Research

Verticillium klebahnii and *V. isaacii* Isolates Exhibit Host-Dependent Biological Control of Verticillium Wilt Caused by *V. dahliae*

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Abstract

Verticillium dahliae, the soilborne fungal pathogen, causes vascular wilt on many economically important crops, resulting in significant yield losses. *V. klebahnii* (isolate PD659) and *V. isaacii* (isolate PD660), two related species that cause few or no symptoms in some hosts, were evaluated as potential biocontrol agents (BCAs) in eggplant, lettuce, and tomato by pre-, post-, and coinoculation with a virulent race 1 isolate of *V. dahliae* (VdLs16). Initial studies demonstrated that the biocontrol efficacy of both BCAs was similar to reference BCA *Talaromyces flavus* (NRRL15936) across all hosts ($\alpha = 0.05$). Subsequent experiments with PD659 against *V. dahliae* isolate Sm113 from eggplant, VdLs16 and VdLs17 isolates from lettuce, and Le1811 isolate from tomato demonstrated a significant biocontrol efficacy in eggplant and tomato but not in lettuce (at 95% confidence interval), suggesting host-dependent effectiveness of *V. klebahnii*. Confocal microscopy using green fluorescent protein-tagged tomato *V. dahliae* isolate Le1811 indicated delayed xylem colonization or lack of pathogen progression into the vascular system in a host-dependent manner on BCA-treated plants. Quantitative analyses of the expression of defense-related genes *PR1a*, *PR5*, acidic extracellular β -1,3-glucanase (*GlucA*), basic intracellular β -1,3-glucanase (*GlucB*), acidic extracellular chitinase (*Chi3*), basic intracellular chitinase (*Chi9*), and cysteine proteases (*cysProteases*) in tomato in the presence or absence of PD659 suggested an elevated expression of defense-related genes in compatible interaction of *V. dahliae*–tomato cultivar Early Pak. *V. klebahnii* (PD659) may delay the entry of *V. dahliae* by competing for space or nutrients during the initial stages of root colonization.

Keywords: biocontrol, disease management, vegetable crops, Verticillium wilt, *Verticillium klebahnii*, *V. isaacii*

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Verticillium dahliae is a destructive soilborne fungal pathogen that causes vascular wilt in more than 400 plant species, including lettuce, strawberry, tomato, eggplant, and other economically important crops (Atallah et al. 2011; Berlinger and Powelson 2000; Pegg and Brady 2002; Subbarao et al. 2017). Despite the broad host range, many isolates display differential virulence on some of these hosts (Gurung et al. 2015). The pathogen produces melanized microsclerotia, which serve as resting structures and remain viable in the soil for over 10 years to initiate disease in the subsequent crops (Klosterman et al. 2009; Vallad and Subbarao 2008). Losses caused by *V. dahliae* exceed several billion dollars worldwide annually (Klosterman et al. 2009; Pegg and Brady 2002).

Historically, the management of soilborne pathogens, including *V. dahliae*, has relied on crop rotation, resistant cultivars, and soil fumigation. However, the unavailability of resistant cultivars, prohibition of methyl bromide (MB) use for agricultural soil fumigation, ineffective cultural practices, broad host range, and prolonged survival of the dormant propagule have all impeded the successful management of this disease (Hayes et al. 2011a; Usami et al. 2017). Soil fumigation using MB was the primary method of controlling *Verticillium* wilt before its complete phase-out from agricultural fumigation in 2015 owing to its ozone-depleting properties and high toxicity to humans, animals, and the environment (Chellemi et al. 2016; Martin 2003). Currently, a few chemical alternatives to MB are available but are not as effective (Duniway 2002; Meadows 2013). Increased demand for organic produce or crops and environmental hazards associated with chemicals has reduced their use in agriculture (Donley 2019).

Biological pest management using natural antagonists and niche competitors has garnered considerable research attention for sustainable crop production (Bale et al. 2008). Biological antagonism works against pathogens through various direct mechanisms such as antibiosis and competition for rhizosphere space and infection sites or nutrients, or indirectly by inducing plant growth and systemic disease resistance (Deketelaere et al. 2017; Fravel 2005; Li et al. 2013; Malandraki et al. 2008; Veloso and Díaz 2012). Even a single biocontrol agent (BCA) functions through several complementary mechanisms (Deketelaere et al. 2017). An induced host defense response triggered by BCAs is one of the established mechanisms for suppressing plant pathogens. Host defense activation via pathogen-associated molecular patterns in cotton (Han et al. 2015), defense-related *pathogenesis-related* (PR) genes *PR1* and *PR4* in eggplant (Angelopoulou et al. 2014), induced plant resistance through salicylic and jasmonate-dependent defense pathways (Gkizi et al. 2016; Tjamos et al. 2005), and damage-associated pattern-triggered production of endopolymethylgalacturonase, endoglucanase, endoxyloglucanase (Gkizi et al. 2016), and so on are proven defense mechanisms against *V. dahliae*.

Fungal antagonists such as *Gibellulopsis nigrescens* (Zare et al. 2007), *Fusarium oxysporum*, and *Talaromyces flavus* (anamorph *Penicillium dangeardii*) have shown efficacy against *Verticillium* wilt in many crops but none is currently employed in commercial agriculture (Angelopoulou et al. 2014; Marois et al. 1984; Veloso and Díaz 2012; Zhu et al. 2013). Application of *Paenibacillus alvei* K165 and nonpathogenic *F. oxysporum* F2 reduced *Verticillium* wilt in eggplant (Angelopoulou et al. 2014) by limiting the germination of microsclerotia in the rhizosphere (Antonopoulos et al. 2008). Microsclerotial germination of *V. dahliae* was also lowered by *Gliocladium roseum* and *T. flavus* strains (Fravel 1989; Keinath et al. 1991; Kim et al. 1988). Similarly, the nonpathogenic

isolate *F. oxysporum* 47 (Fo47) conferred protection against *V. dahliae* and is associated with the expression of defense genes (Veloso and Díaz 2012). *Gibellulopsis nigrescens* significantly reduced the *Verticillium* wilt symptoms in cotton (Zhu et al. 2013). Among several characterized fungal antagonists, *T. flavus* is one of the more broadly used and reliable BCAs to manage *Verticillium* wilt on eggplant, potato, cotton, and tomato (Li et al. 2013; Marois et al. 1982, 1984; Naraghi et al. 2010; Tjamos and Fravel 1995).

Specific isolates or species within the genus *Verticillium* can colonize their hosts with no or minimal vascular discoloration without the external wilt-like symptoms (Chen et al. 2004; Malcolm et al. 2013; Qin et al. 2008; Robinson et al. 2007). *V. tricorpus* suppressed *Verticillium* wilt of potato and cauliflower (Davis et al. 2000; França et al. 2013). Despite *V. tricorpus* being endophytic or weakly pathogenic in potato and offering protection against *V. dahliae* (Davis and Sorensen 1985; Davis et al. 2000), some isolates were highly pathogenic on lettuce (Usami et al. 2011). Using genealogical concordance phylogenetic species recognition (Taylor et al. 2000), three different lineages were recognized within the morphospecies *V. tricorpus*: *V. klebahnii*, *V. isaacii*, and *V. tricorpus* sensu stricto (Inderbitzin et al. 2011). *V. isaacii* Vt305 was effective in reducing *Verticillium* wilt of cauliflower caused by *V. longisporum* in both the greenhouse and the field (Deketelaere et al. 2020). Therefore, it is quite likely that some of the previously reported weakly pathogenic or nonpathogenic isolates of *V. tricorpus* belong to either *V. isaacii* or *V. klebahnii*. In cauliflower, the endophytic *V. isaacii* isolate Vt305 colonized the roots, hypocotyl, and stem without visible wilting and significantly reduced wilt severity caused by *V. longisporum* (Tyvaert et al. 2014).

Previously, we had isolated the endophytic *V. isaacii* (PD660) and *V. klebahnii* (PD659) isolates from the roots of spinach and lettuce, respectively (Short et al. 2015). Both *V. klebahnii* PD659 (Gurung et al. 2015) and *V. isaacii* (PD660) (unpublished) produced no visible symptoms on lettuce and tomato, and inoculated plants seldom exhibited vascular discoloration. Thus, it is essential to explore their endophytic lifestyles in the roots and stems of lettuce, tomato, or other hosts and to test their biocontrol efficacy against pathogenic *Verticillium* spp. In the current study, we tested the biocontrol potential of nonpathogenic *V. isaacii* (PD660) and *V. klebahnii* (PD659) isolates on eggplant, lettuce, and tomato, initially challenging them against a single *V. dahliae* isolate (VdLs16) as pre-, post-, and coinoculated treatments and compared their efficacy with a known BCA, *T. flavus* NRRL15936. Subsequently, the efficacy of isolates PD659 and PD660 was further tested against a panel of *V. dahliae* isolates derived from three different hosts. The specific objectives of the study were to (i) evaluate the biocontrol efficacy of *V. isaacii* (PD660) and *V. klebahnii* isolate (PD659) in reducing *V. dahliae* colonization and wilt in eggplant, lettuce, and tomato; (ii) explore biocontrol mechanisms based on confocal microscopy using green fluorescent protein (GFP)-tagged isolates of *V. dahliae* during the infection process; and (iii) determine whether *V. klebahnii* isolate PD659 induces the plant defense-related genes during the infection process in tomato and explore the possible mode of action.

MATERIALS AND METHODS

Fungal isolates and maintenance

Pathogenic *V. dahliae* isolates VdLs16 (race 1) and VdLs17 (race 2) from lettuce, Sm113 (race 2) from eggplant, and Le1811 (race 2) from tomato were isolated from symptomatic

plants collected from commercial farms in California, United States. The biocontrol isolates PD659 (*V. klebahnii*) and PD660 (*V. isaacii*) were isolated from lettuce roots and spinach plants sampled at Salinas, CA, respectively, as described previously by Short et al. (2015). The reference BCA *T. flavus* strain NRRL15936 was obtained from the United States Department of Agriculture Culture Collection (Peoria, IL, U.S.A.). All fungal isolates were grown on potato dextrose agar (PDA) (Difco Laboratories Inc., Detroit, MI, U.S.A.) and used for inoculation or storage in 25% glycerol at -80°C .

Biocontrol experiments using *V. dahliae* isolate VdLs16 from lettuce

Seed of eggplant cultivar Long Purple, tomato cultivar Early Pak, and lettuce cultivar Salinas were planted in 50-well trays filled with Sunshine Professional Growing Mix 4 (SUN-GRO Horticulture, Canada). In all hosts, 2-week-old seedlings were inoculated with 3 ml of a suspension of 1.0×10^7 conidia/ml in the germination tray, then transplanted into 0.5-liter Styrofoam cups (Amerifoods, USA) filled with steam-sterilized sand/potting soil (2:1 [vol/vol]) mixture.

In the first experiment, five treatment combinations each for the three putative BCAs plus five control treatments (for a total of 20 treatments) were used to evaluate the biocontrol efficacy against *V. dahliae* isolate VdLs16 relative to the control BCA *T. flavus* strain NRRL15936 (Fig. 1) in three

different hosts. Treatments included were (i) inoculation of VdLs16 1 week prior to BCAs, (ii) inoculation of VdLs16 2 weeks prior to BCAs, (iii) coinoculation of BCAs and VdLs16, (iv) inoculation of BCAs 1 week prior to VdLs16, and (v) inoculation of BCAs 2 weeks prior to VdLs16. Seedlings treated with sterile distilled water served as negative controls, while plants inoculated only with VdLs16, PD659, PD660, and strain NRRL15936 served as positive controls. Following seedling transplantation, individual cups were arranged in a randomized complete block design in factorial arrangement using three replications, each with at least seven plants (21 total plants). The entire experiment was repeated once. Seeding, inoculation, disease rating, and standard agronomic practices were performed the same way across experiments (Puri et al. 2017).

Biocontrol experiments using host-dependent isolates

The second experiment was conducted to test the efficacy of both BCAs (PD659 and PD660) using the treatment that reduced Verticillium wilt the most in the experiment above. Each host was inoculated with isolates obtained from infected root or stem tissues of the corresponding host: eggplant isolate Sm113 on Long Purple eggplant, lettuce isolates VdLs16 and VdLs17 on Salinas lettuce, and tomato isolate Le1811 on Early Pak tomato. In this experiment, BCAs were introduced on 2-week-old seedlings and later challenged by individual

FIGURE 1

Relative marginal effect (RME) and median disease severity scores combined across eggplant, lettuce, and tomato. Nonoverlapping confidence interval (CI) lines on the top of the bars represent statistically significant treatment effects at 95% CI. Numbers inside the bars represent median disease severity, while the color of bars represents timing of treatments. The treatment annotations represent the order and the interval in which inoculations of the hosts were performed. For example, PD659-1w-VdLs16 represents PD659 applied 1 week before VdLs16, and VdLs16-2w-PD659 represents VdLs16 applied 2 weeks before PD659, and so on. The plus (+) sign represents coinoculation at the same time. Tf = *Talaromyces flavus* NRRL15936. Both PD659 (*Verticillium klebahnii*) and VdLs16 (*V. dahliae*) were obtained from lettuce, while isolate PD660 (*V. isaacii*) was collected from spinach.

pathogenic isolates at 2 weeks after BCA treatments. The BCAs were introduced by immersing the seedling roots in a suspension of 1.0×10^7 conidia/ml for 10 min; then, seedlings were transplanted into Styrofoam cups as described above. The pathogenic isolates were inoculated by drenching the soil with a 5-ml inoculum suspension containing 1.0×10^7 conidia/ml/cup. Seedlings that received water treatment at the time of BCA inoculation (2-week-old seedlings) but were challenged with the pathogenic isolate 2 weeks later (4-week-old seedlings) served as positive controls. The water-treated plants served as negative controls. The experimental design was a randomized complete block design with three replications each as above, and the experiment was repeated once.

GFP transformants

V. dahliae isolate Le1811 (race 2) from tomato was transformed with a GFP gene using the *Agrobacterium tumefaciens*-mediated transformation (ATMT) protocol (Maruthachalam et al. 2013). Briefly, conidia from a 7-day-old culture were harvested, diluted to approximately 1.0×10^6 conidia/ml, and used for ATMT. The transformation was carried out using a T-DNA binary vector (pSK2241) encoding a hygromycin B resistance gene (*hph*) under the control of *Aspergillus nidulans* trpC promoter, and *Zoanthus* sp. green fluorescent protein (*ZsGFP*) gene under the control of an *F. verticillioides* translation elongation factor 1- α promoter. The vector was transformed into *Agrobacterium tumefaciens* strain EHA105 by electroporation and by using random insertional mutagenesis for *V. dahliae* transformation. The putative GFP transformants were selected on PDA supplemented with hygromycin (50 μ g/ml) and examined under a trinocular bright field/fluorescence microscope (Olympus BX60) equipped with a GFP filter (peak excitation wavelength at 489 nm, peak emission wavelength at 508 nm). The transformants were also examined for morphological features (colony growth and microsclerotia production) and pathogenicity on Long Purple eggplant, Early Pak tomato, and Salinas lettuce (data not presented) along with the wild-type strain. A transformed isolate with characteristics similar to the wild type was selected for inoculation followed by confocal microscopy.

Sample collection for confocal microscopy

Fourteen-day-old seedlings of Long Purple eggplant and Beefsteak tomato were inoculated with BCA PD659 using root dip, as described above. The GFP-tagged isolate Le1811 was inoculated 2 weeks postinoculation (wpi) with PD659 or sterile water treatment via soil drenching. At least three plants from each treatment were uprooted, washed free of soil, and used in confocal microscopy at 17 and 34 days after Le1811 inoculation. Several thin vertical or transverse sections were prepared from stem tissues sampled approximately 1 cm from the soil line to track pathogen growth within the plant. Cross-sections were placed on a glass slide, covered with a glass coverslip number 1.5 (approximately 0.17 mm), and observed under a confocal microscope (Leica LAS-AF V.3) at the Department of Plant Pathology, University of California, Davis. Default settings on the confocal microscope such as the acquisition of images (format = 512 by 512 pixels, scan speed = 400 Hz) and the channel settings were utilized. The GFP setting for the GFP signal (fluorescence emission maximum at 532 nm) and autofluorescence of 680 nm was used. The

captured digital images acquired were further processed using GIMP-2.10 (<https://www.gimp.org/>).

RNA extraction and reverse-transcription quantitative real-time PCR

To monitor plant defense responses in the presence or absence of BCA PD659, the expression of selected *PR* marker genes was monitored using quantitative real-time PCR over time. Briefly, the susceptible Early Pak tomato was first inoculated with BCA PD659 or treated with water 14 days after germination using the root-dip method. Fourteen days after inoculation, seedlings from both sets were challenged with the *V. dahliae* isolate Le1811 from tomato using soil drench, as described above. The water-treated plants served as a negative control. Shoot tissues from all three treatment combinations (water, water followed by Le1811, and PD659 followed by Le1811) were collected using destructive sampling of actively growing tips of three plants per replication at 24 h, 48 h, 1 week, and 2 weeks after inoculation of Le1811. Collected tissues were frozen immediately in liquid nitrogen and stored at -80°C until use. In total, 36 samples (three treatments by four time points by three replications) were collected.

The reverse-transcription quantitative real-time PCR (RT-qPCR) was performed following the method described by Puri et al. (2016). Briefly, total RNA was extracted using the SV Total RNA Isolation Kit (Promega Corp., Madison, WI, U.S.A.) and purified by treatment with DNase I (NEB, Ipswich, MA, U.S.A.) according to the manufacturers' protocols. An RT reaction was performed on 1 μ g of total RNA using SuperScript III reverse transcription (Invitrogen, Carlsbad, CA, U.S.A.). cDNA was diluted sixfold and used as a template for RT-qPCR using a LightCycler 480 II (Roche Life Science, Inc.). Three technical replicates were performed for each sample in 20- μ l reactions containing 2 μ l of cDNA template and 250 nm forward and reversed primers using SYBR Green PCR Master Mix (Applied Biosystems, Foster, CA, U.S.A.). PCR conditions included one cycle of preincubation at 50°C followed by 95°C , each for 2 min; then, 45 cycles of 95°C for 30 s and 59°C for 1 min; followed by melt curve analysis. The differential expression of selected *PR* genes was performed using previously standardized primer pairs. The primer sequences for tomato (*Lycopersicon esculentum*) acidic extracellular β -1,3-glucanase (*GluA*), basic intracellular β -1,3-glucanase (*GlucB*), acidic extracellular chitinase (*Chi3*), basic intracellular chitinase (*Chi9*), and *PR* gene *SIPR5* were retrieved from Aimé et al. (2008) or Barda et al. (2015); *PR1a* from Block et al. (2005); cysteine protease (*cysProtease*) from Balaji et al. (2008); and the reference gene *actin* from Herman et al. (2008). The constitutively expressed *actin* gene was used as a reference to calculate relative expression with respect to mock-inoculated samples using REST software, version 2009 (Pfaffl et al. 2002).

Disease assessment and data analysis

Disease severity (DS) was assessed at 8 to 10 wpi based on the discoloration of the vascular tissue of the taproot using a 0-to-5 subjective ordinal scale (Hayes et al. 2011b). Nonparametric analyses using PROC MIXED for analysis of variance (ANOVA)-type statistics of ranked mean, and LD_CI macro for relative marginal effects (RME) of each treatment at 95% confidence intervals (CI), were performed in SAS v9.4 (SAS Institute) (Shah and Madden 2004). Two treatments were

considered significantly different when RME values had non-overlapping CIs (Brunner and Langer 2000; Shah and Madden 2004). A higher RME value refers to higher DS. Orthogonal contrast analyses were used to compare the biocontrol effect of each treatment versus disease caused by the pathogenic isolate VdLs16. The Pearson Correlation Coefficients (r) and correlograms among disease incidence, severity, plant height, and biomass were calculated using a ggscatter function in R package 'ggpubr.'

The biocontrol effect was quantified based on the decreased rate of disease (DRD) using the following formula: $DRD = (1 - S_i/S_m) \times 100\%$, where S_i = the average DS of plants that received biocontrol treatment and S_m = the average severity of plants inoculated with pathogenic isolates.

RESULTS

Pathogenicity

None of the biocontrol strains tested in the experiments caused any visible symptoms of Verticillium wilt in any hosts (Fig. 1).

Biocontrol efficacy of *V. klebahnii* (PD659) and *V. isaacii* (PD660) against *V. dahliae* isolate VdLs16 across three hosts

An ANOVA-type statistical analysis (ANOVA-F) using nonparametric methods showed significant differences among biocontrol treatments, host responses, and treatment $\times$ host interactions ($P < 0.0001$). The results from the two independent experiments were similar. Because experiments were not a significant source of variation ($P = 0.6263$) (Table 1), the data from two independent experiments were combined for further analysis.

The RME indicated a significant effect of BCA strain PD659 against the *V. dahliae* isolate VdLs16 across the three hosts when introduced 2 weeks in advance of the pathogenic *V. dahliae* isolate VdLs16 (PD659-2w-VdLs16) (Fig. 1). This treatment, by far, had the lowest RME that was significantly different (RME = 0.36, CI = 0.31 to 0.41) from other treatments, except when PD659 was introduced 1 week prior to the inoculations with *V. dahliae* isolate VdLs16 (PD659-1w-VdLs16, RME = 0.44, CI = 0.37 to 0.51) (Fig. 1). Treatments involving PD660 and *T. flavus* strain NRRL15936 also had significantly lower RME than VdLs16 alone, and all other treatments inoculated with VdLs16 prior to the inoculations with BCAs (Fig. 1). Overall, PD659 reduced Verticillium wilt severity the most relative to either PD660 or *T. flavus* when inoculated 2 weeks in advance of the pathogen. RME values were not significantly different between PD660 and *T. flavus* strain regardless of the treatment orders. All treatments where VdLs16 was introduced earlier than the biocontrol had

consistently higher RME (0.60 to 0.68). Individual BCAs had the lowest RME (0.24 to 0.26), followed by BCAs introduced 2 weeks (0.36 to 0.51) and 1 week (0.44 to 0.55) prior to inoculations with *V. dahliae* isolate VdLs16. Plants inoculated with VdLs16 had the highest (0.77) and significantly different RME than all other treatments (CI = 0.67 to 0.83) (Fig. 1).

The median DS was zero for all BCAs (NRRL15936, PD659, and PD660), which was not significantly different than the water control in all hosts (Fig. 1). The median DS of treatments that received BCA as coinoculation, or BCA inoculated 1 and 2 weeks in advance of inoculations with VdLs16 was lower (2.0) than that caused by VdLs16 (Fig. 1). The orthogonal contrasts between VdLs16 versus all other possible treatment combinations were highly significant ($P < 0.001$), except for VdLs16 versus VdLs16-1wk-PD659 ($P = 0.1697$) and VdLs16 versus VdLs16-2wk-PD659 ($P = 0.091$) (Table 2). These results provide additional evidence that the introduction of any of the BCAs (i.e., PD659, PD660, or *T. flavus* strain NRRL15936) as either coinoculation or 1 and 2 weeks in advance of the pathogenic strain significantly reduce Verticillium wilt caused by the pathogenic strain.

Biocontrol efficacy on lettuce, tomato, and eggplant against VdLs16

The BCAs *V. klebahnii* (PD659), *V. isaacii* (PD660), and *T. flavus* (NRRL15936) on individual hosts (i.e., eggplant, lettuce, and tomato) showed a host-dependent disease suppression of Verticillium wilt caused by VdLs16. Of these, PD659 had higher efficacy than either PD660 or *T. flavus* strain NRRL15936 in all hosts (Fig. 2). In eggplant, PD659 inoculated 2 weeks before VdLs16 had the highest DRD (82.0%), followed by 1 week (72.9%) and coinoculation (63.3%), and also had consistently higher efficacy in lettuce (100%) and tomato (61.1%) (Fig. 2).

TABLE 2

Orthogonal contrasts of *Verticillium dahliae* isolate VdLs16 versus biocontrol treatments combined across the three hosts

Contrasts	df _N ^a	df _D	F value	P value
VdLs16 versus PD659	1	659	308.44	<0.0001
VdLs16 versus PD660	1	659	273.97	<0.0001
VdLs16 versus NRRL15936	1	659	230.15	<0.0001
VdLs16 versus PD659 + VdLs16	1	659	21.8	<0.0001
VdLs16 versus PD660 + VdLs16	1	659	18.05	<0.0001
VdLs16 versus NRRL15936 + VdLs16	1	659	30.57	<0.0001
VdLs16 versus PD659-1wk-VdLs16	1	659	20.27	<0.0001
VdLs16 versus PD660-1wk-VdLs16	1	659	39.52	<0.0001
VdLs16 versus NRRL15936-1wk-VdLs16	1	659	31.92	<0.0001
VdLs16 versus PD659-2wk-VdLs16	1	659	50.5	<0.0001
VdLs16 versus PD660-2wk-VdLs16	1	659	18.05	<0.0001
VdLs16 versus NRRL15936-2wk-VdLs16	1	659	57.31	<0.0001
VdLs16 versus VdLs16-1wk-PD659	1	659	1.89	0.1697
VdLs16 versus VdLs16-1wk-PD660	1	659	9.42	0.0022
VdLs16 versus VdLs16-1wk-NRRL15936	1	659	12.25	0.0005
VdLs16 versus VdLs16-2wk-PD659	1	659	2.68	0.091
VdLs16 versus VdLs16-2wk-PD660	1	659	8.47	0.0037
VdLs16 versus VdLs16-2wk-NRRL15936	1	659	4.55	0.0332

^a df_N = numerator degree of freedom and df_D = denominator degree of freedom.

TABLE 1

Analysis of variance (ANOVA) using ANOVA F-type statistics of biocontrol treatments on the ranked mean of disease severity

Effect	df _N ^a	df _D	F value	P value
Experiment	1	659	0.24	0.6263
Treatments	19	659	266.10	<0.0001
Hosts	2	659	41.95	<0.0001
Treatment $\times$ host	38	659	230.20	<0.0001

^a df_N = numerator degree of freedom and df_D = denominator degree of freedom.

In eggplant, the disease suppression by PD659 was higher than by PD660 regardless of the inoculation treatments. Similarly, disease suppression by PD659 was higher than by *T. flavus* strain NRRL15936, except when this strain was coinoculated with VdLs16 that suppressed Verticillium wilt to a higher degree than PD659 (70.8 versus 63.3%). In lettuce, the efficacy of PD659 (100%) was significantly higher than either with PD660 (53.5%) or *T. flavus* strain NRRL15936 (79.5%) when inoculated 2 weeks in advance of VdLs16. Although the overall efficacy of all BCAs used were lower in tomato, PD659 still had higher efficacy (37.6 to 60.1%) than PD660 (27.9 to 37.0%) and *T. flavus* strain NRRL15936 (25.5 to 27.9%) (Fig. 2).

Likewise, *V. isaacii* strain PD660 also showed a higher biocontrol efficacy on eggplant and lettuce than in tomato against VdLs16. In lettuce, disease reduction was 97.5% when PD660 was inoculated 1 week in advance of VdLs16, which was even higher than with PD659 (60.9%) for the same treatment. Similarly, DRD with PD660 was higher than with PD659 in coinoculation treatments (51.7 versus 16.9%). A comparable biocontrol efficacy was observed between PD660 and *T. flavus* strain NRRL15936 in all three hosts, except the coinoculation treatment in tomato and inoculation of PD660 1 week prior in lettuce, which had significantly higher efficacy than other combinations (Fig. 2).

The standard BCA *T. flavus* strain NRRL15936 alone ensured consistently higher biocontrol efficacy (42.3 to 79.5%) in eggplant and lettuce for all three inoculation treatments (coinoculation, 1 week, and 2 weeks in advance of inoculations with VdLs16) than in tomato (22.6 to 27.9%). It had significantly high efficacy when coinoculated in eggplant (70.8%) and preinoculated 2 weeks prior in lettuce (79.5%). All BCAs had at least 16.9% DRD regardless of the hosts or

time of BCA inoculation (Fig. 2). However, inoculations of VdLs16 before the BCAs resulted in very low or nonsignificant DRD (data not shown).

Biocontrol efficacy of PD659 and PD660 on eggplant, lettuce, and tomato against corresponding isolates from each host

The biological control efficacy of *V. klebahnii* (PD659) and *V. isaacii* (PD660) was further confirmed by challenging each host with an isolate from the corresponding host. The ANOVA indicated highly significant differences among the biocontrol treatments in each host ($P < 0.001$) (Table 3). Plants inoculated with BCAs developed no Verticillium wilt symptoms aboveground and vascular tissue discoloration on the taproots.

Biocontrol efficacy against Sm113 in eggplant. Both PD659 and PD660 reduced Verticillium wilt in eggplant challenged with the eggplant isolate Sm113 (Fig. 3). Plants inoculated with Sm113 alone exhibited clear Verticillium wilt symptoms such as severe stunting, dried leaves that curled upwards, and

TABLE 3

Nonparametric analysis of variance (ANOVA) statistics on Verticillium wilt responses on biocontrol treatments to host-dependent isolates

Treatment effects	ANOVA-type statistic			
	df _N ^a	df _D	F	P value
Eggplant	2.76	55.7	61.55	<0.0001
Lettuce	7.37	154	16.74	<0.0001
Tomato	2.83	153	247.7	<0.0001

^a df_N = numerator degree of freedom and df_D = denominator degree of freedom.

FIGURE 2

Biocontrol efficacy of *Verticillium klebahnii* (isolate PD659), *V. isaacii* (isolate PD660), and *Talaromyces flavus* (strain NRRL15936) evaluated as the decreased rate of disease on A, eggplant; B, lettuce; and C, tomato caused by the *V. dahliae* isolate (VdLs16) from lettuce. The horizontal line inside the bar represents the standard error of the mean from two independent experiments.

extensive vascular discoloration of the taproot and stem compared with the treatments that received PD659, PD660, or water (Fig. 3A). Plants were significantly taller in BCA treatments than plants inoculated with Sm113 and Water-2w-Sm113 inoculation but significantly lower than in uninoculated controls (Fig. 3B). The RME values for both biocontrol strains were significantly lower than the pathogenic isolates inoculated at either 2 weeks (Sm113) or 4 weeks (Water-2w-Sm113) (Fig. 3C). The differences between PD659 and PD660 ($P < 0.05$) for RME or plant height were not significant.

Plant height had highly significant positive correlation to total plant biomass (root + shoot) ($r = 0.93$, $P < 0.001$) (Fig. 4A). Both biomass and plant height were negatively correlated with DS (Fig. 4BE) and disease incidence (Fig. 4CF). However, a highly significant positive correlation existed between DS and disease incidence ($r = 0.97$, $P < 0.001$) (Fig. 4D), suggesting that either variable can be used to assess the effectiveness of treatments.

Biocontrol efficacy against VdLs16 and VdLs17 in lettuce. Consistent with the previous experiments, lettuce inoculated with *V. dahliae* isolates VdLs16 (race 1) and VdLs17 (race 2) exhibited foliar wilt symptoms and taproot vascular tissue discoloration in susceptible Salinas lettuce. The VdLs17-inoculated plants had a higher RME than those inoculated

with VdLs16 but values were not significantly different ($P < 0.05$). Even though RME values for the different treatments were varied, they were not significantly different from each other when inoculated with VdLs17 ($P < 0.05$). Both PD659 (RME = 0.37) and PD660 (RME = 0.36) had significantly lower DS and RME values than the VdLs16-positive control when inoculated on 2-week-old seedlings (VdLs16, RME = 0.65) but not for seedlings inoculated at 4 weeks (Water-2w-VdLs16, RME = 0.40). In the case of VdLs17, the DS was reduced moderately when BCAs were applied 2 weeks before VdLs17 inoculation but the RME was not significantly different between control and treated plants (Fig. 5).

Biocontrol efficacy against Le1811 in tomato. Early Pak tomato inoculated with the tomato *V. dahliae* isolate Le1811 developed Verticillium wilt symptoms, including plant stunting, chlorosis, and wilting of lower leaves (Fig. 6A). Vascular tissue discoloration of the taproot and stem was also observed. In general, a more prominent plant canopy and significantly taller plants were associated with both BCAs (PD659 and PD660) than with Le1811, though they weren't significantly different from the Water-2w-Le1811 treatment (Fig. 6B). The RME values indicated a significant reduction in Verticillium wilt severity by both BCAs regardless of the time of pathogenic isolate inoculation (Fig. 6C).

FIGURE 3 Biological control effect of *Verticillium klebahnii* (PD659) and *V. isaacii* (PD660) against *V. dahliae* isolate Sm113 from the eggplant. **A**, Morphological changes; **B**, plant height; and **C**, relative marginal effect (RME) (numbers inside the bars represent mean disease severity) and in response to various treatment combinations were shown. RME was calculated using LD_CI macros (Shah and Madden 2004) in SAS 9.4. Pictures were taken at the time of disease rating at approximately 8 weeks postinoculation with the pathogenic isolate. Four-week-old seedlings were inoculated with pathogenic isolates Sm113 that received either biocontrol (PD659 or PD660) or water treatment at 14 days after germination. Seedlings were 2 weeks old at the time of inoculations with PD659, PD660, and Sm113 but were 4 weeks old in all other treatments.

FIGURE 4

Correlation among plant height, biomass, disease severity, and disease incidence of all treatments in eggplant. Four-week-old seedlings were inoculated with isolate Sm113 that had received either biocontrol (PD659 or PD660) or water treatment at 14 days after germination. **A**, Biomass versus plant height; **B**, disease severity versus plant height; **C**, disease incidence versus plant height; **D**, disease severity versus disease incidence; **E**, disease severity versus biomass; and **F**, disease incidence versus biomass.

FIGURE 5

Relative marginal effect (RME) values of *Verticillium klebahnii* (PD659) and *V. isaacii* (PD660) against isolates VdLs16 and VdLs17 from lettuce. RME values were calculated using LD_CI macros (Shah and Madden 2004) in SAS 9.4. Numbers inside the bars represent mean disease severity. Both biocontrol and control isolates were first inoculated to 2-week-old seedlings using root dipping. Two weeks after inoculation (4-week-old seedlings), pathogenic isolates VdLs16 and VdLs17 were inoculated to seedlings that received either biocontrol (PD659 or PD660) or water treatment at 2 weeks old. Seedlings were 2 weeks old at the time of inoculations with PD659, PD660, VdLs16, or VdLs17 but were 4 weeks old in all other treatments.

Confocal microscopy of *V. dahliae* progress on eggplant and tomato

Pathogen colonization in the vascular systems of eggplant and tomato following inoculations of the GFP-tagged *V. dahliae* in the presence or absence of BCA *V. klebahnii* PD659 were determined by confocal microscopy. In eggplant, at 17 days postinoculation (dpi), the colonization of vascular tissue at the stem base (1 cm above the soil line) was visible slightly in the

presence of biocontrol (Fig. 7A) compared with the visibly higher vascular tissue colonization in treatments without BCA (Fig. 7B). However, at 34 dpi, vascular tissue colonization levels were indistinguishable between the treatments (Fig. 7C and D). In tomato, however, no visible colonization of the vascular tissues was evident in the BCA-treated plants (Fig. 7E). In contrast, a moderate level of colonization was distinct in the absence of biocontrol (Fig. 7F) at 17 DAI. A similar trend occurred at 34 DAI, where only a few colonized vessels were observed in

FIGURE 6 Biological control effect of *Verticillium klebahnii* (PD659) and *V. isaacii* (PD660) against isolate Le1811 from tomato. **A**, Morphological changes; **B**, plant height; and **C**, relative marginal effects (RME) (numbers inside the bars represent mean disease severity). RME were calculated using LD_CI macros (Shah and Madden 2004) in SAS 9.4. Confidence intervals for PD659-2w-Le1811, PD660-2w-Le1811, and Water-2w-Le1811 treated with the two biocontrol agents were 0.5079 and 0.5999, 0.5501 and 0.6367, and 0.6369 and 0.7095, respectively, while for Le1811 they were 0.9119 and 0.9275. Pictures were taken at the time of disease rating at approximately 8 weeks postinoculation of the pathogenic isolate. Four-week-old-seedlings were inoculated with pathogenic strain Le1811 that received either biocontrol (PD659 or PD660) or water treatment at 14 days.

BCA-treated plants (Fig. 7G), whereas the vascular tissue was extensively colonized in plants inoculated with the Le1811 isolate alone (Fig. 7H).

Altered expression of defense response genes to biocontrol treatments

The putative BCA *V. klebahnii* isolate PD659, when inoculated 2 weeks in advance of the pathogen, prevented recognition of the pathogen, as indicated by the lack of induced resistance gene expression in biocontrol-treated tomato plants. None of the genes evaluated (i.e., *PR1a*, *PR5*, *GlucA*, *GlucB*, *Chi3*, *Chi9*, and *cysProtease*) had significant expression or upregulation in biocontrol-treated plants at all time points (24 h postinoculation [hpi] to 2 wpi) relative to the expression in mock-inoculated plants (Fig. 8).

However, in plants inoculated with the pathogenic *V. dahliae* isolate Le1811, significant upregulation of the PR genes (i.e., *PR1a*, *PR5*, *GlucA*, *GlucB*, *Chit3*, *Chit9*, and *cysProtease*) at various time points relative to their expression in water-treated plants was observed (Fig. 8). *PR1a* was the most highly upregulated gene at 24 hpi (18.8-fold, $P < 0.01$) and 48 hpi (19.6-fold, $P = 0.086$), followed by *PR5* (7.4-fold, $P = 0.067$) and *GlucB* (10.7-fold, $P < 0.05$), respectively. However, the differences in expression were not significant at 1 or 2 wpi, except for *PR5* and *GlucA*, which exhibited increased expression in response to Le1811 at 2 wpi ($P < 0.05$) (Fig. 8).

DISCUSSION

The populations of *V. dahliae* were predominantly composed of race 1 and race 2 in lettuce and tomato, until recently. A third race was identified in tomato from Japan (Usami et al. 2017). Resistance against *V. dahliae* race 1 is available in several hosts, including lettuce and tomato (Alexander 1962; Hayes et al. 2007), and against race 2 only in wild tomato species *Solanum neorickii* (Usami et al. 2017). Partial resistance, however, is available against it in lettuce (Sandoya et al. in press). Despite the availability of resistance against race 1 in lettuce, no commercial cultivars with resistance have been deployed. The phase-out of widely used soil fumigant MB left few effective chemical alternatives for disease management (Duniway 2002; Meadows 2013). This calls for the integration of complementary management options. BCAs could be a dependable alternative for managing *V. dahliae* (Fravel 2005) provided locally adapted strains with efficacy are identified. Therefore, we assessed one isolate each from *V. klebahnii* (PD659) and *V. isaacii* (PD660) for their biocontrol potential against Verticillium wilt caused by *V. dahliae* in eggplant, lettuce, and tomato. We determined that the nonpathogenic *V. isaacii* (PD660) and *V. klebahnii* (PD659) isolates protected crops against virulent strains of *V. dahliae* in a host-dependent manner, with higher efficacy when inoculated in advance of the pathogenic strain. Previous studies have also demonstrated biocontrol efficacy of certain

FIGURE 7 Transverse sections of eggplant and tomato stem colonized by a green fluorescent protein (GFP)-expressing isolate of *Verticillium dahliae* Le1811 in the presence or absence of biocontrol treatments. The stem base at 1 cm above the soil line was sampled at 17 (upper) and 34 (lower) days after inoculation (DAI) with the GFP-tagged isolate for imaging. Biocontrol treatments received PD659 inoculation on 2-week-old seedlings followed by inoculations with the GFP-tagged isolate Le1811 after 14 days on **A** and **C**, Long Purple eggplant and **E** and **G**, Beefsteak tomato. Controls **B** and **D**, eggplant and **F** and **H**, tomato were first inoculated with water followed by the pathogenic isolate at the same time as biocontrol agent-inoculated plants received the GFP-tagged isolate Le1811. Imaging was done using a Lecia confocal laser-scanning microscope at 10x, where Tile Scan Acquisition Mode was used for all images except for H.

strains of *V. tricornis* and *V. isaacii* (formerly *V. tricornis*) in Verticillium wilt suppression of eggplant, lettuce, cauliflower, and potato via endophytism, cross-protection, and induced resistance (Davis et al. 2000; Qin et al. 2008; Robinson et al. 2007; Tyvaert et al. 2014). The known fungal antagonist *T. flavus*, which was used as a reference BCA in the present study, also exhibits hyperparasitism to a broad spectrum of soilborne pathogens or directly kills microsclerotia of *V. dahliae* (Fravel 1989; Marois et al. 1982, 1984; Nagtzaam et al. 1998; Yuan et al. 2017; Zeise and Kersten 2000). Thus, the higher or comparable efficacy of PD659 and PD660 relative to *T. flavus* strain NRRL15936 positions them as potentially valuable BCAs for Verticillium wilt management. These strains are much better adapted to the agricultural ecosystems from which they were isolated and would not encounter the problems of a BCA that is unadapted to the system.

Although it was not possible to track the colocalization of pathogenic and BCA strains on the root surface or inside the roots during infection, it is evident that these BCAs excluded the pathogenic strains by competing for the same ecological niche, space, and nutrients in a host-dependent manner. A significantly higher biocontrol efficacy against host-dependent isolates in eggplant and tomato relative to lettuce is likely due to host-specific endophytic behavior of these BCAs on different hosts (Malcolm et al. 2013; Tyvaert et al. 2014). In lettuce, the pathogenic strain may take advantage of dense feeder root architecture that has not been entirely occupied by BCAs and initiate infection at later stages. Qin et al. (2008) demonstrated variability in biocontrol efficacy and suggested specificity of nonpathogenic *V. tricornis* isolates VdLs432, VdLs441, and VdLs443 in lettuce. These isolates, which were subsequently reclassified as *V. isaacii* (Inderbitzin et al.

2011), were highly effective in reducing DS caused by *V. dahliae* isolate VdLs17 but not VdLs16 (Qin et al. 2008). The infection of *V. isaacii* isolates was dependent on the crop species and cultivars (Gurung et al. 2015). The *V. isaacii* isolate Vt305 (previously classified as *V. tricornis*) from cauliflower effectively reduced the DS caused by *V. longisporum* in cauliflower (Tyvaert et al. 2014). Other known fungal antagonists (i.e., genera *Trichoderma*, *Talaromyces*, or bacterial endophytes such as *Bacillus*, *Pseudomonas*, and so on) were effective in providing cross-protection against various soilborne pathogens, including *V. dahliae*, *Rhizoctonia solani*, and *Sclerotinia sclerotiorum*, in a host-dependent manner (Eljounaidi et al. 2016; Madi et al. 1997). Therefore, the interactions between different *Verticillium* BCAs and host species have played a role in determining their efficacy. The disparity in the effectiveness of these BCAs in the first and second experiment in lettuce was likely due to the age of seedlings when they first received the pathogen. In the first experiment, comparisons were made with lettuce seedlings that were inoculated with the pathogen at 2 weeks after germination while, in the second experiment, seedlings were 4 weeks old when they were inoculated. This interaction signifies that the infection establishment occurs more efficiently and rapidly on younger seedlings than in older seedlings and likely plays a critical role in the overall disease progression. Thus, seedling transplanting rather than direct seeding may be an alternative strategy to reduce Verticillium wilt in lettuce fields with a history of consistently high Verticillium wilt incidence.

Furthermore, the efficacy of BCAs against soilborne plant pathogens largely depends on application timing and delivery mechanism (Angelopoulou et al. 2014; El-Hassan and Gowen 2006). We found a significantly high efficacy of PD659 when

FIGURE 8 Relative expression of defense response genes in Early Pak tomato in the presence and absence of biocontrol agents (BCAs) and normalized with tomato *actin* as the reference gene. BCA PD659 was inoculated 2 weeks before pathogenic *Verticillium dahliae* isolate Le1811. Leaf tissues were harvested at 24 and 48 h after pathogen inoculation (HPI) and 1 and 2 weeks after pathogen inoculation (WPI) on BCA or water-treated plants. Mean expression and error was determined using the relative expression software REST (Pfaffl et al. 2002). Quantitation cycle values from three biological replicates, each combined from three technical replicates, were examined. Genes at least twofold upregulated and significant at $P < 0.09$, 0.05 , and 0.001 are indicated by *, **, or ***, respectively.

introduced 2 weeks in advance of the pathogenic isolates in the greenhouse. A similar interaction was observed in vitro when endophytic strain Dvd-E6 inoculated before or together with the pathogenic *V. dahliae* strain Vd1 and led to the complete exclusion of pathogenic strain from tomato roots (Shittu et al. 2009). Qin et al. (2008) observed better protection against Verticillium wilt of lettuce when individual isolates of *V. isaacii* were inoculated 1 week in advance of the pathogenic strain rather than a coinoculated treatment. Preinoculation of a nonpathogenic *V. albo-atrum* group 2 isolate 4 days prior to the pathogenic *V. tricorpus* reduced wilt severity more than the simultaneous inoculation of both in potato (Robinson et al. 2007). Other fungi such as *Gibellulopsis nigrescens* (reclassified from *Verticillium* by Zare et al. [2007]) isolate CVn-WHg, and nonpathogenic *V. dahliae* isolate CVD-WHw reduced Verticillium wilt incidence significantly (77.9 to 95%) when inoculated 4 days before a highly virulent *V. dahliae* isolate CVD-AYb in cotton (Zhu et al. 2013). *V. albo-atrum* strain WCS850 effectively reduced the Dutch elm disease and is now available as a commercial formulation for injection into tree trunks (Postma and Goossen-van de Geijn 2016).

When direct seeding is not possible, lettuce is generally transplanted into the fields in coastal California. Thus, the introduction of these BCAs during transplant production in seeding trays at least 2 weeks prior to transplanting likely protects plants against Verticillium wilt in the field. Seed coating of beneficial microbes in vegetables and other crops is widely practiced to promote plant vigor and protect against many diseases (Rocha et al. 2019), including *V. dahliae* (Müller and Berg 2008) and *V. longisporum* (Deketelaere et al. 2020). Integration of *Bacillus subtilis* as seed treatment with carriers such as glucose and peat provided better disease suppression against *F. oxysporum* f. sp. *lentils* (El-Hassan and Gowen 2006). Thus, it is worth investigating the possibility of developing a seed-priming technique using PD659 in lettuce.

Instead of a universal mechanism by which nonpathogenic isolates of *Verticillium* spp. protect against pathogenic *V. dahliae* strains in all systems, there could be multiple modes of action. We observed a significant increase in plant biomass and plant height that directly correlated with low disease incidence and DS in the presence of BCAs. The reduced Verticillium wilt severity in tomato, eggplant, and other solanaceous crops was associated with increased plant vigor due to increased nitrogen and phosphorus uptake after using arbuscular mycorrhizal fungus (Demir et al. 2015; Karagiannidis et al. 2002). BCA *Purpureocillium lilacinum* QLP12 promoted seed germination (76.7%), bud growth (79.4%), chlorophyll content (47.83%), and root activity (182.02%), which altogether led to a significant decline in Verticillium wilt incidence by 83.82% in eggplant (Lan et al. 2017). Thus, the applied BCAs could have contributed to better nutrient uptake, healthier plants, better plant canopy, and reduced *V. dahliae* colonization. In addition, a strong correlation between biomass and plant height in both eggplant and tomato could be taken as an indicator of low Verticillium wilt incidence and severity and, thus, can be utilized to avoid labor-intensive disease scoring in the greenhouse or the field.

Several factors, including ecological fitness, host resistance, pathogen inoculum load, and so on, might play a role in a BCA's success (Deketelaere et al. 2017). *T. flavus* showed better protection when coinoculated in eggplant because it is capable of producing glucose oxidase, which has a lethal effect on the microsclerotia (Kim et al. 1988; Stosz et al. 1996) and inhibits microsclerotial germination (Marois et al.

1982). The lower and similar efficacy in tomato and nonsignificant interaction to inoculation time points in lettuce against VdLs16 signifies a host-dependent interaction of *T. flavus*. Integrating enhanced GFP in biocontrol and Discosoma Red Fluorescent Protein reporter genes in pathogenic strains, Pantelides et al. (2009) concluded that *F. oxysporum* strain F2 suppressed *V. dahliae* via increasing competition for space or nutrients on the root surface as the primary mode of action. It is quite likely that competition for space and nutrients was the primary mode of action observed in this study for *V. isaacii* and *V. klebahnii* rather than antagonism, as in *T. flavus*.

Many plant vascular system colonizing endophytes also induce PR proteins that activate systemic acquired resistance or induced systemic resistance in *Verticillium* interactions in hosts such as tomato, eggplants, and *Arabidopsis* (Antonopoulos et al. 2008; Tjamos et al. 2005; van Loon et al. 2006). For example, pepper plants inoculated with *V. dahliae* following inoculations with the nonpathogenic *F. oxysporum* (Fo47) showed a higher relative expression of PR-1 protein (*CABPR1*), a class II chitinase (*CACHI2*), and a sesquiterpene cyclase in root and stem tissues (Veloso and Díaz 2012). The soil application of 5% dry mycelium of *Penicillium chrysogenum* provided resistance to *V. dahliae* in cotton by inducing peroxidase and lignin deposition in hypocotyls (Dong et al. 2003). The *Paenibacillus alvei* K165 and the nonpathogenic *F. oxysporum* F2 enhanced the expression of *PR1* and *PR4* in eggplant as a *V. dahliae* defense response (Angelopoulou et al. 2014). In contrast, our results showed an opposite association, where the application of BCAs decreased the level of PR protein-related gene expression in pathogen-challenged treatments. Such upregulation of defense-related genes agrees with a host-susceptibility model or constitutive resistance rather than induced resistance (Mandelc et al. 2013). The host plants without BCA were easily infected by *V. dahliae* and quickly mounted a robust defensive response via upregulation of induced resistance genes at the early infection stage (until 48 h) but, ultimately, failed as the pathogen was established. Such noticeable changes in protein levels or expression of other known plant defense proteins, including PR-5, PR-10, chitinase, and β -glucanase in cotton and PR-2, PR-4, and so on in oilseed rape, were induced significantly in susceptible plants but only a modest variation was seen in a resistant plant in response to *V. dahliae* (McFadden et al. 2001; Robb et al. 2012; Xu et al. 2011). A systemic increase in plant defense proteins PR-1, chitinase (PR-3), β -1,3-glucanase (PR-2), and a thaumatin-like protein (PR-5) was significant in susceptible hop plants in response to a highly virulent *V. albo-atrum* strain (Cregeen et al. 2015). The PR-1 protein upregulated 153-fold in susceptible but 1.5-fold in resistant hops plants at 10 dpi (Cregeen et al. 2015). Robb et al. (2007) found a similar phenomenon, where genes strongly expressed in susceptible interactions were downregulated in tolerant tomato in response to *V. dahliae*. Klosterman et al. (2011) reported a similar relationship, where *PR-3*, *PR-5*, and a cysteine protease (*LsCP2*) were expressed only in symptomatic lettuce leaves at 3 wpi with *V. dahliae*. The PR protein genes *Chi3*, *Chi9*, *GluA*, *GluB*, and *PR-1a* had significantly low expression in the plants or cells inoculated with biocontrol strain *F. oxysporum* Fo47 (Aimé et al. 2008). Thus, it is likely that the biocontrol strains excluded *V. dahliae* from the root surface (Shittu et al. 2009; Tyvaert et al. 2014), preventing pathogen recognition. This assumption was strongly supported by our tracking of the GFP-tagged pathogen, where only limited colonization was observed in both eggplant and tomato vascular

systems in the presence of BCAs. Despite these studies conducted on the most susceptible cultivars of each host, the BCAs reduced the disease caused by the most virulent strains. Combining BCAs with even moderately resistant cultivars of individual crops may dramatically reduce the incidence of *Verticillium* wilt on these crops. Thus, priming tomato plants with BCA PD659 could reduce disease establishment and provide cross-protection by limiting the pathogen's niche resources.

In conclusion, we evaluated the biocontrol efficacy of *V. isaacii* isolate PD660 and *V. klebahnii* isolate PD659, two species that are known to be endophytes and are occasionally isolated from asymptomatic lettuce or spinach plants (Gurung et al. 2015). Both BCAs resulted in significant control of the highly virulent isolate VdLs16 or other host-dependent isolates derived from the three crops evaluated in this study. Of the treatments evaluated, *V. klebahnii* isolate PD659, when introduced 2 weeks before *V. dahliae* inoculation, showed a significantly high biological control effect, compared with *T. flavus* and *V. isaacii*. Competitive exclusion might be the primary mode of action where disease initiation was delayed in a host-dependent manner. In light of these findings, seed coating of dicotyledonous crops with *V. klebahnii* isolate PD659 or soil-plug inoculation at least 2 weeks before transplanting could provide an alternative management tool against *Verticillium* wilt, which will eventually be a part of an integrated *Verticillium* wilt management. However, further research and optimization tests will be needed to develop these BCAs as a seed coating. We recognize that genetic exchanges between pathogenic and biocontrol *Verticillium* spp. strains have not been evaluated yet and, thus, should be taken under consideration before a large-scale introduction of these novel nonpathogenic strains on a commercial scale.

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