a	815 b
	Isolate 653	0.62 b	0.81 c	529 b	433 a	962 b
***P. wasabiae* isolate PwO405**	**Water control**	1.80 c	0.13 ab	157 a	1288 b	1445 c
	Isolate 653	1.72 c	0.28 b	219 a	1206 b	1426 c

[z] Values followed by a different letter indicate significant differences among treatments using Tukey's test ($P = 0.05$).

Fig. 1. Symptoms observed on 'Russet Burbank' potato plants after inoculation with (a) water control representing natural senescence; (b) *Pectobacterium carotovorum* subsp. *carotovorum* isolate Ec101 alone; (c) *P. wasabiae* isolate PwO405 alone; (d) *V. dahliae* isolate 653 alone; (e) isolate Ec101 co-inoculated with isolate 653; and (f) isolate PwO405 co-inoculated with isolate 653.

Table 2. Area under lesion progress curve (AULPC) values and ratios of final to initial mass of 'Russet Burbank' potato slices inoculated with two isolates of *Pectobacterium* (5 x 10^6 CFU) and a noninoculated control[z]

***Pectobacterium* treatment**	**AULPC**	**Final mass/ initial mass**
Noninoculated control	0 a	0.99 a
***P. carotovorum* subsp. *carotovorum* isolate Ec101**	304 b	0.81 b
***P. wasabiae* isolate PwO405**	289 b	0.75 c

[z] Tuber slices were inoculated with 5 x 10^6 CFU and incubated at 82°F under 90 to 100% relative humidity.

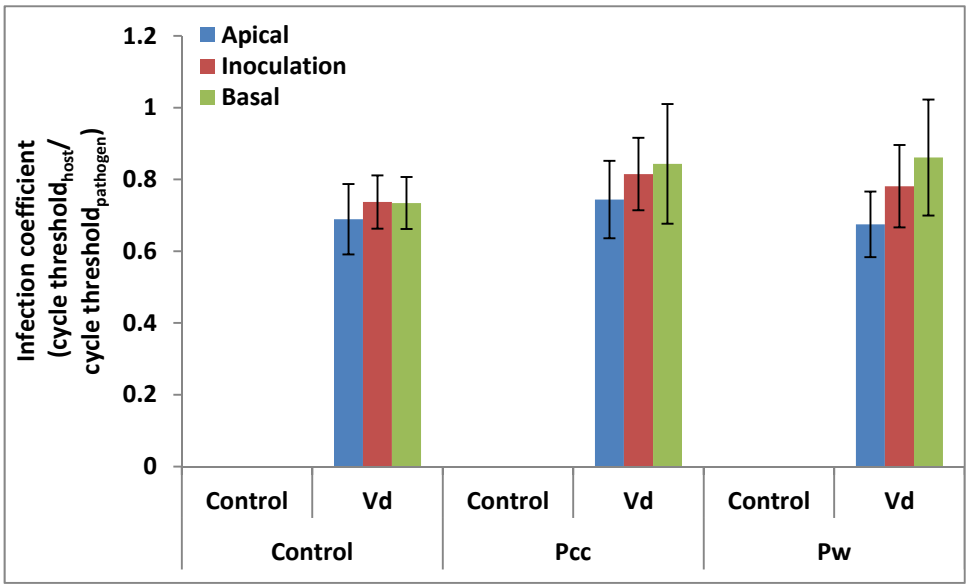

Fig. 2. *Verticillium dahliae* infection coefficient values of 'Russet Burbank' potato plants inoculated with *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc) isolate Ec101, *P. wasabiae* (Pw) isolate PwO405, or a water control in the presence and absence of *V. dahliae* (Vd) isolate 653. Tissues were sampled from apical, inoculation, and basal portions of plants. Infection coefficient = cycle threshold$_{host}$/cycle threshold$_{pathogen}$. Error bars represent standard deviations.

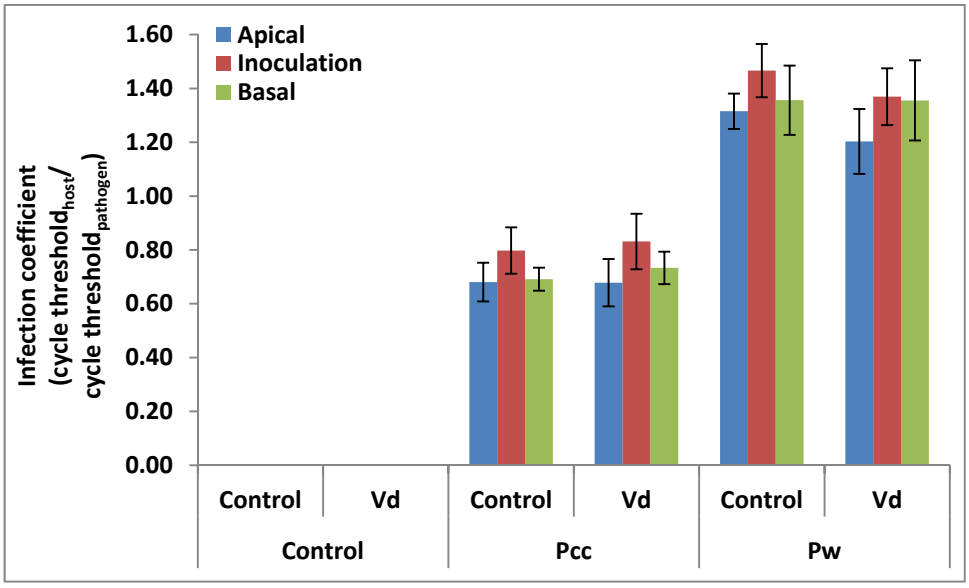

Fig. 3. *Pectobacterium* infection coefficient values of 'Russet Burbank' potato plants inoculated with *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc) isolate Ec101, *P. wasabiae* (Pw) isolate PwO405, or a water control in the presence and absence of *V. dahliae* (Vd) isolate 653. Error bars represent standard deviations. Tissues were sampled from apical, inoculation, and basal portions of plants. Infection coefficient = cycle threshold$_{host}$/cycle threshold$_{pathogen}$. Error bars represent standard deviations.