Volatile Compounds Emitted by Diverse *Verticillium* Species Enhance Plant Growth by Manipulating Auxin Signaling

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Some volatile compounds (VC) play critical roles in intra- and interspecies interactions. To investigate roles of VC in fungal ecology, we characterized how VC produced by *Verticillium* spp., a group of broad-host-range soilborne fungal pathogens, affect plant growth and development. VC produced by 19 strains corresponding to 10 species significantly enhanced the growth of *Arabidopsis thaliana* and *Nicotiana benthamiana*. Analysis of VC produced by four species revealed the presence of diverse compounds, including those previously shown to affect plant growth. Using *A. thaliana*, we investigated the mechanism underpinning plant growth enhancement by *Verticillium dahliae* VC. Allometric analysis indicated that VC caused preferential resource allocation for root growth over shoot growth. Growth responses of *A. thaliana* mutants defective in auxin or ethylene signaling suggested the involvement of several components of auxin signaling, with TIR3 playing a key role. AUX1, TIR1, and AXR1 were also implicated but appeared to play lesser roles. Inhibition of auxin efflux using 1-naphthylphthalamic acid blocked VC-mediated growth enhancement. Spatial and temporal expression patterns of the auxin-responsive reporter *DR5::GUS* indicated that the activation of auxin signaling occurred before enhanced plant growth became visible. Results from this study suggest critical yet overlooked roles of VC in *Verticillium* ecology and pathology.

All organisms employ multiple mechanisms to recognize, communicate with, and manipulate their neighbors. Secretion

of semiochemicals (= infochemicals), diverse types of metabolites that evoke specific behavioral or physiological responses in receiving organisms, is one such mechanism (Law and Regnier 1971). Certain classes of primary and secondary metabolites with high vapor pressure under ambient conditions function as volatile signals. Because volatile compounds (VC) can travel through water, air, and porous soils (Effmert et al. 2012), they can mediate both short- and long-distance organismal interactions even when water as a medium is absent. Plants, animals, and microbes have evolved to produce and recognize semio-VC, and VC-mediated signaling serves diverse vital ecological functions (Baldwin 2010; Bitas et al. 2013; Herrmann 2010; Kaiser 2006; Li et al. 2016; Schenkel et al. 2015). For example, plants attacked by insect herbivores release specific VC to attract parasitoids for pest control (Baldwin 2010). Animals use specific VC to recognize foods, mates, and threats (Bergström 2008). Similarly, accumulating evidence implicates microbial VC in antagonism, mutualism, and regulation of cellular and developmental processes (Bitas et al. 2013; Schmidt et al. 2015).

VC produced by a number of plant-associated fungi have been shown to affect plant physiology, growth, or development. VC released by ectomycorrhizal fungi *Laccaria bicolor* and truffles induced lateral root growth in both host and nonhost plants (Ditengou et al. 2015; Splivallo et al. 2009). Volatile sesquiterpene (-)-thujopsene, which was shown to be emitted by *L. bicolor*, was sufficient to stimulate lateral root formation (Ditengou et al. 2015). Similarly, VC produced by multiple *Trichoderma* spp. enhanced the growth of *Arabidopsis thaliana* (Hung et al. 2013; Lee et al. 2016). When 6-pentyl-2H-pyran-2-one, an unsaturated lactone produced by several *Trichoderma* spp., was exogenously applied at concentrations of 50 to 175 μ M, it enhanced plant growth (Garnica-Vergara et al. 2016; Kottb et al. 2015). However plant growth was inhibited when the concentration reached 2 mM. The growth of *A. thaliana* was enhanced by VC emitted by 30 phylogenetically diverse bacterial and fungal species (Sánchez-López et al. 2016). *Trichoderma* VC have been shown to induce systemic resistance in *A. thaliana* and tomato (Martínez-Medina et al. 2017), illustrating multiple roles of fungal VC in association with plants. Diverse roles of fungal VC in plant interactions were reviewed by Li et al. (2016).

Here, we investigated how VC produced by all known *Verticillium* spp. affect plant growth and development, as well as the mechanism underpinning resulting changes. Members of the genus *Verticillium* cause Verticillium wilt, a destructive

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soilborne disease on numerous crops and other economically or environmentally important plants around the world (Inderbitzin et al. 2011; Pegg and Brady 2002). Among the 10 species described (Inderbitzin et al. 2011), *Verticillium dahliae*, *V. alfalfae*, and *V. longisporium* are most destructive, causing billions of dollars of crop loss worldwide (Inderbitzin and Subbarao 2014;

Pegg and Brady 2002). The importance of other newly erected species is only now becoming apparent (Gurung et al. 2015; Kasson et al. 2014; Short et al. 2015). Their soil habitat, long-term persistence in infested soils, and ability to infect a broad spectrum of plants, as well as the scarcity of resistant host germplasm, make effective control of *Verticillium* wilt very challenging (Agrios 2005; Chawla et al. 2012). Research on *Verticillium* VC was driven, in part, by our previous study on VC produced by *Fusarium oxysporum*, another major soilborne fungal pathogen that causes Fusarium wilt. Most *F. oxysporum* strains enhanced the growth of *A. thaliana* and tobacco via VC production; however, VC produced by some strains inhibited or killed *A. thaliana* (Bitas et al. 2015). Genetic and histochemical analyses revealed the involvement of auxin signaling—in particular, polar auxin transport (PAT) and the functions of AUX1 and TIR1—in causing growth enhancement and increased lateral root formation by *F. oxysporum* VC (Bitas et al. 2015). The present study was aimed at expanding our knowledge of VC-mediated plant–fungus interactions and their roles in the ecology and pathology of soilborne fungi. A better understanding of this underexplored process will help develop novel disease management strategies.

RESULTS

Volatile compounds produced by diverse *Verticillium* spp. enhanced the growth of *A. thaliana* and *Nicotiana benthamiana*.

To investigate the effect of *Verticillium* VC on plant growth and development, seedlings of *A. thaliana* ecotype Col-0 were exposed to VC produced by 19 strains corresponding to 10 *Verticillium* spp. (Table 1; Supplementary Figure S1). After 14 days of cocultivation, seedlings exposed to *Verticillium* VC displayed significantly higher shoot fresh weight than those grown in the absence of *Verticillium* (Fig. 1), with the magnitude of increase ranging from 3.3-fold (*V. isaacii* PD343) to 5.9-fold (*V. zaregamsianum* PD736). Seedlings exposed to VC also developed more leaves (approximately 9) than control plants

Table 1. *Verticillium* strains used in this study

Strain ^a	Species	Host of origin	Location of isolation
PD343	<i>Verticillium isaacii</i>	Artichoke	California, United States
PD753	<i>V. isaacii</i>	Spinach seed	Washington, United States
PD401	<i>V. klebahnii</i>	Lettuce	California, United States
PD659	<i>V. klebahnii</i>	Lettuce	California, United States
PD690	<i>V. tricorpus</i>	Tomato	United Kingdom
PD703	<i>V. tricorpus</i>	Carnation	The Netherlands
PD736	<i>V. zaregamsianum</i>	Lettuce	Japan
PD739	<i>V. zaregamsianum</i>	Stock	Japan
PD670	<i>V. albo-atrum</i>	Potato	Wisconsin, United States
PD693	<i>V. albo-atrum</i>	Potato	United Kingdom
PD621	<i>V. nubilum</i>	Potato	United Kingdom
PD702	<i>V. nubilum</i>	Potato	United Kingdom
PD489	<i>V. alfalfae</i>	Alfalfa	United States
PD626	<i>V. nonalfalfae</i>	Hop	United Kingdom
PD810	<i>V. nonalfalfae</i>	Petunia	Slovenia
PD687	<i>V. longisporium</i> lineage D3	Horseradish	Germany
PD348	<i>V. longisporium</i> lineage D1	Cauliflower	California, United States
PD322	<i>V. dahliae</i>	Lettuce	California, United States
PD413	<i>V. dahliae</i>	Lettuce	California, United States

^a These strains were chosen among those used for a genus-wide phylogenetic analysis of *Verticillium* (Inderbitzin et al. 2011), with the exception of *V. dahliae* PD413.

Fig. 1. Growth response of *Arabidopsis thaliana* to volatile compounds (VC) produced by *Verticillium* spp. Mean shoot fresh weights of ecotype Col-0 seedlings after 14 days of cocultivation with no fungus (Control) and 19 strains corresponding to 10 *Verticillium* spp. are shown. Vi = *Verticillium isaacii*, Vk = *V. klebahnii*, Vt = *V. tricorpus*, Vz = *V. zaregamsianum*, Vaa = *V. albo-atrum*, Vn = *V. nubilum*, Va = *V. alfalfae*, Vna = *V. nonalfalfae*, Vl = *V. longisporium*, and Vd = *V. dahliae*. Values shown correspond to the mean ± standard error of data from three replicates ($n = 15$). Asterisks indicate significant differences between VC-treated and control seedlings according to Tukey's test at $P = 0.05$. The inset shows Col-0 seedlings after cocultivation with no fungus (left) and *V. dahliae* PD413 (right).

(approximately 7). VC emitted by all *Verticillium* strains enhanced the growth of *Nicotiana benthamiana*, ranging from 2.3-fold (*V. alfalfae* PD489) to 3.2-fold (*V. dahliae* PD322) (Supplementary Fig. S2). However, the number of leaves did not increase.

Growth responses of eight *Arabidopsis* ecotypes to VC produced by two *V. dahliae* strains, PD322 and PD413, were also measured (Supplementary Fig. S3). All of the ecotypes exhibited significantly increased shoot biomass compared with controls. With the exception of C24, which only displayed increased lateral root elongation, all ecotypes exposed to VC displayed significantly higher lateral root number and density than control plants.

Growth enhancement by *V. dahliae* VC became visible as early as 5 to 7 days after cocultivation, and the degree of growth enhancement correlated with the duration of VC exposure.

To determine how the growth of *A. thaliana* changed during cocultivation with *V. dahliae* strains PD312 and PD413, seedlings were collected at multiple time points for shoot weight measurement starting at day 3 (Fig. 2). Measurement of root fresh weight started at day 7, because accurate weight measurement at earlier time points was difficult. Differences in shoot growth between VC-exposed and control plants became notable between days 5 and 7 (Fig. 2A). Similarly, enhanced root growth (Fig. 2B) and lateral root development (Supplementary Fig. S4)

were apparent at day 7. As expected from the growth curves (Fig. 2), VC-exposed plants exhibited constantly higher relative growth rate of shoots and roots compared with those of control plants (Table 2). Between days 10 and 14, a sharp increase in biomass occurred, resulting in threefold (shoot) and ninefold (root) increases at day 14 (Fig. 2).

To further investigate the relationship between the degree of growth enhancement and the duration of VC exposure, *V. dahliae* culture was removed at 5, 7, and 10 days after cocultivation; however, *A. thaliana* seedlings were not harvested until day 14 (Supplementary Fig. S5). Five days of VC exposure were insufficient to enhance growth. However, when VC exposure was extended to 7 days, 90% (PD322) and 70% (PD413) increases in shoot fresh weight were observed. After 10 and 14 days of VC exposure, two- and fourfold increases were observed, respectively.

Other changes in *A. thaliana* caused by *V. dahliae* VC.

In addition to significantly increased lateral root number and density in response to *V. dahliae* VC, leaf surface area, total root length, and root branching also increased (Fig. 3). However, the primary root length (Fig. 3B) and leaf chlorophyll content (Fig. 3D) did not change significantly compared with controls. Comparison of leaf relative water content (Fig. 3E) showed that increased shoot fresh weight was not due to increased water accumulation.

To investigate the coordination of growth between shoots and roots in response to *Verticillium* VC, we calculated the allometric coefficient, *k*, an approach that has been widely used to study resource allocation (Crookshanks et al. 1998; Hunt 2017; Jin et al. 2009; Ristova and Busch 2014; Shipley and Meziane 2002; Veresoglou et al. 2012; Wright and Westoby 2000). A value of *k* > 1 indicates that roots grow faster than shoots, suggesting more resource allocation to root growth. Compared with the *k* of control plants, plants exposed to *V. dahliae* VC displayed significantly higher *k* values (Fig. 4).

Several components of auxin signaling are involved in *V. dahliae* VC-mediated growth enhancement.

Both auxin and ethylene affect many aspects of plant growth and development, including primary root elongation, lateral root development, and apical hook formation (Muday et al. 2015; Růžicka et al. 2007). The formation of highly branched roots (Fig. 3F) and preferential enhancement of root growth (Fig. 4) in response to *V. dahliae* VC suggested the involvement of these hormones in regulating plant responses. To test this hypothesis, we screened *A. thaliana* mutants impaired in PAT, auxin signaling, or ethylene signaling. The mutants included *pin-formed 1* (*pin1-7*), *auxin-resistant 1* (*aux1-7*), *transport inhibitor response 3* (*tir3-101*), *transport inhibitor response 1* (*tir1-1*), *auxin-resistant 1* (*axr1-3*), *ethylene response 1* (*etr1-1*), and *ethylene-insensitive 2* (*ein2*). The *tir3-101* mutation caused aberrant morphologies, including vitreous leaves, no lateral root formation, and restricted leaf expansion and petiole

Fig. 2. Growth of *Arabidopsis thaliana* during 14 days of cocultivation with two *Verticillium dahliae* strains. **A**, Shoot and **B**, root fresh weights of Col-0 at multiple time points during cocultivation with no fungus (Control) and *V. dahliae* strains PD322 and PD413 are shown. Values shown correspond to the mean $\pm$ standard error of data from three replicates ($n = 15$). Asterisks indicate significant differences between volatile compound-treated and control samples according to Tukey's test at $P = 0.05$.

Table 2. Influence of *Verticillium dahliae* volatile compounds on the relative growth rate of shoots (RGRS) and the relative growth rate of roots (RGRR) of *Arabidopsis thaliana* ecotype Col-0 at different stages of cocultivation

Treatments	RGRS ($\mu\text{g}^{-1} \text{day}^{-1}$) per day range					RGRR ($\mu\text{g}^{-1} \text{day}^{-1}$) per day range		
	3-5	5-7	7-10	10-12	12-14	7-10	10-12	12-14
Control	0.296	0.172	0.127	0.124	0.131	0.098	0.137	0.096
PD322	0.327	0.432	0.221	0.272	0.230	0.256	0.347	0.306
PD413	0.374	0.346	0.189	0.222	0.352	0.181	0.329	0.453

elongation, which is consistent with the critical role of TIR3 in growth and development (Gil et al. 2001; Guo et al. 2013). Growth of this mutant was not enhanced by *V. dahliae* VC (Fig. 5). Three other mutants, including *aux1-7*, *tir1-1*, and *axr1-3*, positively responded to *V. dahliae* VC but the degree of growth enhancement was lower than that in Col-0 (Fig. 5). In contrast, the responses of *pin1-7*, *etr1-1*, and *ein2* were indistinguishable from that of Col-0 (Fig. 5). Our previous study also showed the involvement of auxin signaling in mediating growth enhancement by *F. oxysporum* VC but did not include *axr1-3* and *tir3-101* (Bitas et al. 2015). Growth responses of these mutants to VC from two *F. oxysporum* strains used in our previous study, NRRL 26379 and NRRL 38335, were evaluated. The *tir3* mutant failed to respond to *F. oxysporum* VC, and the growth of *axr1-3* was enhanced by *F. oxysporum* VC but the degree of enhancement was lower than that in Col-0 (Supplementary Fig. S6).

To analyze spatial and temporal patterns of auxin response to *V. dahliae* VC, we employed a transgenic Col-0 carrying the auxin-responsive reporter *DR5::GUS* (Jefferson et al. 1987; Ulmasov et al. 1997). After 7 days of cocultivation with *V. dahliae*, higher β -glucuronidase (GUS) activity was observed in root tips compared with control plants (Fig. 6; Supplementary Fig. S7). GUS expression was highly induced in the primary root cap and meristem and was also observed in stele and epidermal cells (Fig. 6B). Lateral root primordia of VC-treated plants, but none in control plants, displayed GUS activity (Fig. 6A). After 14 days of cocultivation, differences between treatments were less pronounced (Supplementary Fig. S8). Most of the root tips of VC-exposed plants (85% for PD322 and 96% for PD413) only displayed very weak staining, while 98% of the root tips of control plants fell into this category.

PAT is important for growth enhancement by *V. dahliae* VC.

To determine whether PAT is critical for *V. dahliae* VC-mediated growth enhancement, we evaluated plant growth in

the presence of different concentrations of 1-naphthylphthalamic acid (NPA), an auxin efflux inhibitor that has been shown to disrupt lateral root development and leaf expansion by blocking the establishment of optimal auxin concentrations required for normal leaf growth and lateral root initiation (Casimiro et al. 2001; Ljung et al. 2001; Reed et al. 1998). After growing *A. thaliana* on Murashige and Skoog (MS) medium supplemented with varying concentrations of NPA in the presence and absence of *V. dahliae*, both lateral root density and total leaf surface area were assessed (Fig. 7). In the presence of 1 μ M NPA, enhanced lateral root formation by *V. dahliae* VC was still observed at days 7 and 14 (Fig. 7A) but the degree of

Fig. 4. Allometric analysis of *Arabidopsis thaliana* growth pattern after exposure to volatile compounds produced by *Verticillium dahliae*. Shoot and root fresh weights of Col-0 seedlings were measured after 7, 10, 12, and 14 days of cocultivation with no fungus (Control) and *V. dahliae* strains PD322 and PD413. The relationship between ln shoot fresh weight and ln root fresh weight of Col-0 is shown. Each value corresponds to the mean of data from three replicates ($n = 15$). Inset shows the allometric coefficient (k) as the slope of the linear regression model through the transformed weight means; r^2 indicates the fitness of the linear regression model to a set of observations. Statistical analysis was performed to demonstrate significant differences in k value among the treatments.

Fig. 3. Assessment of multiple changes in *Arabidopsis thaliana* upon exposure to *Verticillium dahliae* volatile compounds (VC). **A**, Total leaf surface area; **B**, primary root length; **C**, total root length; **D**, leaf chlorophyll content; and **E**, leaf relative water content of Col-0 seedlings after 14 days of cocultivation with no fungus (Control) and *V. dahliae* strains PD322 and PD413 are shown. Values shown correspond to the mean $\pm$ standard error of data from three replicates ($n = 15$). Different letters indicate significant differences among treatments according to Tukey's test at $P = 0.05$. **F**, Root architectures of representative plants (from left to right: control, PD322, and PD413) are shown. Scale bar = 1 cm.

enhancement was lower than that of those grown in the absence of NPA. The degree of enhancement was further reduced as the concentration of NPA increased. In the presence of 10 μ M NPA, lateral root formation in VC-exposed plants was indistinguishable from that in control plants. To confirm that NPA disrupted PAT, we analyzed GUS activity in the *DR5::GUS* reporter line cultured on NPA-amended medium after 7 days of cocultivation with *V. dahliae*. In the presence of 10 μ M NPA, *V. dahliae* VC failed to increase auxin in primary roots, and patterns in VC-exposed plants were comparable with those in control plants (Supplementary Fig. S9). Although the total leaf surface area was also negatively affected by NPA, it still was significantly larger in VC-exposed plants than in control plants, even in the presence of 10 μ M NPA (Fig. 7B), suggesting that additional mechanisms may be involved in regulating leaf expansion in response to VC. We did not notice any differential GUS expression in leaves among all of the treatments.

Analysis of VC produced by *Verticillium* spp.

As an initial step toward the identification of *Verticillium* VC that affect plant growth and development, we analyzed VC produced by five strains corresponding to four species via gas chromatography–mass spectrometry (GC-MS) (Table 3; Supplementary Fig. S10). The compounds produced by all five strains were 2-methyl-1-propanol and phenylethyl alcohol. Earlier studies reported that these two compounds did not affect plant growth (Hung et al. 2014; Naznin et al. 2013). Some compounds, including 2-methyl-1-butanol, 3-octanone, 3-octanol, 1-octen-3-ol, and himachala-2,4-diene, were produced by at least three strains. Some strain-specific compounds were detected. For instance, *V. longisporium* PD687 produced 2, 3-butanediol, acetoin, 1-hexanol, and 3-ethyl-4-methyl-3-penten-2-one. *V. albo-atrum* PD670 emitted two unique sesquiterpenes, including β -caryophyllene and α -amorphene. Interestingly, *V. isaacii* PD753 produced methyl salicylate, a signaling molecule that controls long-distance, systemic plant defense responses.

Fungal CO₂ contributes to enhancing plant growth but is not the only factor.

CO₂ produced via microbial respiration has been shown to contribute to enhanced plant growth (Ditengou et al. 2015; Kai and Piechulla 2009; Meldau et al. 2013; Naznin et al. 2013; Piechulla 2017). KOH or Ba(OH)₂ has been employed to trap CO₂ (Bitas et al. 2015; Kai and Piechulla 2009; Lee et al. 2016; Meldau et al. 2013). In the presence of 0.1 M KOH, the growth of control plants was severely impaired (Fig. 8A), suggesting that photosynthesis is required for growth in the presence of 0.25% sucrose. The growth of *V. dahliae* VC-exposed plants was also reduced under the same condition (Fig. 8B) but, when compared with control plants, their growth was still significantly increased (Fig. 8). We supplemented 3% sucrose to plant culture medium to eliminate the need for photosynthesis. In the presence of 3% sucrose, the growth of control plants was similar between the no-KOH and KOH treatments (Fig. 8B), and the degree of VC-mediated growth enhancement remained unaffected (PD322) or slightly but not significantly reduced (PD413) compared with that observed in the absence of KOH (Fig. 8B). Some *Verticillium* strains that caused significantly different degrees of growth enhancement (Fig. 1) were indistinguishable in colony size (indirectly suggesting similar metabolic activities and amounts of CO₂ produced). Examples include *V. isaacii* PD343 and PD753 and *V. longisporium* PD687 and PD348 (Fig. 1). As for *V. tricorpus*, although the colony size of PD690 was larger than PD703, the degree of growth enhancement by PD690 VC was lower. These results suggested the involvement of compounds other than CO₂ in growth enhancement.

DISCUSSION

Both beneficial and pathogenic microbes produce VC that affect plant fitness, growth, or development (Bailly and Weisskopf 2012; Bitas et al. 2013; Chung et al. 2016; Cordovez et al. 2017; Hung et al. 2015; Li et al. 2016), suggesting that this mechanism may play critical roles in microbial ecology and interaction with plants. Similar to VC produced by root pathogen *F. oxysporum* (Bitas et al. 2015), VC produced by all described *Verticillium* spp. also significantly affected the growth and development of *A. thaliana* and tobacco (Figs. 1, 2, and 3). Growth responses of *A. thaliana* to hormone signaling mutants and to inhibition of auxin transport, as well as spatial and temporal patterns of auxin response, indicate that VC produced by both fungi work through auxin signaling. Further delineation of the molecular mechanism underlying VC-mediated growth manipulation may guide the development of novel strategies for boosting plant growth while helping control pathogens. However, as discussed below, there exist a number of complex questions that need to be investigated so as to realize this potential.

Potential roles of VC produced by soilborne fungi in rhizosphere competence and root infection.

Although many studies (Bailly and Weisskopf 2012; Kanchiswamy et al. 2015a; Li et al. 2016; Morath et al. 2012; Sánchez-López et al. 2016) have shown that microbial VC enhance the growth of both shoots and roots, this study is the first to reveal that fungal VC shift resource allocation from shoots to roots (Fig. 4). Such manipulation results in accelerated root growth and development, likely benefiting *Verticillium* spp. The rhizosphere attracts diverse microbes due to the availability of

Fig. 5. Growth and developmental responses of *Arabidopsis thaliana* hormonal signaling mutants to *Verticillium dahliae* volatile compounds. **A**, Shoot fresh weights of Col-0 (WT) and its mutants defective in auxin signaling (*pin1-7*, *aux1-7*, *tir3-101*, *tir1-1*, and *axr1-3*) or ethylene signaling (*etr1-1* and *ein2-1*) after 14 days of cocultivation with no fungus (Control) and *V. dahliae* strains PD322 and PD413 are shown. **B**, Lateral root densities of Col-0 and auxin signaling mutants are shown. Values shown correspond to the mean $\pm$ standard error of data from three replicates ($n = 15$). Different letters indicate significant differences among the treatments according to Tukey's test at $P = 0.05$.

rich nutrients such as root exudates, dead root cells, and soil nutrients mineralized by plants and rhizosphere microbes (de Weert and Bloemberg 2007). Increased nutrient availability, resulting from VC-induced increase in root biomass and lateral root formation, would certainly support the growth of *Verticillium* spp. Additional benefit may come from root developmental changes. *Verticillium* spp. enter the vascular system of roots through physical wounds created at the sites of lateral root formation (Fradin and Thomma 2006; Klosterman et al. 2009). Increased formation of lateral roots upon exposure to *Verticillium* VC (Figs. 3 and 6) could facilitate infection by creating more potential entry points to the vascular system. Similar changes in root development were also observed upon exposure to *F. oxysporum* VC (Bitas et al. 2015). Evaluation of other root-associated fungi will reveal whether VC-mediated manipulation of root growth and development represents a conserved mechanism underlying rhizosphere competence and root colonization.

Which VC manipulate plant growth and development?

This is one of the most critical questions that should be pursued to understand the roles and underlying mechanisms of VC-mediated manipulation of plants. Some microbial VC have been shown to affect plant growth through application of their synthetic versions (Hung et al. 2015; Kanchiswamy et al. 2015b; Li et al. 2016). However, their roles in natural habitats remain to be validated. Because microbes release many VC

simultaneously, with their composition and amounts being highly dynamic (Bitas et al. 2013; Hutchinson 1973; Kanchiswamy et al. 2015b; Korpi et al. 2009; Morath et al. 2012), application of synthetic versions may be inadequate for identifying which VC regulate plant responses in natural habitats. Characterization of the fitness and behavior of microbial mutants defective in the synthesis of candidate VC would be needed.

The four *Verticillium* spp. analyzed produced diverse VC (Table 3), including some that have been shown to affect plants such as 3-octanone, 1-octen-3-ol, 2, 3-butanediol, acetoin, 1-hexanol, and β -caryophyllene. 3-Octanone and 1-octen-3-ol, two commonly produced fungal VC, inhibited *A. thaliana* growth and also induced an oxidative burst, leading to bleaching (Hung et al. 2014; Splivallo et al. 2007). Both 2,3-butanediol and acetoin are well-known plant-growth-enhancing compounds produced by many bacteria (Blom et al. 2011; Ryu et al. 2003), and the latter was recently found to be produced by *V. longisporium* (Rybakova et al. 2017). Sesquiterpene β -caryophyllene enhanced the growth of lettuce and turnip plants (Minerdi et al. 2011; Yamagiwa et al. 2011) but did not affect *A. thaliana* (Ditengou et al. 2015).

In addition to manipulating plants, *Verticillium* VC may also affect other microbes. For example, 1-octen-3-ol has been shown to control fungal spore production and germination (Chitarra et al. 2004; Nemčovič et al. 2008). *V. longisporium* increased the production of antimicrobial VC, including 1-butanol, upon exposure to biocontrol agent *Paenibacillus polymyxa*

Fig. 6. β -Glucuronidase (GUS) activity in roots of transgenic *Arabidopsis thaliana* Col-0 containing *DR5::GUS*. Plants were stained for GUS activity after 7 days of cocultivation with no fungus (Control) and *Verticillium dahliae* strains PD322 and PD413. **A**, Root tips and lateral root primordia expressing GUS are marked with light and dark arrows, respectively. Scale bar = 500 μ m. **B**, Enlarged primary root (PR) and lateral root (LR) tips of control plants and those exposed to *V. dahliae* volatile compounds are shown. Scale bar = 100 μ m.

(Rybakova et al. 2017). This alcohol was also produced by *V. longisporium* PD687 (Table 3). Some sesquiterpenes, including β -caryophyllene, α -amorphene, 4,11-selinadiene, and himachala-2,4-diene (Table 3), may function as chemical weapons to suppress the growth of competing microbes. These compounds displayed antimicrobial and insect-repellent activities (Kramer and Abraham 2012).

How do plants process semio-fungal VC?

Our results (Figs. 6 and 7), as well as previous studies, (Bailly et al. 2014; Bitas et al. 2015; Garnica-Vergara et al. 2016; Zhang et al. 2007), indicated the involvement of auxin signaling in mediating growth enhancement and modulation of root growth by microbial VC. The *TIR3* gene, which encodes a protein related to the *Drosophila* protein Calossin/Pushover (Xu et al. 1998), is critical for *A. thaliana* response to VC emitted by both *F. oxysporum* and *V. dahliae* (Fig. 5). Although the precise mode of action and function of *TIR3* remains to be unveiled, *TIR3* has been shown to affect basipetal auxin transport and auxin signaling (Guo et al. 2013; Ruegger et al. 1997). *TIR3* affects the level of PIN1 in leaf pavement cells and root cells and also regulates PIN1 protein distribution (Gil et al.

2001; Guo et al. 2013). However, considering that the *pin1* mutant responded to *V. dahliae* VC just like Col-0 (Fig. 5), the lack of response to *V. dahliae* VC in the *tir3* mutant probably is not due to the disruption of PIN1 functionality by this mutation. AUX1, TIR1, and AXR1 appeared to play minor roles in modulating plant response to *V. dahliae* VC. However, in our previous study (Bitas et al. 2015), the VC from *F. oxysporum* failed to enhance the growth of the *aux1* and *tir1* mutants, suggesting that the two pathogens manipulate auxin signaling through both common and pathogen-specific components.

Auxin accumulation had been induced before growth response became evident but subsequently went down (Fig. 6). Similarly, VC from both *Bacillus subtilis* and *F. oxysporum* induced *DR5::GUS* expression after 7 to 8 days of cocultivation; however, its expression subsequently went down to the level indistinguishable from that in control plants (Bitas et al. 2015; Zhang et al. 2007). The data showing that the magnitude of growth enhancement correlates with the duration of VC exposure suggest that auxin accumulation during the early stage of cocultivation may not be sufficient. In agreement with several previous studies (Bailly et al. 2014; Bitas et al. 2015; Garnica-Vergara et al. 2016; Zhang et al. 2007), PAT is also

Fig. 7. Inhibition of polar auxin transport interrupted *Verticillium dahliae* volatile compound-mediated growth enhancement in *Arabidopsis thaliana*. **A**, Lateral root density and **B**, total leaf surface area of Col-0 seedlings cocultivated with no fungus (Control) and *V. dahliae* strains PD322 and PD413 for 7 and 14 days in the presence of 1, 5, and 10 µM 1-naphthylphthalamic acid (NPA) are shown. The same volume of dimethyl sulfoxide (DMSO) was used for all treatments, including the control. Values shown correspond to the mean $\pm$ standard error of data from three replicates ($n = 15$). Different letters indicate significant differences among the treatments according to Tukey's test at $P = 0.05$.

required for *V. dahliae* VC-mediated change in root development (Fig. 7). However, even in the presence of 10 μ M NPA, the leaf surface area of plants exposed to *V. dahliae* VC still increased compared with that of control plants, raising the possibility that *V. dahliae* VC-mediated leaf expansion involves other signals. Production of volatile hormone methyl salicylate by *V. isaacii* PD753 (Table 3) raised the possibility that fungal VC may affect additional hormonal signaling pathways. However, because only one of five strains analyzed produced this compound, its role in modulating growth is unclear. Although growth responses of *etr1* and *ein2* to *V. dahliae* and *etr1* to *F. oxysporum* VC were comparable with that of Col-0 (Fig. 5), whether ethylene signaling is involved in this process requires inclusion of more mutants.

MATERIALS AND METHODS

Fungal cultures and plant materials.

Verticillium strains used in this study are shown in Table 1. *F. oxysporum* strains NRRL 26379 and NRRL 38335 were obtained from the USDA ARS Culture Collection (Peoria, IL). All strains were maintained as conidia suspension in 20% glycerol at -80°C and were revitalized by inoculating on half-strength potato dextrose agar (PDA; Becton, Spark, MD) at room temperature. Seed of *A. thaliana* ecotypes were purchased from Lehle Seed Co. (Round Rock, TX). The mutants of Col-0 used in this study, including CS237 (*etr1-1*), CS3074 (*aux1-7*), CS3075 (*axr1-3*), CS3798 (*tir1-1*), CS3928 (*tir3-101*), CS8844 (*ein2*), and SALK_047613 (*pin1-7*), were obtained from the Arabidopsis Biological Resource Center at the Ohio State University. Because homozygous *pin1-7* mutant is infertile, only seedlings that formed an elongated primary root (= homozygous mutants) were used for evaluating their response to fungal VC. Transgenic Col-0 containing *DR5::GUS* (Jefferson et al. 1987) was provided by D. Desveaux at the University of Toronto. M. Axtell at Penn State provided *N. benthamiana* seed.

I-plate assay to assess the effect of *Verticillium* VC on plant growth.

A bipartite Petri plate (I-plate; Fig. 1), which only allows VC-mediated interaction between *Verticillium* spp. and plants, was used as described in earlier studies (Bitas et al. 2015; Naznin et al. 2013; Paul and Park 2013). Seed treatment was conducted as described by Bitas et al. (2015). Seed of Nd-0 and Kin-0 were stratified for 1 week to increase germination rate. MS medium (Sigma-Aldrich, St. Louis) supplemented with 0.8% (wt/vol) agar and 0.25% (wt/vol) sucrose, with the exception of the experiment shown in Figure 8, was used for growing plants. One plug of fungal culture on PDA (5 mm in diameter) and 5-day-old plant seedlings were transferred to each I-plate on the same day. Growth chamber conditions for cocultivation were 22°C , 12 h of light (4,500 lux, $60 \mu\text{mol photons m}^{-2} \text{s}^{-1}$), and 60% relative humidity.

Analysis of growth, root development, and physiological changes caused by *Verticillium* VC.

Shoot and root fresh weight, leaf surface area, primary and total root length, lateral root density, chlorophyll content, and relative water content ($[(\text{fresh weight} - \text{dry weight})/(\text{turgid weight} - \text{dry weight})]$) were measured as previously described (Bitas et al. 2015). Primary root length was measured via analysis of photographed roots using ImageJ. Seedlings were collected at 3, 5, 7, 10, 12, and 14 days of cocultivation with *V. dahliae* strains PD312 and PD413 to measure shoot fresh weight. Root fresh weight was measured only for the samples collected at days 7, 10, 12, and 14. The method described by Hiscox and Israelstam (1979) was employed to measure chlorophyll content. Relative growth rate was calculated using the formula $(\ln W_2 - \ln W_1)/dt_{(2-1)}$. Shoot and root fresh weights at days 7, 10, and 14 were used to calculate the allometric growth coefficient, k , using the equation $\log R_w = \log b + k \log S_w$, where b is a constant and R_w and S_w correspond to root and shoot fresh weights, respectively (Hunt 2017).

Table 3. Volatile compounds produced by five *Verticillium* strains

N ^b	Compound ^c	Formula	RIcal ^d	RIref ^e	Verticillium strains analyzed ^a				
					PD322 (Vd)	PD413 (Vd)	PD687 (Vl)	PD670 (Vaa)	PD753 (Vi)
1	2-Methyl-1-propanol	C ₄ H ₁₀ O	1068	1068	✓	✓	✓	✓	✓
2	3-Methyl-1-butanol	C ₅ H ₁₂ O	1181	1182	...	✓	✓	...	...
3	2-Methyl-1-butanol	C ₅ H ₁₂ O	1190	1191	✓	...	...	✓	✓
4	3-Pentenol	C ₅ H ₁₀ O	1218	1204	...	✓	...	...	...
5	3-Methyl-3-buten-1-ol	C ₅ H ₁₀ O	1344	—	...	...	✓	...	✓
6	3-Octanone*	C ₈ H ₁₆ O	1229	1230	✓	✓	...	...	✓
7	Acetoin*	C ₄ H ₈ O ₂	1267	1268	...	...	✓	...	...
8	1-Hexanol*	C ₆ H ₁₄ O	1333	1335	...	...	✓	...	...
9	4-Methyl-5-hexen-2-ol	C ₇ H ₁₄ O	1344	—	...	✓	...	...	...
10	3-Octanol	C ₈ H ₁₈ O	1359	1359	✓	✓	...	...	✓
11	4-Methyl-6-hepten-3-ol	C ₈ H ₁₆ O	1379	—	...	✓	...	...	...
12	1-Octen-3-ol*	C ₈ H ₁₆ O	1411	1411	✓	✓	...	✓	✓
13	3,5,5-Trimethyl-2-hexene	C ₆ H ₁₈	1449	—	...	✓	...	...	...
14	2,3-Butanediol*	C ₄ H ₁₀ O ₂	1547	1581	...	...	✓	...	...
15	3-Ethyl-4-methyl-3-penten-2-one	C ₈ H ₁₄ O	1573	—	...	...	✓	...	...
16	Himachala-2,4-diene	C ₁₅ H ₂₄	1583	—	✓	✓	...	✓	...
17	β -Caryophyllene*	C ₁₅ H ₂₄	1602	1601	...	...	...	✓	...
18	α -Amorphene	C ₁₅ H ₂₄	1690	—	...	...	...	✓	...
19	Azulene	C ₁₅ H ₂₄	1723	—	✓	...	...	...	...
20	Methyl salicylate*	C ₈ H ₈ O ₃	1767	1765	...	...	...	...	✓
21	Phenylethyl alcohol	C ₈ H ₁₀ O	1871	1871	✓	✓	✓	✓	✓

^a Vd = *Verticillium dahliae*, Vl = *V. longisporium*, Vaa = *V. albo-atrum*, and Vi = *V. isaacii*. Symbol ✓ denotes the presence of compound.

^b Numbers correspond to gas chromatography peaks, numbered according to the order of elution through the Rtx-Wax capillary column used.

^c Identity of volatile compounds (VC) present in the headspace of 11-day-old *Verticillium* cultures is shown. Headspace VC were extracted ($n = 2$) using solid phase microextraction. Mass spectra and retention index of each compound were compared with reference data in the NIST library for identification. Asterisks (*) indicate compounds with known or suggested effect on plant growth based on literature review.

^d Linear retention index (RI) of each compound determined using a mixture of alkane standards (C₇ to C₄₀).

^e Linear RI of each compound in the literature is shown; — indicates that linear RI of these compounds are not available in the literature.

NPA treatment.

MS medium was amended with NPA (Sigma-Aldrich) dissolved in dimethyl sulfoxide (DMSO) to determine whether PAT is critical for mediating the effect of *Verticillium* VC on plant growth and development. MS medium in the control treatment contained only the same volume of DMSO.

Assessment of GUS activity and staining quantification.

Histochemical staining for GUS activity in transgenic *A. thaliana* Col-0 seedlings containing *DR5::GUS* was performed as previously described (Bitas et al. 2015). Staining patterns were observed using a stereomicroscope (Olympus

SZ60) and a compound microscope (Nikon 104) and imaged using the Olympus DP26 camera. Resulting images were imported into Adobe Photoshop (Adobe Systems, San Jose, CA) to quantify stain intensity. Quantification of stain intensity was performed using the ImageJ method.

Assessment of the effect of CO₂ produced by fungal culture on plant growth.

KOH was used to trap CO₂ in a tripartite Petri plate (Y-plate). Cocultivation was performed as described above, except that only four *A. thaliana* seedlings per plate were used due to space limitations. The third compartment was filled with 8 ml of 0.1 M KOH

Fig. 8. Growth responses of *Arabidopsis thaliana* to *Verticillium dahliae* volatile compounds on media with different amounts of sucrose. Col-0 seedlings grown on medium supplemented with low (0.25%) and high (3%) sucrose were cocultivation with no fungus (Control) and *V. dahliae* strains PD322 and PD413 in the absence and presence of 0.1 M KOH for 14 days. **A**, Representative images of treatments in the presence of 0.1 M KOH and **B**, shoot fresh weights after individual treatments are shown. Values shown correspond to the mean $\pm$ standard error of data from three replicates ($n = 12$). Different letters indicate significant differences among the treatments within each group according to Tukey's test at $P = 0.05$.

or water. After 14 days of cocultivation, shoot fresh weight was measured.

Analysis of VC produced by *Verticillium* spp.

After sealing vials containing PDA slant inoculated with individual strains with breathable film, they were incubated at 22°C for 11 days. After replacing the film with a screw cap containing gas-tight silicone/Teflon septa, they were incubated at 22°C for 1 day to accumulate VC in headspace. For VC extraction, the solid phase microextraction fiber was inserted into the vial for 1 h. To determine which fiber coating works best for capturing VC (Stoppacher et al. 2010), the following materials were tested: 100 µm polydimethylsiloxane (PDMS), 85 µm carboxen (CAR)/PDMS, 65 µm PDMS/divinylbenzene (DVB), 50/30 µm DVB/CAR/PDMS, and 7 µm PDMS (Sigma-Aldrich). Because the largest number of compounds was extracted using the DVB/CAR/PDMS 50/30-µm fiber coating, this fiber was chosen for capturing VC produced by *Verticillium* spp. VC released from PDA were also analyzed to help identify which GC peaks corresponded to background signals.

Extracted VC were analyzed using a nontargeted GC-MS method employing the QP 2010 Ultra GC-MS system (Shimadzu, Japan). An Rtx-Wax capillary column (60 m by 0.25 mm by 0.25 µm) (RESTEK, USA) was used for GC. The VC bound to the fiber were desorbed for 2 min using a split/splitless injector (splitless mode). The temperature program was as follows: 35°C for 5 min, then ramped to 230°C at 5°C/min, and kept for 15 min. Helium was used as the carrier gas with a constant flow rate of 1 ml/min. The injection, transfer line, and ion source temperatures were all set at 230°C. An electron ionization source set at 70 eV was used. Data acquisition started at 1.5 min and the mass scan range was 35 to 500 m/z. To calculate retention indices of individual compounds, 1 µl of a mixture of alkane standards (C₇ to C₄₀ containing each alkane in n-hexane at 100 mg/liter) was injected. Putative identity of individual VC was determined by comparing their mass spectral profiles and retention index values with those archived in the NIST 11 library.

Statistical analysis.

The experimental design was completely randomized, consisting of three replications for each treatment. Each experiment was repeated at least twice. Analyses of variance for data were performed using Minitab 17.3 (Minitab Inc., State College, PA). The significance of treatment was determined by the magnitude of the *F* value (*P* = 0.05). When a significant *F* test was obtained for treatment, separation of the means was conducted using Tukey's test at *P* = 0.05.

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