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Temporal dynamics of the survival of *Verticillium dahliae* microsclerotia with or without melanin in soils amended with biocontrol agents

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Abstract *Verticillium dahliae* is a soilborne pathogen that causes wilt in many economically important crops. It produces melanized microsclerotia for its long-term survival in soil. Accurate quantification of viable microsclerotia in soil prior to planting is essential for predicting the risk of wilt. Melanin is believed to help microsclerotia resist to or tolerate abiotic stresses. We studied the temporal dynamics of both melanized and melanin-deficient microsclerotia of *V. dahliae* in four types of soils with or without addition of two biocontrol products (one based on *Trichoderma viride*; and the other based on two *Bacillus* strains: *B. subtilis* and *B. amyloliquefaciens*). Of the four soil types, two were from wheat and maize (non-host to *V. dahliae*) fields and the other two from sites with history of continuous cropping of cotton and pepper (both susceptible to *V. dahliae*). Results showed that the survival of microsclerotia in soils over time can be satisfactorily described by a negative exponential decline model. Microsclerotium mortality was much greater in the soils from wheat and maize field than from cotton and pepper, irrespective of biocontrol agents. Similarly, mortality of melanin-deficient microsclerotia was much greater than melanized microsclerotia. Both biocontrol products resulted in additional mortality of microsclerotia, especially *Bacillus* spp. There were significant interactions between soil origin, microsclerotium type and biocontrol treatment in affecting microsclerotium mortality. These results demonstrated that melanin contributes to the long-time survival of microsclerotia in soil and suggested that combination of biocontrol with rotation with non-host crops can be effective in reducing the number of viable microsclerotia of *V. dahliae* in soil.

Keywords Microsclerotium survival · Melanized microsclerotia · Rotation · Biocontrol

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Introduction

Verticillium wilt, caused by the soilborne fungus *Verticillium dahliae*, is an important fungal disease of many economically important crops (Pegg and Brady 2002; Inderbitzin and Subbarao 2014). Once plants are infected, control options become limited (Fradin and Thomma 2006; Klosterman et al. 2009). The disease cycle of *V. dahliae* consists of a parasitic stage in which the plant (in particular vascular tissues) is colonized, and

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a brief saprophytic stage, followed by a dormant stage in which the pathogen can survive for years in the soil as microsclerotia (Klosterman et al. 2009).

Microsclerotia, the thick-walled fungal structures, are primary inocula of the disease. Microsclerotia of *V. dahliae* obtained under field and *in vitro* conditions contain melanin, a black or dark pigment that can be found in many organisms, including bacteria, fungi and mammals (Nosanchuk and Casadevall 2003). Although melanin is not essential for microsclerotium production (Bell et al. 1976), a laboratory study showed that melanin-deficient microsclerotia from the tricyclazole treatment were more susceptible to cell wall degrading enzymes, suggesting that melanin is important for microsclerotium survival (Hawke 1994). Other laboratory studies showed that melanized microsclerotia were highly resistant to environmental stresses *in vitro*, such as extreme temperatures and UV irradiation (Jabnoun-Khiareddine et al. 2009; Hawke 1994; Shang et al. 2013). However, the effects of melanin on microsclerotium survival have yet been studied in field soils.

Continuous cropping of susceptible hosts for a long period often leads to an increase of soilborne diseases (Manici and Caputo 2010; Chen et al. 2011b; Mazzola and Manici 2012). One reason for such an increase in continuous monocultures is the increased level of pathogen inocula (Mazzola and Manici 2012). Continuous cropping of *V. dahliae*-susceptible potato cultivars for five seasons increased the density of microsclerotia by 60%-70%, compared to crops of resistant potato cultivars (Davis et al. 1994). Changes in the soil microbiota may also be responsible for increased disease risks in continuous cropping systems (Larkin 2008; Mazzola and Manici 2012; Inderbitzin et al. 2017). Crop rotation with non-host crops or resistant cultivars is a cultural technique to reduce soilborne disease development.

Effective use of biocontrol agents (BCAs) is an important component of sustainable agriculture (Martin and Bull 2002). Many bacterial strains, such as isolates from *Bacillus* spp., have been shown to be antagonists of plant pathogens (Chowdhury et al. 2013; Bisutti et al. 2017; Yu et al. 2019) as well as some fungi, such as *Trichoderma* spp. (Kredics et al. 2003; Freeman et al. 2004; Porras et al. 2007; Dubey et al. 2011; Morán-Diez et al. 2019). However, biocontrol efficacy under field conditions is often variable because of the complex ecological processes involved in biocontrol of plant diseases (Cunniffe and Gilligan 2011; Xu and Jeger 2013a, b).

In this paper, we report results from a study focusing on the effects of melanin, soil origins and BCAs on the survival dynamics of *V. dahliae* microsclerotia. Specifically, we used microsclerotia produced from three strains – two mutants producing melanin-deficient microsclerotia and one wild type strain with melanized microsclerotia. The survival of the microsclerotia over time was investigated in four types of soils (two from *V. dahliae* hosts, the other two from non-hosts) with or without amendment of one of the two BCAs: *Trichoderma viride* and *Bacillus* spp. (*B. subtilis* and *B. amyloliquefaciens*).

Materials and methods

Soil origins Soils were collected from four different fields in Yangling, Shaanxi province, China for use in the experiments: (1) a greenhouse plot where pepper had been continuously grown for at least eight years, (2) an experimental field where cotton had been continuously grown for at least 10 years [the field was also artificially inoculated with *V. dahliae* microsclerotia five years ago], (3) a field with a continuous monoculture history of wheat, and (4) a field with a continuous monoculture history of maize. Cotton and pepper are both susceptible to *V. dahliae*, and wheat and maize are not known to be hosts of *V. dahliae*. Soils in the top 25 cm layer were collected from each field at the end of April 2017, just before the start of cotton or pepper sowing in the region. These fields were close to each either with similar soil physical characteristics, belonging to the Orthic Anthrosols type (Huang et al. 2017): 2.5% sand, 59.5% silt and 38% clay, and pH of about 8.2.

Biocontrol agents Two commercial powder-formulated BCAs, *Trichoderma viride* (2×10^{10} cfu/g, Weiyuan Bio-technology Co., Ltd, Guangzhou, China) and a mixture of *Bacillius subtilis* (4×10^{9} cfu/g) and *B. amyloliquefaciens* (1×10^{9} cfu/g) (Kunhe Bio-technology Co., Ltd, Tianjin, China) were used. The *T. viride* product was used at the rate of 10 g per kg of soil and the *Bacillus* spp. mixture at 20 g per kg of soil. The rates used were five times of the manufacturers' recommended rates in order to increase the probability of successful biocontrol outcomes. These two products are currently used widely in China.

Mutant generation and microsclerotium production *Verticillium dahliae* strain JY isolated from cotton in

Shaanxi province, China (Hu et al. 2013) was used; this strain produces melanized microsclerotia and hence was used as a wild-type (WT) strain to generate melanin-deficient mutants. Mutants deficient with *VDAG_00190* or *VDAG_0366* were generated followed the published protocols (Paz et al. 2011; Fan et al. 2017). Both the genes are essential for DHN melanin biosynthesis in *V. dahliae* (Duressa et al. 2013). Strains, plasmids and primers used are given in Tables S1 and S2. Pathogenicity assays were performed on the susceptible eggplant cv. Ziguanqie (Shaanxi Qinxing Seeds CO., LTD, China) with a root dipping-inoculation method (Hu et al. 2015) (Fig. S2). Microsclerotia of the WT strain and two mutant strains were collected from BMM (Basal Agar Modified Medium, BMM) plates, water-sieved with 38 μm sieves (Hu et al. 2013, 2014), and air-dried in a laminar flow hood before experimentation.

Placing microsclerotia in soils Each BCA was added to each of the four samples of collected soils, and mixed thoroughly; sterilized water was then used to adjust the soil moisture level to 60% of soil moisture capacity. Two kilograms of soils of an appropriate origin were added to a single pot (height 38 cm × top diameter 30 cm). Small nylon bags (3 × 4 cm, pore size 20 μm) were prepared and each filled with 0.05 g (about 1.5×10^4) microsclerotia of one strain; 1 g of the experimental soil was also taken from the pot and mixed with microsclerotia to enhance the contact between soil and microsclerotia. The nylon bags were then sealed before they were buried in the center of each pot. There were four packets of microsclerotia for each strain, given a total of 12 packets in a single pot. The pots were placed in a glasshouse compartment (set to maintain 24 °C) with the surface covered with a black polyethylene film to prevent direct sunlight and rapid loss of soil moisture.

Estimating viability of microsclerotia One packet of microsclerotia from each strain in each pot was taken out of the soil 3, 5, 7, and 9 weeks after the treatment for estimation of microsclerotium viability. In addition, samples of microsclerotia at time zero (i.e. at the time of treatment) were also taken for estimation of the viability. In each sample (packet), 25 microsclerotia were randomly picked out with a botanical needle under a stereo dissecting microscope and then transferred to a sterilized plate containing solutions (sterile water, pH = 8.0) conducive for germination of microsclerotia and cultured in 25 °C for 20 h (Hu et al. 2014). It was not possible to delay the assessment any longer (hence to assess delayed germination) because the hyphae originating from those germinated microsclerotia made the assessment of individual microsclerotia at late times unreliable.

Statistical analysis There were three factors in the study: four types of soils, three *V. dahliae* strains and three biocontrol treatments (control and two BCAs). For each combination of soil and biocontrol treatment, there were three replicates (pots); a completely randomized design was used. Within each of the 36 pots, microsclerotia from each of the three strains were sealed within each nylon bag (3 × 4 cm, pore size 20 μm). Viability of microsclerotia was assessed five times: 0, 3, 5, 7 and 9 weeks after the biocontrol treatment. The experiment was first conducted during March 8th to June 25th in 2017 and repeated from March 15th to July 2nd in 2017.

The main objective of the present study was to investigate the effects of melanin, soils and BCAs on the microsclerotium mortality. Thus, we first modelled the temporal dynamics of viable microsclerotia for each strain in each pot in each experiment to estimate the rate of microsclerotium mortality. Then, we applied analysis of variance (ANOVA) to the estimated mortality values in order to assess the treatment effects.

Preliminary analysis indicated that a negative exponential decline model could satisfactorily describe the observed temporal patterns of viable microsclerotia for most of the data sets based on percentage of the variability explained and the distribution of the residuals. This negative exponential decline model is of the form: $\ln(\text{survival}\%+1) = \ln(a) - b \times \text{time}$, where 'a' and 'b' are parameters to be estimated. In the model, parameter ''b' is the rate of microsclerotium mortality over time, and 'time' represented sampling time (number of weeks from applying treatment to sampling microsclerotia for viability assessment). Microsclerotium germination was assumed to be 100% at time zero and hence all subsequent germination data were adjusted accordingly if the germination was not 100% for time zero. Parameter 'a' was estimated as ln(101) (= 4.615) from the time zero point. The negative exponential model was fitted to the data obtained from a single pot for each individual treatment combination.

Analysis of variance (ANOVA) was then used to assess the effect of treatment factors on parameter 'b'.

In ANOVA, a split plot design was used where strain effect was assessed within pots whereas soil and BCA effects were assessed based on individual pots. Because the normal distribution assumption required for ANOVA was not met, a permutation test (with 1000 permutations) was used to determine the significance level of treatment effects. In addition, for each factor the overall effect was decomposed into several single orthogonal degree-of-freedom comparisons. For the soil type, the three comparisons were: (wheat + maize) vs. (cotton + pepper), wheat vs. maize, and cotton vs. pepper. For the strain, the two comparisons were WT vs. two mutants, and the differences between the two mutants. For the biocontrol treatment, the two comparisons were untreated vs. BCA treated, and the differences between the two BCA treatments. All analyses were carried out in R (version 3.4.0) and permutation test was done with the lmPerm package.

Results

Generation of melanin deficient mutants in V. dahliae Deletion of *VdPKS* (*VDAG_00190*) and *VdT4HR* (*VDAG_03665*) genes in the strain JY generated the mutant strains of Δ*VdPKS* and Δ*VdT4HR*, respectively (verified data are given in Fig. S1). Compared with the dark colony of the WT strain, Δ*VdPKS* mutant was albino and the color of Δ*VdT4HR* mutant was orange-brown after incubation for two weeks on Czapek Dox agar medium (Fig. 1a). The two mutants can produce microsclerotia after incubation on BMM plates at 20 °C for 4 weeks (Fig. 1b). Microscopic assessment showed that Δ*VdPKS*, Δ*VdT4HR* and WT strains did not differ significantly in microsclerotium diameter size, with the respective average of 41.2 μm, 41.7 μm and 43.6 μm, respectively. A large number of melanized microsclerotia were observed in the WT strain, whilst Δ*VdPKS* and Δ*VdT4HR* formed hyaline and orange-brown microsclerotia, respectively (Fig. 1b), indicating that melanin productions in the Δ*VdPKS* and Δ*VdT4HR* mutants were disrupted. The virulence of Δ*VdPKS* and Δ*VdT4HR* mutants were slightly reduced, but the difference was not statistically significant (Fig. S2).

Survival of microsclerotia in soils Proportions of microsclerotia germinated decreased over time for all treatment combinations (Table 1), even in those treatments without addition of commercial BCAs. The extent of the microsclerotium decrease over time varied greatly with specific treatment combinations (Table 2). The combination of cotton-growing soil, without BCA addition and microsclerotia from the WT strain, showed the lowest mortality. On the other hand, the combination of wheat or maize-growing soil, *Bacillus* spp. treatment, and Δ*VdPKS* microsclerotia had the greatest mortality.

The negative exponential model fitted the observed data well for most of the data sets (Fig. 2), except for the Δ*VdPKS* microsclerotia subjected to the *Bacillus* spp. treatment in wheat/maize soil. The lack of fit for these specific cases were due to the fact that the mortality rate was much higher than the fitted – viability was close to zero at the second sampling point (week 3) (Table 1).

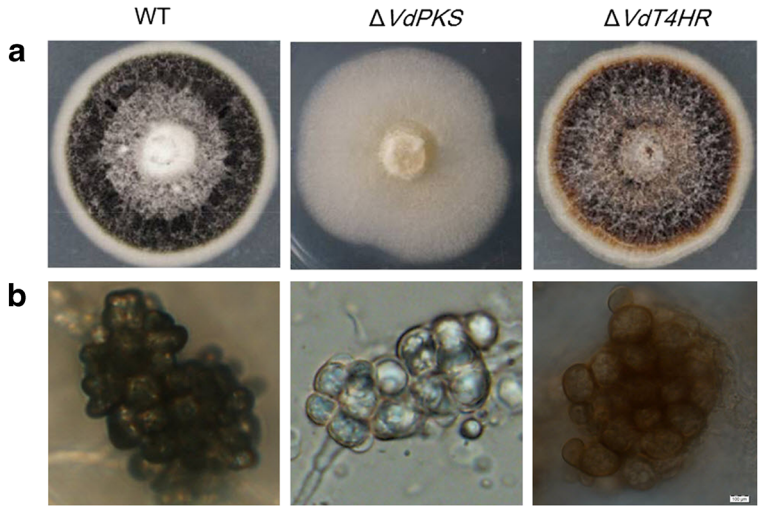

Fig. 1 Colony appearance (**a**) of *Verticillium dahliae* wildtype (WT) strain JY and the two DHN melanin deficient mutants on Czapek Dox agar medium after incubation for two weeks at 25 °C, and their microsclerotia (**b**) after incubated for four weeks on BMM plates. Bar = 30 μm

Table 1 Average survival (%) of *Verticillium dahliae* microsclerotia over time in soils from different cropping sites amended with or without specific biocontrol agents

Soil	Biocontrol	Fungal strain	Time (weeks)			
			3	5	7	9
Cotton	*Bacillus*	Δ*VdPKS*[a]	28.67 ± 2.81[d]	7.33 ± 1.23	0.00	0.00
		Δ*VdT4HR*[b]	38.00 ± 2.25	14.67 ± 2.46	3.33 ± 1.61	0.67 ± 0.67
		WT[c]	54.00 ± 3.54	33.33 ± 3.68	21.33 ± 4.22	12.67 ± 4.55
	Control	Δ*VdPKS*	57.33 ± 3.68	40.00 ± 4.84	26.00 ± 5.14	8.67 ± 3.92
		Δ*VdT4HR*	70.67 ± 2.46	54.67 ± 4.34	41.33 ± 4.22	26.67 ± 3.21
		WT	92.67 ± 1.23	86.00 ± 1.71	79.33 ± 2.81	70.67 ± 3.68
	Trichoderma	Δ*VdPKS*	40.00 ± 3.10	17.33 ± 2.67	1.33 ± 0.84	0.00
		Δ*VdT4HR*	46.67 ± 1.98	23.33 ± 2.81	8.67 ± 2.40	2.67 ± 1.33
		WT	60.67 ± 3.49	43.33 ± 3.49	31.33 ± 3.33	20.67 ± 4.55
Pepper	*Bacillus*	Δ*VdPKS*	8.67 ± 1.23	0.00	0.00	0.00
		Δ*VdT4HR*	15.33 ± 1.23	1.33 ± 0.84	0.00	0.00
		WT	39.33 ± 1.23	18.00 ± 1.71	6.67 ± 1.33	0.00
	Control	Δ*VdPKS*	38.67 ± 1.33	21.33 ± 1.69	8.00 ± 1.46	0.00
		Δ*VdT4HR*	51.33 ± 1.23	32.00 ± 1.03	14.67 ± 1.33	2.00 ± 0.89
		WT	84.00 ± 1.03	73.33 ± 0.84	63.33 ± 1.23	50.00 ± 0.89
	Trichoderma	Δ*VdPKS*	16.67 ± 1.61	0.67 ± 0.67	0.00	0.00
		Δ*VdT4HR*	25.33 ± 1.33	4.00 ± 1.03	0.00	0.00
		WT	50.00 ± 1.71	27.33 ± 2.81	13.33 ± 1.98	4.00 ± 1.46
Maize	*Bacillus*	Δ*VdPKS*	0.67 ± 0.67	0.00	0.00	0.00
		Δ*VdT4HR*	6.67 ± 0.84	0.00	0.00	0.00
		WT	18.00 ± 0.89	3.33 ± 0.67	0.00	0.00
	Control	Δ*VdPKS*	17.33 ± 1.69	4.67 ± 1.61	0.00	0.00
		Δ*VdT4HR*	32.00 ± 1.03	12.67 ± 1.23	0.67 ± 0.67	0.00
		WT	46.00 ± 0.89	24.67 ± 1.23	11.33 ± 0.67	2.00 ± 0.89
	Trichoderma	Δ*VdPKS*	6.67 ± 0.84	0.00	0.00	0.00
		Δ*VdT4HR*	16.00 ± 1.03	1.33 ± 0.84	0.00	0.00
		WT	27.33 ± 0.67	8.00 ± 1.03	0.00	0.00
Wheat	*Bacillus*	Δ*VdPKS*	3.33 ± 1.23	0.00	0.00	0.00
		Δ*VdT4HR*	13.33 ± 0.84	0.00	0.00	0.00
		WT	24.00 ± 1.46	6.67 ± 0.84	0.00	0.00
	Control	Δ*VdPKS*	25.00 ± 2.29	9.33 ± 0.84	0.00	0.00
		Δ*VdT4HR*	40.00 ± 1.03	19.33 ± 0.67	4.00 ± 1.03	0.00
		WT	56.67 ± 0.67	32.67 ± 0.67	18.00 ± 0.89	9.33 ± 0.84
	Trichoderma	Δ*VdPKS*	12.00 ± 1.03	0.00	0.00	0.00
		Δ*VdT4HR*	22.00 ± 0.89	5.33 ± 1.33	0.00	0.00
		WT	31.33 ± 1.23	12.67 ± 0.67	3.33 ± 0.67	0.00

[a] *V. dahliae* mutant strains with the deletion of *VdPKS* gene (*VDAG_00190*), producing hyaline microsclerotia

[b] *V. dahliae* mutant strains with the deletion of *VdT4HR* gene (*VDAG_03665*), producing orange-brown microsclerotia

[c] The wildtype *V. dahliae* strain JY, producing melanized microsclerotia

[d] Average percentage of viable microsclerotia and its standard error from six replicates (two experiments, each with three replicate pots)

Table 2 Repeated measures analysis of variance for the mortality of microsclerotia in four soils under the treatment of biocontrol agents. Statistical significance was determined based on the permutation test

Terms	d.f.[a]	SS[b]	As % of total SS	*P* value
At the pot level				
Treatments	2	1.999	46.3	< 0.001
Control vs. BCAs[c] (T1)	1	1.893		< 0.001
Bacillus vs. *Trichoderma*	1	0.106		< 0.001
Soils[d]	3	2.181	50.5	< 0.001
(Wheat + maize) vs. (cotton + pepper) (T2)	1	1.610		< 0.001
Wheat vs. maize	1	0.050		< 0.001
Cotton vs. pepper	1	0.520		< 0.001
Treatments x Soils	6	0.131	3.0	< 0.001
T1 x T2	1	0.111		< 0.001
Others	5	0.020		< 0.001
Residual	24	0.011		
Within pot				
Between repeated experiments	1	0.040	1.6	
Strains[e]	2	1.873	72.7	< 0.001
WT vs. mutants (T3)	1	1.714		< 0.001
Δ*VdT4HR* vs. Δ*VdPKS*	1	0.159		< 0.001
Strains x Treatment	4	0.070	2.7	< 0.001
T3 x T1	1	0.047		< 0.001
Others	3	0.023		< 0.001
Strains x Soils	6	0.184	7.1	< 0.001
T3 x T2	1	0.141		< 0.001
Others	5	0.043		< 0.001
Strains x Soils x Treatment	12	0.106	4.1	< 0.001
T3 x T2 x T1	1	0.054		< 0.001
Others	11	0.052		< 0.001
Residual	179	0.302		

[a] Degree of freedom

[b] Sum of squares

[C] Biocontrol agents used in this study, including *Trichoderma* spp. and *Bacillus* spp

[d] Two types of soils were from wheat and maize field (both crops are not susceptible to *V. dahliae*) and the other two from sites with history of continuous cropping of cotton and pepper (both susceptible to *V. dahliae*)

[e] Two mutants of Δ*VdT4HR* and Δ*VdPKS* and one wildtype (WT) strain

All treatment factors and their interactions were highly significant ($P < 0.001$) in affecting parameter 'b' estimates with the main effects of the three treatment factors accounting for most of the variability in the parameter (Table 2). Figure 3 shows the average 'b' estimates for each combination of three treatment factors.

The overall differences among four soil origins accounted for 50.5% of the total variability in parameter 'b' at the pot level (Table 2). Microsclerotia in soils collected from cotton and pepper fields survived much better than in soils from wheat and maize fields; this comparison explained nearly 74% of the differences among the four types of soils. Average estimates of parameter 'b' were 0.307, 0.446, 0.571 and 0.528 for soils collected from cotton, pepper, maize and wheat fields, respectively. There was also a large ($P < 0.001$) difference between soils from cotton and pepper fields: microsclerotia survived better in the soil from cotton than from pepper. The main effect of BCA treatments accounted for 46.3% of the total variability at the pot

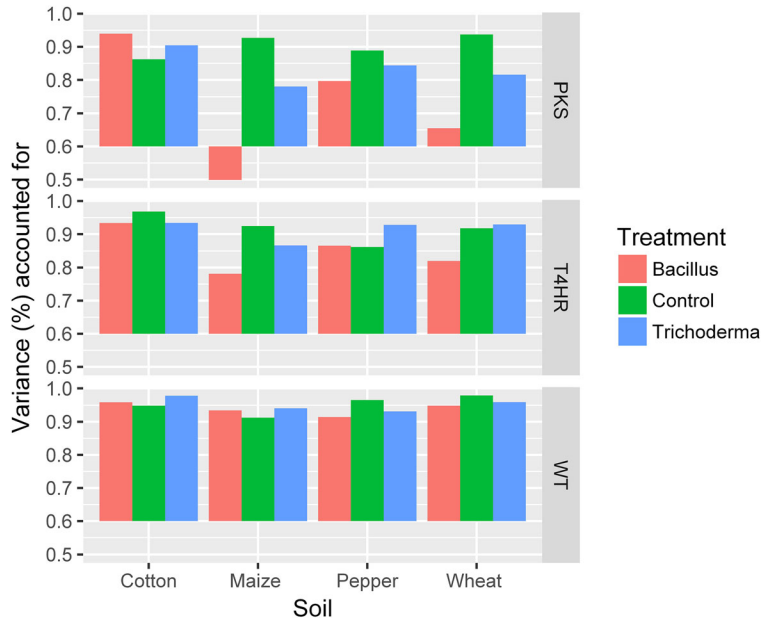

Fig. 2 Average percentage of variance accounted for by the fitted negative exponential models [ln(survival%+1) = ln(a) – b*time] describing the temporal survival of melanized (WT), orange-brown (Δ*VdT4HR*) and hyaline (Δ*VdPKS*) microsclerotia of *Verticillium dahliae* in soils collected from cotton, pepper, maize, and wheat planting sites with or without addition of one of two biocontrol products (*Bacillus* spp. or *Trichoderma* spp.). In the model, parameter ''b' is the mortality rate of microsclerotia over time and parameter 'a' was estimated as ln(101) (= 4.615) from the time zero point. The negative exponential model was fitted to the data obtained from a single pot for each individual treatment combination

level (Table 2): most (94.7%) of the effect was due to the difference between the control and the BCA treatments. Amendment with *T. viride* or *Bacillus* spp. led to increased microsclerotium mortality (Fig. 3): average estimate of parameter 'b' was 0.556, 0.502 and 0.331 for the *Bacillus* spp., *T. viride* and untreated samples, respectively. Strains differed significantly in the mortality rate of microsclerotia over time, accounting for

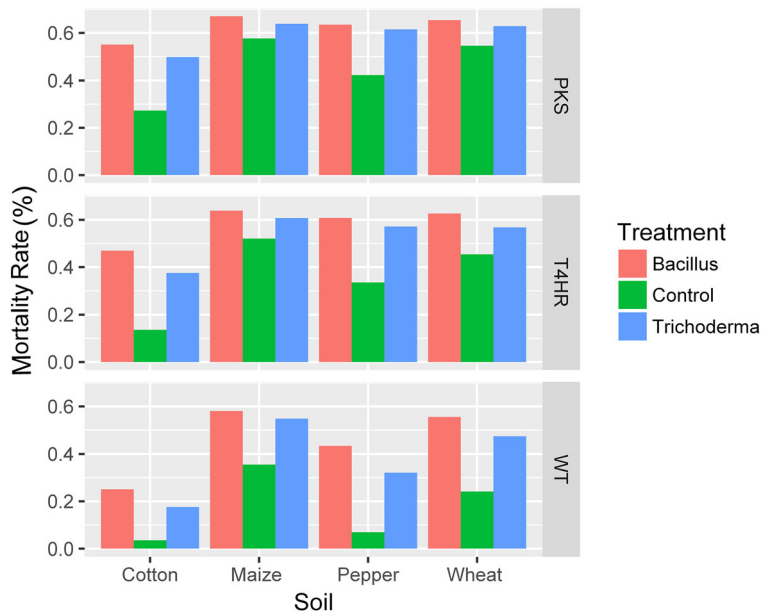

Fig. 3 Average values of the mortality parameter of fitted negative exponential models describing the temporal dynamics of melanized (WT), orange-brown (Δ*VdT4HR*) and hyaline (Δ*VdPKS*) microsclerotia of *Verticillium dahliae* in soils collected from cotton, pepper, maize, and wheat planting sites with or without addition of one of two biocontrol products (*Bacillus* spp. or *Trichoderma* spp.)

72.7% of the total variability within pots (Table 2). Most (91.5%) difference was attributable to the difference of the WT strain with the two mutants. Average parameter 'b' estimate was 0.337, 0.493 and 0.559 for the WT, Δ*VdT4HR* and Δ*VdPKS* strains, respectively.

There were significant interactions between treatment factors in influencing the mortality of microsclerotia in soils (Table 2). These interactions primarily involved the following single-degree comparisons: *Verticillium* host vs. non-host, untreated vs. BCA-treated, and WT vs. mutant strains (Table 2). These interactions can be seen from Figs. 3 and 4. Key features of these interactions included: (1) microsclerotia from the WT strain survived much better in soils from cotton and pepper fields, and (2) mortality of microsclerotia from the two mutants was much greater in the presence of BCAs when compared with the WT strain.

Discussion

Understanding the temporal dynamics of viable *V. dahliae* microsclerotia in soil in the presence and absence of additional inputs of biocontrol organisms is important for managing Verticillium wilt diseases. This is particularly true given that broad-spectrum soil fumigants have been banned in many regions and are facing uncertain future in other regions. Such knowledge will enable prediction of inoculum levels (hence wilt risks) at times when plants are most susceptible to the pathogen (e.g. planting), considering inoculum threshold for specific crops, previous crops and biocontrol treatments. The present study demonstrated that microsclerotium survival is affected by cropping history, biocontrol organisms and presence/absence of melanin in microsclerotia. Treatments with BCA significantly increased microsclerotium mortality, and melanin deficient microsclerotia were much more sensitive to *T. viride* and *Bacillus* spp. agents.

The rate of microsclerotium mortality was much lower in soils previously grown with *V. dahliae* susceptible plants than in soils grown with non-hosts, irrespective of presence/absence of melanin in microsclerotia and BCA amendments. The density of *V. dahliae* microsclerotia is shown to increase over time in soils continuously grown with plants susceptible to *V. dahliae* for many seasons because of the release of microsclerotia from infected host debris (Davis et al. 1994; Subbarao and Hubbard 1996; Xiao et al. 1998; Wu and Subbarao 2014; Short et al. 2015). This increased inoculum density is one of main reasons for increased wilt occurrence in monoculture systems. Recently, gradually more attention has been paid to study whether and, if so, how changes in soil microbiota and phytotoxic effects of allelochemicals affected development of soilborne diseases in monocultures (Liu et al. 2017; Zhu and Morel 2018; Hu et al. 2019). Reduced diversity and abundance of beneficial microbes appear

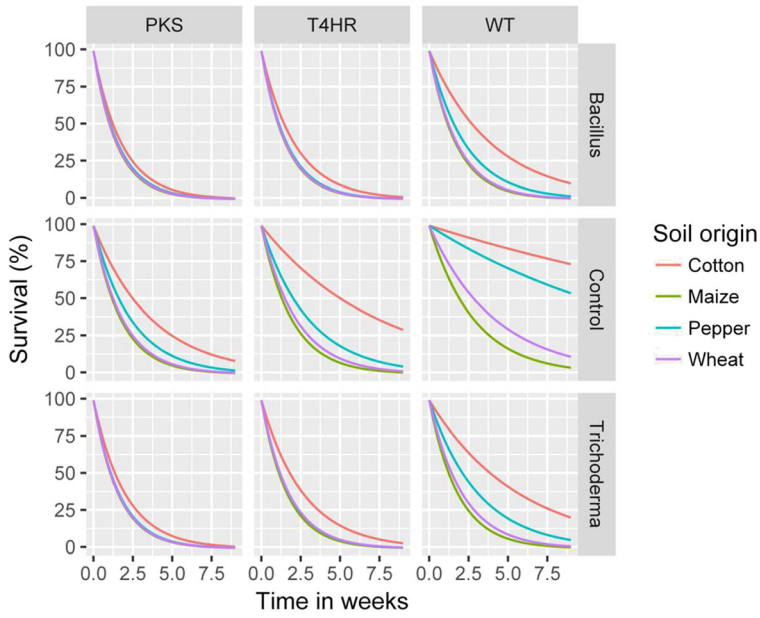

Fig. 4 Fitted negative exponential models describing the temporal survival of melanized (WT), orange-brown (Δ*VdT4HR*) and hyaline (Δ*VdPKS*) microsclerotia of *Verticillium dahliae* in soils collected from cotton, pepper, maize, and wheat planting sites with or without addition of one of two biocontrol products (*Bacillus* spp. or *Trichoderma* spp.)

to correlate with disease-prone soils (Liua and Herbert 2002; Chen et al. 2011b). Allelochemicals released by plant leaching, root exudation and residue decomposition may have negative effects on the current crops (Weir et al. 2004; Jilani et al. 2008), probably through changes in the rhizosphere microbial population structure (Wu and Wang 2006; Kong et al. 2008; Nayyar et al. 2009; Hu et al. 2019), affecting pathogen infection potential (Chen et al. 2011a; Zhu and Morel 2018). In the present study, the higher survival rate of microsclerotia in cotton and pepper soils suggest that soils from non-*Verticillium* hosts may have microbes and/or allelochemicals that reduce the survival of *V. dahliae* microsclerotia. One explanation for the greater survival rate of microsclerotia in cotton soils may be because this field was artificially inoculated with a high density of *V. dahliae* microsclerotia before, which could have reduced the abundance of those microbes contributing to wilt suppression. Present results support potential roles of crop rotation in reducing soilborne diseases in general (Cook 1988; Subbarao and Hubbard 1996). Indeed, rotating cauliflower with broccoli resulted in long-term reduction of *V. dahliae* microsclerotia and using corn as a rotation crop led to the best control of Verticillium wilt of potato (Davis et al. 1996; Xiao et al. 1998).

Use of *T. viride* and *Bacillus* spp. significantly reduced the survival potential of *V. dahliae* microsclerotia. The time needed to achieve 50% mortality was within 3–5 weeks of BCA treatments. Such an effective biocontrol efficacy is likely attributable to two factors. Firstly, there is a good contact between BCA propagules and microsclerotia since microsclerotia in the nylon bags were well mixed with the soils amended with BCA. Germination of melanized microsclerotia of *V. dahliae* was completely suppressed after exposure to liquid cultures of *Trichoderma* spp. for 30 min (Daami-remadi 2009). Secondly, the final rates of the two BCA products applied were up to 10^{11} cfu per kg of soil, which is much higher than recommended rates. We used such a higher rate in order to achieve a certain degree of biocontrol as one of our objectives was to compare the effect of melanin on the susceptibility of microsclerotia to biotic stresses (i.e., BCAs). In the present study, *Bacillus* spp. achieved greater efficacies than *Trichoderma* spp.; this could be due to the alkaline pH (pH = 8.2) and good water retention properties of the used soils (Wu et al. 2002; Zhang et al. 2011), both of which would favor *Bacillus* spp. over *Trichoderma* spp. (Burpee 1990; Ahlem et al. 2012; Bisutti et al. 2017).

Melanin-deficient microsclerotia suffered from much greater mortality than microsclerotia with melanin irrespective of soil origins and biocontrol treatments. The high mortality in melanin deficient microsclerotia is probably related to the loss of a melanin barrier that may prevent cell wall degrading enzymes produced by microorganisms from degrading their cell wall. For a given soil type, melanin deficient microsclerotia were also more susceptible to BCAs than microsclerotia with melanin. These results demonstrated that melanin can protect microsclerotia from attacks by microbial antagonists. Melanin can also protect *V. dahliae* microsclerotia from UV irradiation, extreme temperatures and enzyme digestion *in vitro* (Hawke 1994). The poor survival of melanin deficient *V. dahliae* microsclerotia in soils may partially explain why non-melanized microsclerotia are rarely observed under natural field conditions.

Present results suggested that rotation with crop species that are not susceptible to *V. dahliae* would be effective in reducing the level of microsclerotia in soils. For example, in continuous monoculture of cotton, present results predict that it would take nearly 21, 90 and 137 weeks for the viability of melanized microsclerotia to reduce to 50%, 5% and 1%, respectively, under constant conditions. In contrast, the corresponding values are 2, 9 and 13 weeks in continuous monoculture of maize soils. Thus, one-year rotation of maize would be sufficient to kill nearly all microsclerotia in soils. Of course, this is the best scenario as soil from long-term monoculture of maize is expected to have greater suppressive effect than from a single season of maize. Nevertheless, it does illustrate the potential of crop rotation for reducing soilborne diseases, particularly for those crops that can tolerate high pathogen inoculum thresholds. Furthermore, present models can also be used to predict best times for applying BCAs relative to planting in order to reduce the level of microsclerotia below threshold values. Significant interactions between soil and biocontrol treatment suggest that the efficacy of BCAs depends on specific soil conditions, most likely due to differences in microbial community structure although physio-chemical properties cannot be excluded either. Future research should be directed to understand what factors in soils from continuously cropped monoculture of *V. dahliae* non-hosts are mainly responsible for increased mortality of *V. dahliae* microsclerotia.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals This article does not contain any research involving human or animal participants.

Informed consent Not applicable.

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