Interactive Effects of Two Soilborne Pathogens, *Phytophthora capsici* and *Verticillium dahliae*, on Chile Pepper

S. Sanogo

Department of Entomology, Plant Pathology, and Weed Science, New Mexico State University, Las Cruces 88003.
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ABSTRACT

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Phytophthora capsici and *Verticillium dahliae* are two mycelial microorganisms associated with wilt symptoms on chile pepper (*Capsicum annuum*). Both pathogens occur in the same field and can infect a single plant. This study examined the nature of the co-occurrence of *P. capsici* and *V. dahliae*. Chile pepper plants were inoculated with each pathogen separately or with both pathogens concomitantly or sequentially. In concomitant inoculations, plants were inoculated with a mixture of zoospores of *P. capsici* and conidia of *V. dahliae*. In sequential inoculations, plants were inoculated with zoospores of *P. capsici* 4 days prior to inoculation with conidia of *V. dahliae*, or plants were inoculated with conidia of *V. dahliae* 4 days prior to inoculation with zoospores of *P. capsici*. Stem necrosis and leaf wilting were visible 3 to 4 days earlier in plants inoculated with both *P. capsici* and *V. dahliae* than in plants inoculated with *P. capsici* alone. Stem necrosis and generalized plant wilting were observed in plants inoculated with *P. capsici* alone, and stem necrosis, generalized plant wilting, and vascular discoloration were ob-

served in plants inoculated with both *P. capsici* and *V. dahliae* by 21 days after inoculation. These symptoms were not observed in control plants or plants inoculated with *V. dahliae* alone. The frequency of recovery of *V. dahliae* from stems was ≈ 85 to 140% higher across inoculum levels when plants were inoculated with both *P. capsici* and *V. dahliae* than when plants were inoculated by *V. dahliae* alone. Similarly, the frequency of recovery of *V. dahliae* from roots was ≈ 13 to 40% higher across inoculum levels when plants were inoculated with both *P. capsici* and *V. dahliae* than when plants were inoculated by *V. dahliae* alone. There was no apparent antagonism between the two pathogens when they were paired on growth media. In general, when *P. capsici* and *V. dahliae* were paired on growth media, mycelial growth of each pathogen grown alone was not significantly different from mycelial growth when the pathogens were paired. Results suggest that wilt development is hastened by the presence of both *P. capsici* and *V. dahliae* in the same plants. The presence of *P. capsici* and *V. dahliae* in the same inoculum court enhanced infection and colonization of chile pepper by *V. dahliae*.

Additional keywords: coexistence, co-infection, Verticillium wilt.

Phytophthora blight caused by *Phytophthora capsici*, and Verticillium wilt caused by *Verticillium dahliae*, are two diseases which constitute a limiting factor to profitable production of many crops (6,14) in the United States and worldwide. Phytophthora blight and Verticillium wilt are the most prevalent diseases on chile pepper (*Capsicum annuum*) in New Mexico (17) and, on this crop, *P. capsici* and *V. dahliae* display differential infection patterns (16). *P. capsici* causes root rot and blight of stems and fruit. Infection of the root and lower portion of the stem leads to plant wilting, which is the most conspicuous symptom of the disease. *V. dahliae* invades roots without causing rotting, and colonizes the vascular tissues in roots and stems. Visual symptoms of plant infection by *V. dahliae* include stunting, defoliation, and wilting, with necrosis of vascular tissues.

Phytophthora blight and Verticillium wilt may severely affect physiologic processes in pepper plants and reduce crop yield. In plants inoculated with *P. capsici*, CO₂ exchange and leaf conductance were reduced by $\approx 74\%$ 4 days after inoculation (1). Xie et al. (21) reported that Phytophthora blight decreased yield of green chile pepper by 55% and combined yield of green and red chile pepper by 36%. Similarly, Biles et al. (2) reported that yield of green chile pepper was reduced by 48 to 55% in fields infested by *P. capsici*.

Although the impact of Verticillium wilt on chile pepper yield has not been quantified, several physiological processes such as

respiration, transpiration, and photosynthesis are affected in plants infected with *V. dahliae* and, therefore, yield reduction may be incurred (9,10,19). For example, in pepper plants, CO₂ uptake was reduced by 34 and 50% at 40 days and 95 days, respectively, after inoculation with *V. dahliae* (19). Infection of pepper plants by *V. dahliae* reduced photosynthesis through defoliation and chlorophyll degradation (10), and led to a decrease in leaf water potential along with a buildup of proline and total sugars, and a pronounced decrease in starch and protein levels in leaves and concentration of abscisic acid in xylem (9).

Plants infected with *P. capsici* and *V. dahliae* are found in the same field, and both pathogens also can infect a single plant (17). In a 3-year etiological survey of wilt symptoms in 59 chile pepper fields (17), plants infected with *V. dahliae* and *P. capsici* were found in 80 and 100% of the fields, respectively, and the two pathogens were found in 80% of the fields, and occurred together in some wilted plants in 12% of the fields. The occurrence of both pathogens within a single plant has raised the question about their interactive effects on plant infection. In a previous study (17), it was postulated that co-infection may hasten the development of plant wilt if *V. dahliae* and *P. capsici* interact in a synergistic fashion. Little is known about the nature of these pathogenic interactions. An increased understanding of the nature of interactions between *P. capsici* and *V. dahliae* may help elucidate ecological factors affecting these pathogens, and may have direct application in designing an effective control strategy for wilt on chile pepper.

The objective of this study was to evaluate the effects of inter-specific interactions between *P. capsici* and *V. dahliae* on infection of chile pepper and on their respective vegetative growth.

Corresponding author: S. Sanogo; E-mail address: ssanogo@nmsu.edu

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MATERIALS AND METHODS

Maintenance of cultures and inoculum preparation. An isolate of *V. dahliae* and isolate PWB-24 of *P. capsici* (3) recovered from a naturally field-infected chile pepper plant in New Mexico were used in this study. These two pathogenic isolates were maintained on water agar in 9-cm-diameter plastic petri plates. Inoculum used in plant infection experiments consisted of conidia of *V. dahliae* and zoospores of *P. capsici*. To produce conidia, a 1-cm mycelial plug of *V. dahliae* grown on water agar was added to 100 ml of sterilized Czapek-Dox broth contained in a 250-ml Erlenmeyer flask. All flasks were placed on a rotary shaker and incubated at room temperature (23 to 25°C) in darkness for 7 to 10 days. Then, the contents of the flask were passed through three layers of cheesecloth to separate conidia from mycelial plugs and mats. The number of conidia in the filtered suspension was estimated with a hemacytometer and adjusted to 4×10^6 conidia/ml.

Production of zoospores of *P. capsici* was performed as follows. A 1-cm mycelial plug of *P. capsici* grown on water agar was placed onto a 9-cm-diameter petri plate containing V8 juice agar and incubated in the dark at 25°C. After 5 days, six mycelial plugs (1 cm in diameter) were cut from the V8 juice agar culture. The plugs were placed in 20 ml of sterile distilled water in a 9-cm-diameter petri plate and maintained in the dark at 25°C for 3 days to induce sporangia formation. The plates then were transferred to 10°C for 60 min and returned to 25°C for 90 min to trigger the release of zoospores. The contents of petri plates then were passed through three layers of cheesecloth to remove agar plugs. The number of zoospores was counted using a hemacytometer, and inoculum concentration was adjusted to 2×10^3 zoospores per ml.

Plant production and inoculation. Seed of the chile pepper cv. Joe Parker, which is known to be susceptible to both *P. capsici* and *V. dahliae* (3), were planted in sterilized vermiculite in 6-cell flats in the greenhouse. At the first true leaf stage, seedlings were transplanted individually into 12-cm round plastic pots filled with a sterilized mixture of Terra-Lite Metro Mix 360 (W. R. Grace & Co., Memphis, TN), sand, and perlite (2:1:1 per volume). Seedlings were fertilized once a week by adding to each pot a 150-ml solution of 20% N, 20% P₂O₅, and 20% K₂O fertilizer (Scotts Co., Marysville, OH) containing 3.94% ammoniacal nitrogen, 6.05% nitrate, 10.01% urea, 0.05% Mg, 0.0068% B, 0.0036% Cu (chelated Cu), 0.05% Fe (chelated Fe), 0.025% Mn (chelated Mn), 0.0009% Mo, and 0.0025% Zn (chelated Zn). Plants were inoculated by dispensing 5 or 10 ml of inoculum suspension on top of the soil in each pot around the base of each plant. The addition of 5 or 10 ml of conidial suspension of *V. dahliae* generated two inoculum levels of 2×10^7 (low level, V1) and 4×10^7 (high level, V2) conidia per pot, respectively. Similarly, the addition of 5 or 10 ml of zoospore suspension of *P. capsici* yielded two inoculum levels of 1×10^4 (low level, P1) and 2×10^4 (high level, P2) zoospores per pot, respectively. For seedlings serving as control (noninoculated), no inoculum suspension was added to the soil in each pot.

Simultaneous and sequential inoculations. Plant production, inoculum production, and plant inoculation were done as described above. Two sets of experiments were conducted. In the first set, seedlings at the one- to four-flower-bud stage were inoculated with *V. dahliae*, *P. capsici*, and a mixture of both pathogens. There were nine treatments consisting of two inoculum levels of *P. capsici* (P1 = low and P2 = high, as described above) and two inoculum levels of *V. dahliae* (V1 = low and V2 = high, as described above), four combinations of both pathogens at each of the two inoculum levels (P1V1, P1V2, P2V1, and P2V2), and a control treatment (noninoculated). The nine treatments were evaluated in a randomized complete block design on a greenhouse bench. Each treatment was replicated six times, with a pot being a

replicate. Experiments were terminated 21 days after inoculation and were completed three times.

In the second set of experiments, treatments were identical to those in the first set of experiments described above, except that plants were inoculated with *V. dahliae* 4 days prior to being inoculated with *P. capsici*, or plants were inoculated with *P. capsici* 4 days prior to being inoculated with *V. dahliae*. Overall, 13 treatments were established consisting of two inoculum levels of *P. capsici* (P1 = low and P2 = high), two inoculum levels of *V. dahliae* (V1 = low and V2 = high), eight combinations of both pathogens at each of the two inoculum levels (P1BV1, P1BV2, P2BV1, P2BV2, V1BP1, V2BP1, V1BP2, and V2BP2), and a control (C) treatment (noninoculated). The letter "B" signifies "before" and indicates which pathogen and inoculum level were used first in the sequential inoculations. For example, inoculation of plants with *P. capsici* at low inoculum level (P1) 4 days before inoculation with *V. dahliae* at low inoculum level (V1) is denoted as P1BV1. Similarly, inoculation of plants with *V. dahliae* at low inoculum level (V1) 4 days before inoculation with *P. capsici* at low inoculum level (P1) is denoted as V1BP1. Each treatment was replicated six times, with a pot being a replicate. The experiments were terminated 21 days after inoculation with *P. capsici* and were completed twice.

Assessment of plant infection. In both sets of experiments, plant infection was assessed in the following two ways: (i) severity of disease symptoms on aboveground parts and (ii) plant colonization. Three plants were allocated for assessment of disease severity, and the other three plants were used to gauge plant colonization. Severity of disease symptoms on aboveground plant parts was evaluated using a scale as follows: 0 = no visual disease symptom, 1 = vascular discoloration or stem necrosis, 2 = vascular discoloration and stem necrosis, 3 = wilting and no vascular discoloration, 4 = wilting and vascular discoloration, and 5 = dead. Vascular discoloration was assessed by sectioning each plant longitudinally. Presence or absence of vascular discoloration was recorded.

To evaluate plant colonization by the pathogens, three plants were uprooted from each treatment, and a piece of tap root at least 6 cm long was recovered. Each plant was washed free of soil and secondary roots and leaves were removed. The plant was surface sterilized by submersion in 0.5% sodium hypochlorite in a 250-ml graduated cylinder for 2 min. The plant was rinsed in sterile distilled water, dried between two layers of sterile paper towels, and cut at the crown to separate root and stem parts. Each part was sectioned into 1.5-cm segments with a surgical blade, which was dipped in 95% ethyl alcohol and flame sterilized between segments. Each segment was split longitudinally, yielding two sections, which were plated onto 2% water agar medium (20 g of agar per liter of distilled water) in 9-cm-diameter plates with the cut surface on the medium. The segments were examined for *P. capsici* or *V. dahliae* after 10 to 14 days of incubation at 25°C. Percentage of root and stem segments yielding *P. capsici* alone, *V. dahliae* alone, or both microorganisms was recorded.

Pathogen interaction on growth media. Mycelial growth of *V. dahliae* and *P. capsici* was evaluated on four solid media: 10% V8 juice agar (100 ml of clarified V8 juice, 20 g of agar, and 900 ml of distilled water), potato dextrose agar (39 g per liter of distilled water), 2% water agar (20 g of agar per liter of distilled water), and Czapek-Dox agar (49 g per liter of distilled water). On each solid medium, *V. dahliae* and *P. capsici* were grown separately or paired by placing a 1-cm plug of each isolate 2 cm apart. Two sets of experiments were conducted. In the first set of experiments, *V. dahliae* and *P. capsici* were placed concomitantly on each medium for 72 h or 7 days, after which radii of colonies were measured. In the second set of experiments, *V. dahliae* was placed on each medium 7 days before being paired with *P. capsici*, and colony radii were measured 72 h after pairing of the isolates. In both sets of experiment, all plates were placed in a

plastic box and incubated in darkness at 25°C. In each experiment, radius of mycelial growth from plugs was measured from the edge of the plug to the margin of mycelial growth. Three radius measurements were made on mycelial growth from each plug and averaged per plate. Each set of experiments was conducted in a completely randomized design, with five to six replications per treatment and a petri plate as a replicate. Each experiment was completed at least three times.

To test whether volatile substances were produced and involved in the outcome of the interaction between *P. capsici* and *V. dahliae*, plates with the four solid media described above (V8 juice agar, potato dextrose agar, water agar, and Czapek-Dox agar) were used. Plates containing *V. dahliae* were inverted and placed over plates containing *P. capsici*. Similarly, plates containing *P. capsici* were inverted and placed over plates containing *V. dahliae*. The two plates were secured in place with parafilm which was overlaid with two layers of white masking tape. All plates were maintained in darkness at 25°C. Radius of mycelial growth was measured as described above after 7 days of incubation. The experiment was conducted in a completely randomized design with six replications, with a petri plate as a replicate. The experiment was conducted three times.

Data analysis. The variables measured in this study consist of (i) disease severity, (ii) percentage of root and stem colonization, (iii) and radius of mycelial growth. Data collected on radius of mycelial growth and percentage of root and stem colonization were analyzed by analysis of variance using the PROC MIXED procedure in SAS (SAS Institute, Cary, NC). The data consisting of disease severity ratings were ordinal; therefore, these data were analyzed by nonparametric approaches using SAS macros OWL and LD_CI as outlined by Brunner et al. (4). These macros allow computation of mean rank of treatments, relative treatment effects (RTE), 95% confidence intervals for RTE, and Kruskal-Wallis test statistic (Q_n) for gauging the significance of treatments. The P value associated with the Kruskal-Wallis test statistic (Q_n) is determined using χ^2 distribution. Data from individual experiments were analyzed separately to determine consistency of results and homogeneity of error variance among repetitions of experiments. Error variances were evaluated for homogeneity using procedures outlined by Gomez and Gomez (11). When results were consistent among experiments and the error variances were homogeneous ($P > 0.05$), results from all repetitions of experiments were combined. Arcsine square root transformation was applied to data consisting of percentage (converted to proportion) of root and stem colonization before analysis of variance. All statistical tests were assessed at 5% significance level, and treatment differences were gauged on 95% confidence intervals for RTE and preplanned linear contrasts among treatments for tissue colonization and mycelial growth data.

RESULTS

Simultaneous inoculations. Stem necrosis and leaf wilting were apparent in plants inoculated simultaneously with *P. capsici* and *V. dahliae* within 7 to 10 days, and in plants inoculated with *P. capsici* alone within 10 to 14 days after inoculation. These symptoms were not observed in control plants or plants inoculated with *V. dahliae* alone. By 21 days after inoculation, stem necrosis and generalized plant wilting were observed in plants inoculated with *P. capsici* alone, and stem necrosis, generalized plant wilting, and vascular discoloration were observed in plants inoculated with both *P. capsici* and *V. dahliae*. Whereas vascular discoloration, which is associated with infection by *V. dahliae*, was observed in plants inoculated with both *P. capsici* and *V. dahliae*, this symptom was not observed in plants inoculated with *V. dahliae* alone. Control plants did not display any stem necrosis, vascular discoloration, or wilting. There was a significant difference among treatments ($P < 10^{-8}$) as determined by

Kruskal-Wallis test (Table 1). The RTE for the two inoculum levels of *V. dahliae* used alone (V1 and V2) were identical to that of the control treatment, with an RTE = 0.166 (Table 1). In contrast, in the presence of *P. capsici*, RTE values were 0.623 and 0.656 at low (V1) and high (V2) inoculum levels of *V. dahliae*, respectively, when *P. capsici* was used at a low level (P1). Similarly, RTE values were 0.830 and 0.860 at low (V1) and high (V2) inoculum levels of *V. dahliae*, respectively, when *P. capsici* was used at a high level (P2). As indicated by the overlapping 95% confidence intervals, the RTE were similar between the two inoculum levels of *V. dahliae* (V1 and V2) at each inoculum level of *P. capsici* (P1 or P2).

Frequency of recovery of *P. capsici* from stems of plants inoculated by *P. capsici* alone was ≈ 19 and 29% at low (P1) and high (P2) levels of inoculum of *P. capsici*, respectively (Fig. 1A). In plants inoculated with both *P. capsici* and *V. dahliae*, *P. capsici* never was recovered alone, but always was recovered along with *V. dahliae*, and the levels of recovery were comparable with those from plants inoculated by *P. capsici* alone. Concomitant recovery of *P. capsici* and *V. dahliae* from stems was restricted to segments with necrosis in the lower stem area. The frequencies of isolation of both pathogens ranged from 17 to 21% across the combination of inoculum levels (P1V1, P1V2, P2V1, and P2V2) of both pathogens, and there was no significant difference among these treatments as assessed by linear contrasts among treatments ($P > 0.10$).

Frequency of isolation of *V. dahliae* from stem segments was ≈ 37 and 45% in plants inoculated with *V. dahliae* alone at low (V1) and high (V2) inoculum levels of *V. dahliae*, respectively (Fig. 1A). In contrast, in the presence of *P. capsici* used at a low inoculum level (P1), frequency of isolation of *V. dahliae* was increased to ≈ 57 and 65% at low (V1) and high (V2) inoculum levels of *V. dahliae*, respectively. The frequency of isolation of *V. dahliae* was further augmented to 73 and 83% at low (V1) and high (V2) inoculum levels of *V. dahliae*, respectively, when *P. capsici* was used at a high inoculum level (P2). These frequencies of recovery of *V. dahliae* from stem segments in the presence of *P. capsici* were significantly higher, by 43 to 97%, than the frequency of its recovery when plants were inoculated with *V. dahliae* alone, as revealed by linear contrasts ($P < 0.0001$). Taking into account the levels of concomitant recovery (stem segments with both *V. dahliae* and *P. capsici*), the frequency of recovery of *V. dahliae* was higher, by 85 to 140%, than the frequency of its recovery when plants were inoculated with *V. dahliae* alone.

TABLE 1. Median, mean rank, relative treatment effects (RTE), and 95% confidence intervals (CI) for RTE of severity ratings on chile pepper (cv. Joe Parker) inoculated simultaneously with *Phytophthora capsici* or *Verticillium dahliae*^a

Treatment ^b	Median ^c	Mean rank	RTE	95% CI for RTE
C	0	14	0.166	(0.166,0.166)
P1	1	39.22	0.478	(0.413,0.543)
P1V1	3	51	0.623	(0.517,0.730)
P1V2	3	53.66	0.656	(0.564,0.750)
P2	3	45.38	0.554	(0.490,0.618)
P2V1	4	67.72	0.830	(0.780,0.881)
P2V2	4	70	0.860	(0.830,0.886)
V1	0	14	0.166	(0.166,0.166)
V2	0	14	0.166	(0.166,0.166)

^a Kruskal-Wallis test: test statistic = 70.51, $P < 10^{-8}$.

^b Treatments consisted of two inoculum levels of *P. capsici* (P1 = low and P2 = high), two inoculum levels of *V. dahliae* (V1 = low and V2 = high), four combinations of both pathogens at each of the two inoculum levels (P1V1, P1V2, P2V1, and P2V2), and a control (C) treatment (noninoculated).

^c Disease severity was rated using the following scale: 0 = no visual disease symptom, 1 = vascular discoloration or stem necrosis, 2 = vascular discoloration and stem necrosis, 3 = wilting and no vascular discoloration, 4 = wilting and vascular discoloration, and 5 = dead.

In plants inoculated with *P. capsici* alone, all root segments yielded *P. capsici* (Fig. 1B). In plants inoculated with *V. dahliae* alone, frequency of isolation of *V. dahliae* from roots was ≈ 47 and 69% at the low (V1) and high (V2) inoculum levels of *V. dahliae*, respectively. In simultaneous inoculation with both pathogens, the frequency of root segments yielding *P. capsici* alone ranged from ≈ 3 to 47%, whereas no root segments yielded *V. dahliae* alone. However, both pathogens were recovered from root segments at frequencies ranging from ≈ 53 to 97%. Hence, simultaneous inoculation of plants with both pathogens resulted in greater percentage of root colonization by *V. dahliae* than when plants were inoculated by *V. dahliae* alone.

Sequential inoculations. Stem necrosis and leaf wilting were visible 3 to 4 days earlier in plants inoculated with both *P. capsici* and *V. dahliae* than in plants inoculated with *P. capsici* alone. Stem necrosis and generalized plant wilting were observed in plants inoculated with *P. capsici* alone, and stem necrosis, generalized plant wilting, and vascular discoloration were observed in plants inoculated with both *P. capsici* and *V. dahliae* by 21 days after inoculation. These symptoms were not observed in control plants or plants inoculated with *V. dahliae* alone.

Fig. 1. Frequency of isolation of *Phytophthora capsici* and *Verticillium dahliae* from **A**, stems and **B**, roots of chile pepper inoculated with each pathogen alone or inoculated simultaneously with both pathogens. The abbreviations Pc, Vd, and Pc+Vd denote isolation frequency of *P. capsici* alone, *V. dahliae* alone, and concomitant isolation of *P. capsici* and *V. dahliae*, respectively. Treatments consist of two inoculum levels of *P. capsici* (P1 = low and P2 = high), two inoculum levels of *V. dahliae* (V1 = low and V2 = high), four combinations of both pathogens at each of the two inoculum levels (P1V1, P1V2, P2V1, and P2V2), and a control (C) treatment (noninoculated). Each column is the mean of three individual experiments comprising three replicates per treatment. The bar on each column represents the standard error of the mean. Bars that are too small are not visible.

There was a significant difference among treatments ($P < 10^{-5}$) as determined by Kruskal-Wallis test (Table 2). Whether plants were inoculated with *P. capsici* prior to inoculation with *V. dahliae* or vice versa, the RTE were comparable. The RTE for the two inoculum levels of *V. dahliae* used alone (V1 and V2) were identical to that of the control treatment, with an RTE = 0.115 (Table 2). When plants were inoculated with *P. capsici* before inoculation with *V. dahliae*, RTE values were similar at 0.624 at both low (V1) and high (V2) inoculum levels of *V. dahliae* when *P. capsici* was used at a low (P1) inoculum level. Similarly, when *P. capsici* was used at a high (P2) inoculum level, RTE were 0.746 and 0.773 at low (V1) and high (V2) inoculum levels of *V. dahliae*, respectively. As indicated by the overlapping 95% confidence intervals, the RTE were similar between the two inoculum levels of *V. dahliae* (V1 and V2) at both inoculum levels of *P. capsici* (P1 or P2).

When plants were inoculated with *V. dahliae* before inoculation with *P. capsici*, RTE values were 0.577 and 0.652 at low (P1) and high (P2) inoculum levels of *P. capsici*, respectively, when *V. dahliae* was used at a low inoculum level (V1). Similarly, when *V. dahliae* was used at a high inoculum level (V2), RTE values were 0.605 and 0.746 at low (P1) and high (P2) inoculum levels of *P. capsici*, respectively. As indicated by the overlapping 95% confidence intervals, RTE values were similar between the two inoculum levels of *V. dahliae* (V1 and V2) at both inoculum levels of *P. capsici* (P1 or P2).

Frequency of recovery of *P. capsici* from stem segments inoculated with *P. capsici* alone was ≈ 24 and 29% at low (P1) and high (P2) inoculum levels of *P. capsici*, respectively (Fig. 2). In plants inoculated with both *P. capsici* and *V. dahliae*, *P. capsici* never was recovered alone, but always was recovered along with *V. dahliae*, and the levels of recovery were comparable with those from plants inoculated by *P. capsici* alone. Both *P. capsici* and *V. dahliae* were concomitantly recovered from the stem, and recovery was restricted to segments with necrosis in the lower stem area. The frequencies of isolation of both pathogens ranged from

TABLE 2. Median, mean rank, relative treatment effects (RTE), and 95% confidence intervals (CI) for RTE of severity ratings on chile pepper (cv. Joe Parker) inoculated sequentially with *Phytophthora capsici* or *Verticillium dahliae*^a

Treatment ^b	Median ^c	Mean rank	RTE	95% CI for RTE
C	0	9.5	0.115	(0.115, 0.115)
P1	2	32	0.404	(0.295, 0.512)
P1BV1	3.5	49.2	0.624	(0.457, 0.791)
P1BV2	3.5	49.2	0.624	(0.457, 0.791)
P2	2	32	0.404	(0.295, 0.512)
P2BV1	4	58.6	0.746	(0.606, 0.885)
P2BV2	4	60.8	0.773	(0.682, 0.865)
V1	0	9.5	0.115	(0.115, 0.115)
V1BP1	3	45.5	0.577	(0.424, 0.730)
V1BP2	3.5	51.3	0.652	(0.505, 0.800)
V2	0	9.5	0.115	(0.115, 0.115)
V2BP1	3	47.6	0.605	(0.467, 0.742)
V2BP2	4	58.6	0.746	(0.606, 0.885)

^a Kruskal-Wallis test: test statistic = 55.66, $P < 10^{-5}$.

^b Treatments consisted of two inoculum levels of *P. capsici* (P1 = low and P2 = high), two inoculum levels of *V. dahliae* (V1 = low and V2 = high), eight combinations of both pathogens at each of the two inoculum levels (P1BV1, P1BV2, P2BV1, P2BV2, V1BP1, V2BP1, V1BP2, and V2BP2), and a control (C) treatment (noninoculated). The letter "B" signifies "before" and indicates which pathogen and inoculum level were used first in the sequential inoculations. For example, inoculation of plants with *P. capsici* at a low inoculum level (P1) 4 days before inoculation with *V. dahliae* at a low inoculum level (V1) is denoted as P1BV1. Similarly, inoculation of plants with *V. dahliae* at a low inoculum level (V1) 4 days before inoculation with *P. capsici* at a low inoculum level (P1) is denoted as V1BP1.

^c Disease severity was rated using the following scale: 0 = no visual disease symptom, 1 = vascular discoloration or stem necrosis, 2 = vascular discoloration and stem necrosis, 3 = wilting and no vascular discoloration, 4 = wilting and vascular discoloration, and 5 = dead.

18 to 25% across the combination of inoculum levels (P1BV1, P1BV2, P2BV1, P2BV2, V1BP1, V2BP1, V1BP2, and V2BP2) of both pathogens, and there was no significant difference among these treatments as assessed by linear contrast among treatments ($P > 0.10$).

Frequency of isolation of *V. dahliae* from stem segments was ≈ 41 and 44% in plants inoculated with *V. dahliae* alone at low (V1) and high (V2) inoculum levels of *V. dahliae*, respectively (Fig. 2). In plants inoculated with *P. capsici* prior to inoculation with *V. dahliae*, the frequency of isolation of *V. dahliae* was ≈ 65 and 74% at low (V1) and high (V2) inoculum levels of *V. dahliae*, respectively, when *P. capsici* was used at a low (P1) inoculum level. The frequency of isolation of *V. dahliae* was $\approx 79\%$ at both low (V1) and high (V2) inoculum levels of *V. dahliae*, respectively, when *P. capsici* was used at a high inoculum level (P2). These frequencies of recovery of *V. dahliae* from stem segments in the presence of *P. capsici* were significantly higher, by 59 to 90%, than the frequency of its recovery when plants were inoculated with *V. dahliae* alone, as revealed by linear contrasts ($P < 0.0001$).

In plants inoculated with *V. dahliae* prior to inoculation with *P. capsici*, the frequency of isolation of *V. dahliae* was 71 and 78% at low (P1) and high (P2) inoculum levels of *P. capsici*, respectively, when *V. dahliae* was used at a low (V1) inoculum level (Fig. 2). Similarly, when *V. dahliae* was used at a high inoculum level (V2), the frequency of isolation of *V. dahliae* was 75 and 81% at low (P1) and high (P2) inoculum levels of *P. capsici*, respectively. These frequencies of recovery of *V. dahliae* from stem segments in the presence of *P. capsici* were significantly higher, by 70 to 90%, than the frequency of its recovery when plants were inoculated with *V. dahliae* alone ($P < 0.0001$).

Fig. 2. Frequency of isolation of *Phytophthora capsici* and *Verticillium dahliae* from stems of chile pepper inoculated with each pathogen alone or inoculated sequentially with both pathogens. The abbreviations Pc, Vd, and Pc+Vd denote isolation frequency of *P. capsici* alone, *V. dahliae* alone, and concomitant isolation of *P. capsici* and *V. dahliae*, respectively. Treatments consist of two inoculum levels of *P. capsici* (P1 = low and P2 = high), two inoculum levels of *V. dahliae* (V1 = low and V2 = high), eight combinations of both pathogens at each of the two inoculum levels (P1BV1, P1BV2, P2BV1, P2BV2, V1BP1, V2BP1, V1BP2, and V2BP2), and a control (C) treatment (noninoculated). The letter "B" signifies "before" and indicates which pathogen and inoculum level were used first in the sequential inoculations. For example, inoculation of plants with *P. capsici* at a low inoculum level (P1) 4 days before inoculation with *V. dahliae* at a low inoculum level (V1) is denoted as P1BV1. Similarly, inoculation of plants with *V. dahliae* at a low inoculum level (V1) 4 days before inoculation with *P. capsici* at a low inoculum level (P1) is denoted as V1BP1. Each column is the mean of two individual experiments comprising three replicates per treatment. The bar on each column represents the standard error of the mean. Bars that are too small are not visible.

Whether plants were inoculated with *P. capsici* prior to inoculation with *V. dahliae* or vice versa, there was a comparable increase in the recovery of *V. dahliae* from stems under both scenarios. Taking into account the levels of concomitant recovery (stem segments with both *V. dahliae* and *P. capsici*), the frequency of recovery of *V. dahliae* was higher, by 109 to 144%, than the frequency of its recovery when plants were inoculated with *V. dahliae* alone.

Pathogen interaction on growth media. In simultaneous pairing of *P. capsici* and *V. dahliae*, mycelial growth of *P. capsici* grown alone (P) was not significantly different from mycelial growth of *P. capsici* grown together with *V. dahliae* (vP) across all media ($P > 0.1$). Similarly, mycelial growth of *V. dahliae* grown alone (V) was not significantly different from mycelial growth of *V. dahliae* grown together with *P. capsici* (Vp) across all media ($P > 0.08$). These results were consistent in both pairing time periods (72 h and 7 days) across all growth media (Fig. 3A and B). Upon contact, which occurred between 72 and 84 h, mycelia

Fig. 3. Radial growth of *Phytophthora capsici* and *Verticillium dahliae* grown alone or paired on various growth media for **A**, 72 h and **B**, 7 days. The abbreviations V8, PDA, CZD, and WA denote V8 juice agar, potato dextrose agar, Czapek-Dox agar, and water agar media, respectively. The designations P, vP, V, and Vp represent *P. capsici* grown alone, *P. capsici* grown with *V. dahliae*, *V. dahliae* grown alone, and *V. dahliae* grown with *P. capsici*, respectively. Each column is the mean of three individual experiments comprising five or six replicates per treatment. The bar on each column represents the standard error of the mean. Bars that are too small are not visible.

of *P. capsici* and *V. dahliae* intermingled with no indication of inhibition. By 7 days, *P. capsici* totally overgrew *V. dahliae* on V8 juice agar and potato dextrose agar media; however, the edges of *V. dahliae* colonies were clearly visible within the colonies of *P. capsici*. In contrast, on water agar and Czapek-Dox media, *P. capsici* overgrew *V. dahliae* colonies by 30 to 50%.

When *P. capsici* and *V. dahliae* were paired, 7 days after initial establishment of *V. dahliae* (Fig. 4), mycelial growth of P was not significantly different from mycelial growth of vP across all media ($P > 0.1$). Similarly, mycelial growth of V was not significantly different from mycelial growth of Vp across all media ($P > 0.1$).

Under conditions which were designed to test for production and activity of volatile substances using inverted culture plates (Fig. 5), mycelial growth of P was not significantly different from mycelial growth of vP across all media ($P > 0.07$). Similarly, mycelial growth of V was not significantly different from mycelial growth of Vp across all media ($P > 0.4$).

DISCUSSION

Interactions between *P. capsici* and *V. dahliae* have not been reported previously. This is the first known report focusing on the interactive effects of *P. capsici* and *V. dahliae* on chile pepper. The salient information emanating from this study indicates that the presence of *P. capsici* and *V. dahliae* in the same inoculum court enhances infection and colonization of chile pepper by *V. dahliae*. The notion of plant infection and colonization by one pathogen being facilitated by another pathogen has been well established in many other pathosystems (5,8,12,13). However, this notion is new in the case of chile pepper infection by *P. capsici* and *V. dahliae*. Consequently, no other studies are available for comparison of results.

The methodological approaches used were similar to those used by others who have examined interactions among other mycelial microorganisms in vitro and in planta (5,8,12,13,18,20). The in

vitro studies on growth media were designed to provide a benchmark evaluation of interactions between *P. capsici* and *V. dahliae* under various nutritional environments as represented by the different growth media. Although these media are not comparable with plant tissue, it was assumed that pathogenic interactions could take place in plant tissue of varying nutritional status. Therefore, it was important to ascertain whether pathogenic interactions were influenced under different nutritional environments. Gauged on the consistency of the responses of *P. capsici* and *V. dahliae* across all growth media used, it appears from this study that nutritional environments may not exert a significant effect on the interaction between *P. capsici* and *V. dahliae*.

Outcomes of studies that addressed mycelial interactions among microorganisms have been cataloged by Stahl and Christensen (18) into four types: invasion-lysis, invasion-overgrowth, contact inhibition, and inhibition at a distance. *P. capsici* grew extensively and overgrew colonies of *V. dahliae* on all media. At points of contact, colonies intermingled with no sign of inhibition. Therefore, the interaction between *P. capsici* and *V. dahliae* is of an invasion-overgrowth type following descriptions by Stahl and Christensen (18).

For each pathogen, the levels of inoculum used were selected from previous studies (3,15). Although it is not known whether these levels are comparable with those encountered in chile pepper fields, symptom development under the selected levels mimicked well that under field conditions as observed in previous experiments for seedling screening for disease resistance and pathogen population characterization (3,15). The phenological stage used in this study was selected to represent the stage at which disease onset is observed in production fields. Typically, wilt onset is observed beginning at approximately flowering and onward through fruit set, maturation, and ripening.

Fig. 4. Radial growth of *Phytophthora capsici* and *Verticillium dahliae* grown alone or paired on various growth media when *P. capsici* establishment was delayed 7 days. Radial growth was measured 72 h following pairing of *V. dahliae* with *P. capsici*. The abbreviations V8, PDA, CZD, and WA denote V8 juice agar, potato dextrose agar, Czapek-Dox agar, and water agar media, respectively. The designations P, vP, V, and Vp represent *P. capsici* grown alone, *P. capsici* grown with *V. dahliae*, *V. dahliae* grown alone, and *V. dahliae* grown with *P. capsici*, respectively. Each column is the mean of three individual experiments comprising five or six replicates per treatment. The bar on each column represents the standard error of the mean. Bars that are too small are not visible.

Fig. 5. Radial growth of *Phytophthora capsici* and *Verticillium dahliae* grown alone or together for 7 days on various growth media in inverted culture plate environment to test for production and activity of volatile substances. The abbreviations V8, PDA, CZD, and WA denote V8 juice agar, potato dextrose agar, Czapek-Dox agar, and water agar media, respectively. The designations P, vP, V, and Vp represent *P. capsici* grown alone, *P. capsici* grown with *V. dahliae*, *V. dahliae* grown alone, and *V. dahliae* grown with *P. capsici*, respectively. Each column is the mean of three individual experiments comprising six replicates per treatment. The bar on each column represents the standard error of the mean. Bars that are too small are not visible.

The three sets of experiments conducted on plant infection were designed to represent three scenarios of plant infection by *P. capsici* and *V. dahliae*: simultaneous infection, or sequential infection in which *P. capsici* infection precedes *V. dahliae* infection or vice versa. It is not known which of these scenarios would be considered as the norm under field conditions. Nonetheless, this study has delineated an outcome common to all three scenarios, which is that of a higher infection level by *V. dahliae* in the presence of *P. capsici* than when plants were inoculated with *V. dahliae* alone. In the sequential inoculations, when plants were inoculated with *P. capsici* 4 days prior to inoculation with *V. dahliae* or vice versa, the period of 4 days was chosen because symptoms of plant infection by *P. capsici* were reported at and beyond 4 days of inoculation (1). It was assumed that the 4-day period was sufficient for plant infection by *P. capsici* or *V. dahliae*. *P. capsici* is a strong plant colonizer and probably was the first to invade the root and paved the way for subsequent increased colonization by *V. dahliae*. In this study, *P. capsici* acts as a facilitator for *V. dahliae*. Similar results were reported by others. For example, in peanut (*Arachis hypogaea*), Frank (8) found that *Fusarium solani* predisposed pods to increased rotting activities by *Pythium myriotylum*.

Studies on interactions between microorganisms with differential infection patterns may be faced with several experimental issues, one of which is defining the time for assessing plant infection. Both pathogens can infect plants at any time, but differ in terms of incubation period, a variable that is influenced by host plant, pathogen, and environmental factors (13). Under controlled-environment conditions of greenhouse or growth chamber, symptoms of infection of pepper by *P. capsici* have been observed as early as 4 days (1) to over 7 days (17) after inoculation. In contrast, symptoms of pepper infection by *V. dahliae* were reported within 3 weeks (7,10) to over 6 weeks (13,17) after inoculation. In this study, assessment of interaction between *P. capsici* and *V. dahliae* was made at 21 days after inoculation with *P. capsici* for two reasons. First, preliminary work showed that all plants inoculated with *P. capsici* died beyond 21 days. Second, plant colonization by *V. dahliae* as gauged by development of *V. dahliae* colonies from stem segments was shown to occur within 21 days of inoculation (10) and is corroborated by the results reported herein.

There is an indication that *P. capsici* and *V. dahliae* act synergistically if incubation period and RTE are taken into account. Symptoms of wilting first were observed in plants inoculated with both *P. capsici* and *V. dahliae*. In addition, RTE generally was greater in plants inoculated with both pathogens than in plants inoculated with each pathogen separately. It is concluded that, under condition of coexistence, *P. capsici* and *V. dahliae* may hasten the development of wilt on chile pepper, as previously postulated (17).

The significance of this work is two-pronged: (i) it provides an advanced understanding of the interaction between *P. capsici* and *V. dahliae*, two pathogens with differential infection patterns; and (ii) it demonstrates the plausibility of an increased incidence and hastened development of wilt symptoms in chile pepper under conditions of coexistence of *P. capsici* and *V. dahliae* in the same field. The findings from this study suggest that *P. capsici* acts as a co-factor in infection and colonization of chile pepper by *V. dahliae*. Therefore, in fields infested by *P. capsici* and *V. dahliae*, control measures should be focused on both microorganisms.

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