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Effects of dark septate endophytes on tomato plant performance

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Abstract Non-mycorrhizal fungal root endophytes can be found in all natural and cultivated ecosystems, but little is known about their impact on plant performance. The impact of three mitosporic dark septate endophytes (DSE48, DSE49 and *Leptodontidium orchidicola*) on tomato plant characteristics was studied. Their effects on root and shoot growth, their influence on fruit yield and fruit quality parameters and their ability to diminish the impact of the pathogen *Verticillium dahliae* were investigated. While shoot biomass of young plants was enhanced between 10% and 20% by the endophytes DSE48 and *L. orchidicola* in one of two experiments and by DSE49 in both experiments, vegetative growth parameters of 24-week-old plants were not affected except a reproducible increase of root diameter by the isolate DSE49. Concerning fruit yield and quality, *L. orchidicola* could double the biomass of tomatoes and increased glucose content by 17%, but this was dependent on date of harvest and on root colonisation density. Additionally, the endophytes DSE49 and *L. orchidicola* decreased the negative effect of *V. dahliae* on tomato, but only at a low dosage of the pathogen. This indicates that the three dark septate endophytes can have a significant impact on tomato characters, but that the effects are only obvious at early stages of vegetative and generative development and currently too inconsistent to recommend the application of these DSEs in horticultural practice.

Keywords Dark septate endophytes (DSE) · Fruit quality · *Leptodontidium orchidicola* · *Solanum lycopersicum* · *Verticillium dahliae*

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Introduction

Cultivation of tomato (*Solanum lycopersicum*) has been worldwide increasing because of the variety of cultivars with optimum growth under different conditions and due to the properties of its edible fruit which is an important source of carotenoids, flavonoids, vitamins and minerals (Guil-Guerrero and Rebolloso-Fuentes 2009). However, fungal pathogens are responsible for economically important crop losses (e.g. Fradin and Thomma 2006). The pathogens are mainly controlled by application of fungicides, but the high input of fungicides has negative effects on the environment (Soares and Porto 2009) and leads to resistance of the pathogens (Stammler et al. 2006). Also, the increasing demand of consumers for food with less pesticide residue contamination stimulates the use of alternative control methods. The use of tomato-resistant cultivars is the best method in controlling such diseases, but the occurrence of new races of pathogens has overcome the resistance (e.g. Parlevliet 2002). This circumstance has stimulated the research in alternatives to combat fungal pathogens by biocontrol agents with abilities to suppress diseases caused by soil-borne pathogens (e.g. Whipps 2001; Alabouvette et al. 2009).

Although suppression effects of biocontrol agents can be demonstrated under controlled conditions, less candidates

are available for application in the field due to lack in effectiveness (Ojiambo and Scherm 2006). The complex interaction in the rhizosphere could be one reason affecting the success of biological agents, because they are not able to compete efficiently with other microorganisms in this environment (Conn and Franco 2004). Due to their colonisation capacity and permanence in the plant, indigenous endophytes, which live within host-plant tissues without causing any visible symptoms of disease (Wilson 1995) could posses an advantage over introduced agents and would be therefore much more effective (Hoitink and Boehm 1999; Conn and Franco 2004). Root fungal endophytes form symbiotic associations with a very wide range of plant species showing a high phylogenetic diversity and possess multiple functions (Rodriguez et al. 2009). The plant–fungus genotype and physiology combination determine the outcome of this symbioses varying from parasitism to mutualism (Redman et al. 2001; Schulz and Boyle 2005; Kogel et al. 2006).

Despite variable and complex interactions, fungal endophytes have been related to plant growth, fitness and stress responses. This has been shown many times and in a broad variation of plant–fungal combinations for mycorrhizal fungi such as arbuscular mycorrhizas (phylum Glomeromycota), for fungi belong to the order *Sebacinales* (phylum Basidiomycota) which includes the root endophyte *Piriformospora indica* (Weiss et al. 2004; Varma et al. 1999) and for endophytes of the phylum Ascomycota as *Trichoderma* species (Harman et al. 2004) and dark septate endophytes (DSE) (Jumpponen et al. 1998). DSE showed no impact (Jumpponen 2001), but some investigations revealed positive effects on plant growth and yield (Shivanna et al. 1994; Wu and Guo 2008) and on nutrient uptake (Jumpponen et al. 1998; Upson et al. 2009). Recently, it was shown for one DSE that it enhanced not only root development and plant biomass of a Chinese medicinal plant, but also increased the level of the flavonoid rutin (Wu et al. 2010).

In order to enlarge the number of indigenous putative biological agents, 51 isolates from tomato roots were screened and 14 were analysed in more detail concerning their taxonomic position and their in vitro colonisation ability (Andrade-Linares et al. unpublished). Based on pot culture experiments under green house conditions (Andrade-Linares et al. 2011), three DSEs were selected for further analysis. Phylogenetic analysis of the internal transcribed spacer regions of rDNA and detailed morphological characterization indicated that DSE48 (AM944359) and DSE49 (AM944355) are unknown ascomycetous fungi belonging to the Pleosporales, while DSE135 (AM944358) represents a new *Leptodontidium orchidicola* strain (Andrade-Linares et al. unpublished). In the present study, the effects of these three endophytes were further evaluated. Six experiments were carried out to analyse (1) their impact on vegetative tomato growth characters in young and older plants, (2) their capacity to protect tomatoes against the pathogen *Verticillium dahliae* in order to proof their suitability to be used as biological agents and, (3) their influence on the yield of tomato fruits and their quality concerning taste related compounds such as sugars and titratable acids as well as health promoting carotenoids.

Materials and methods

Experimental design

To investigate the disease suppression effect of the three endophytes, four experiments (A–D) with tomato plants (cv. Hildares F1, Hild Samen GmbH, Marbach, Germany) artificially infested with *V. dahliae* (see below) were carried out in the greenhouse in Großbeeren (52°N, 13°E). Experiment A and B were also used to show the impact of the endophytes on vegetative growth of young plants. Tomato seeds were disinfected with 70% ethanol for 5 min and subsequently two times with 3.5% NaOCl for 15 min. After each disinfection step, the seeds were washed with sterile water. Seeds were pre-germinated on 0.8% water agar at 24°C to select homogenous and not contaminated plants for the experiments. The tomato seedlings were transplanted into pots (1 L) at 1–2 leaf stage and inoculated with the endophytes (see below). The pots were filled with a substrate based on peat (Fruhstorfer Erde Typ P; Archut, Vechta, Germany) chemical analysis (mg per 100 g: N=75, P=75, K=125; pH 5.9) in experiments A and B or with a sterilised mixture of this substrate and quartz sand (EN12620:EN1339; Euroquarz, Dorsten, Germany) at a 1:1 ratio (v/v) in experiment C and D. The substrate was sterilised at 80°C during 3 days. The plants were further cultivated under greenhouse conditions (Table 1). Each treatment consisted of twice three replicates with six plants each (n=36; experiment A and B) or twice three replicates with four plants each (n=24; experiment C and D) arranged in a randomised block design. The pots were watered daily to maintain the substrate moisture and twice a week with nutrient solution (De Kreij et al. 1997; EC=2 dS m^{-1}; mg per L: N (NO_3)=151.4, N (NH_4)=13.5, P=40.5, K=251.4; pH 5.5) dependent on the growth stage through the time during the experiments from 40 to 200 ml. Shoot fresh and dry weights of tomato plants were measured and dry matter contents were calculated after a cultivation time of six (experiment A and B) or 7 weeks (experiment C and D). Disease severity caused by *V. dahliae* was assessed (experiment A–D) on the following scale according to Morgan et al. (1992): 0=no symptoms, 1=slight yellowing of leaf, stunting or wilting, 2=moderate yellowing of leaf,

Table 1 Conditions for tomato cultivation

	A	B	C and D	E and F
Temperature [day/night; °C]	23.9/18.0	24.8/18.5	23.8/19.2	22.5/18.4
Humidity [day/night;%]	63.3/82.4	54.4/69.3	64.6/79.2	70.8/73.1
Mean daily radiation [Mol×m^{-2}×d^{-1}]	40.3	28.9	32.2	34.2
CO_2 concentration [ppm]	466.8	422.8	ND	433.8

Plants were grown and inoculated with endophytes for analysing their impact on growth, on the interaction with *V. dahliae* (A, B, C, D) or on yield and fruit quality (E, F)

ND not determined

stunting or wilting, 3=severe yellowing of leaf, stunting or wilting and 4=leaf death at harvest.

Two experiments (E and F) were carried out under controlled conditions in a climate chamber (York, Mannheim, Germany; 23/20°C, 60/70% relative humidity day/night, 16 h light h 33.7 Mol×m^{-2}×d^{-1}) to assess the impact of the endophytes on vegetative growth of older plants and on fruit yield and quality. Seeds were prepared as mentioned above for experiment A–D, but pre-germination was carried out first in liquid MS medium (Murashige and Skoog 1962) and after 7 days in plant nutrition medium (Shahollari et al. 2007) at 23°C with a photoperiod of 16/8 h (day/night) to improve the root growth and finally the endophyte root colonisation. The 2–3 leaf stage seedlings without any contamination were transplanted into 1.2 L-pots containing sterilised mix of substrate and sand (see above) which was inoculated with the respective endophyte (see below). Each treatment in each experiment included six replicates with one plant each (n=6) arranged in a randomised design. Watering of plants and the nutrient application were done as described above. The plants were cultivated in the climate chamber until flowering and then transferred to the greenhouse (Table 1). Fresh and dry weights of shoots and roots were measured and dry matter content were calculated 22 weeks after endophyte inoculation. Additionally, root length and diameter were estimated with a root scanner (Perfection V700 Photo; Epson, Meerbusch, Germany).

Preparing of the endophyte inoculum and inoculation

Each dark septate endophyte isolate was grown in a 300-ml Erlenmeyer flask with 150 ml liquid complete medium (Pontecorvo et al. 1953) for 3–4 weeks at 25°C and 100 rpm agitation. Fresh mycelium was filtered and washed with sterile distilled water until the liquid became clear to avoid any carryover of medium into the inoculum. The fresh mycelium was weighted and part of it was mixed with sterile tap water by a blender (Model D72, Moulinex, Leipzig, Germany) for 1 min at minimal speed.

Number of propagules of the endophytes was estimated by a Thoma chamber and their viability was checked by plating on potato dextrose agar (PDA; VWR, Berlin, Germany). For inoculation, the suspensions were adjusted with sterile tap water to a concentration corresponding to each experiment. For experiments A–D, 36 tomato plants were treated with each endophyte, respectively, by root dipping (5×10^5 cfu/ml) at 1–2 leaf stage before planting, the control plants (36 plants) were mock-inoculated with tap water. The substrate was also drenched with fresh mycelium suspension of the endophytes (1% *w*/*v*). For experiment E and F, the pots were filled with a substrate sand mixture inoculated with fresh mycelium suspension of the endophytes (1% *w*/*v*), the control plants were mock inoculated in substrate with tap water.

Inoculation of the pathogen *V. dahliae*

The isolate *V. dahliae* (accession number GU060637) was grown in 150 ml sucrose sodium nitrate liquid medium (Sinha and Wood 1968) at 28°C and 100 rpm for 1 week, transferred to 200 ml fresh medium and further incubated for 2 weeks. The culture was thereafter mixed by a blender (Model D72, Moulinex, Leipzig, Germany) for 40 s at minimal speed. Number of conidia was estimated by a Thoma chamber and their viability was checked by plating on PDA (VWR, Berlin, Germany). Two weeks after endophyte treatment, half of the tomato plants for each treatment including the control without endophyte (n=18 for A and B; n=12 for C and D) were inoculated at 3–4 leaf stage with the pathogen by drenching with 30 ml conidia suspension (2×10^5 conidia mL^{-1} in experiment A and B or 7×10^6 conidia mL^{-1} in experiment C and D). Control plants for *V. dahliae* were treated with 30 ml of sterile tap water.

Effect on fruit yield and quality

In experiment E and F, fruit harvest was started 15 weeks after inoculation and was carried out twice a week for 7 weeks. Numbers of red-ripe fruits, fresh and dry weights were monitored and results were summarised in three groups (weeks 15–16, weeks 17–18, weeks 19–21). For quality analysis, red-ripe tomatoes harvested 19 and

21 weeks after inoculation from plants of experiments E and F were manually sorted, mixed and marketable fruits were separated from blossom-end rot fruits according to the CBT scale (Anonymous 1992). Fruits of the colour stage 10–11 were selected for quality analysis carrying out a double estimation with three replicates of a mixture of 12 fruits from approximately ten different plants. Carotenoids were analysed according to Krumbein et al. (2006). Briefly, 1 g calcium carbonate, 30 g sodium sulphate and 30 ml acetone were added to 15 g homogenised tomatoes and mixed for 2 min. The extract was filtered under suction and the solid materials were repeatedly extracted with acetone until the resulting filtrate was colourless. Carotenoid composition and content were determined by HPLC using a C-18 reversed-phase column (Lichosphere 100; 5 μm, 250×4 mm; Merck, Darmstadt, Germany) with an isocratic eluent of 75% acetonitrile, 15% methanol and 10% methylene chloride. In parallel, 50 g of homogenised samples were frozen for acid and reduced sugar (glucose and fructose) analyses. Titratable acid content was determined by potentiometric titration with 0.1 M NaOH, while sugars were enzymatically determined (Krumbein et al. 2004). All results were referred to 100 g fresh weight.

Microscopy

For confirmation of endophyte colonisation, tomato root fragments were sampled 2 and 3 weeks after inoculation, stained with WGA-AF 488 (Molecular Probes, Karlsruhe, Germany) according to Deshmukh et al. (2006). Three plants per treatment and experiment were randomly selected and samples from different parts of the root system were stained. Fifteen root fragments per sample were mounted on glass slides and analysed with an Axioscop 2 Plus Microscope (Zeiss, Germany). WGA-AF 488 was excited with a 488-nm laser line and detected at 505–540. Quantification of the endophytic colonisation was based on a method for arbuscular mycorrhizal fungi (Trouvelot et al. 1986) modified for the root endophyte *P. indica* (Bütehorn et al. 2000). Colonisation was classified from 0 to 5 representing <1%, between 1% and 10%, between 10% and 50%, between 50% and 90% and >90% of fragment colonised by fungal hyphae. Infection frequency (F) and total colonisation intensity (C) were calculated with F=Ne/Nt×100 (Ne, number of fragments colonised by the endophyte; Nt, total number of analysed root fragments), and $C=(95n_5+70n_4+30n_3+5n_2+n_1)/\mathrm{Nt}$ (n_x, number of fragments in infection class x).

Statistical analyses

The STATISTICA programme version 6.0 (StatSoft Inc., Tulsa, OK, USA) was used for all analyses. Disease severity was non-parametrically analysed by Kruskal–Wallis test. Tomato growth parameters and fruit yield were analysed by one-way ANOVA and the Fisher's protected least significant difference (LSD; P=0.05). Data given as percentages (shoot weight losses after pathogen infection) were arcsin transformed before one-way ANOVA with LSD test (P=0.05).

Results

The endophyte impact on vegetative plant growth

Tomato plants of two parallel experiments (experiment A and B) were harvested 6 weeks after planting in order to determine the impact of the endophytes DSE48, DSE49 and *L. orchidicola* on shoot growth of young tomato plants (Table 2). Colonisation of the roots was observed, but not quantified. Shoot fresh weights of DSE48-colonised plants increased by 16% in one experiment, while *L. orchidicola*-colonised plants showed a 14% higher dry matter content compared to controls in the second experiment. The endophyte DSE49, however, showed a significant effect on young tomato plants in both experiments and enhanced shoot fresh weight by 21% and 11% and dry matter content by 5% and 7%. In contrast, experiment E and F showed that the three endophytes affected neither shoot and root biomasses nor leaf numbers of older plants after 22 weeks of cultivation (Table 3). Only inoculation with DSE48 resulted in lower root lengths in experiment E (21% less relative to controls), but this effect on the root was not evident in experiment F. Differences in both experiments were detected for DSE49. Colonised plants had roots with a 15% and 20% larger diameter than those from the controls.

Table 2 Impact of endophytes on young tomato plants

	Experiment A		Experiment B	
Treatment	FW	dmc%	FW	dmc%
Control	63.4±0.5	13.4±0.1	76.5±1.4	11.4±0.1
DSE48	**73.7±2.8**	14.0±1.0	75.8±1.3	11.6±0.6
DSE49	**76.7±2.1**	**14.1±0.1**	**84.8±0.1**	**12.2±0.2**
L. orchidicola	66.7±1.7	13.5±0.4	78.8±1.3	**13.0±0.4**

Plants from two experiments (A and B) were harvested 6 weeks after inoculation with the fungal endophytes DSE48, DSE49 and *L. orchidicola*. Average values of shoot fresh weights (FW) and dry matter contents (dmc%)±standard deviations are shown

Values in bold denote significant differences between colonised and non-colonised control plants (one-way ANOVA according to LSD test; P=0.05; n=18)

Table 3 Impact of endophytes after 22 weeks of cultivation

		Shoot		Root		Shoot/root FW ratio	Root length [cm/g FW]	Root diameter [mm]	Leaf N°
		FW[a] [g/plant]	dmc[b] [%]	FW [g/p]	dmc [%]				
E	control	1733.3±101.6	12.69±1.23	96.3±19.7	8.47±0.84	18.7±3.54	340.7±28.4	0.26±0.01	43±0.9
	DSE48	1654.8±202.4	12.30±0.72	108.2±28.5	8.10±1.11	16.3±4.12	**270.5±55.8**	0.27±0.02	44±2.9
	DSE49	1634.7±53.7	12.62±1.05	82.4±14.9	9.82±1.64	20.6±4.39	370.9±43.7	**0.30±0.01**	44±2.3
	L. orchidicola	1564.1±137.3	12.81±0.94	86.1±9.7	8.32±0.73	18.4±2.39	403.0±69.9	0.26±0.02	44±1.4
F	Control	1669.7±69.8	14.18±2.20	61.8±8.5	13.80±1.43	27.6±4.43	316.7±39.0	0.29±0.03	45±3.0
	DSE48	1576.1±159.6	13.17±1.79	63.2±9.7	12.44±0.90	25.4±3.71	344.9±59.2	0.30±0.05	41±4.1
	DSE49	1695.3±169.2	13.73±1.70	63.8±9.8	15.28±2.45	27.5±6.34	305.9±14.9	**0.35±0.02**	43±4.6
	L. orchidicola	1635.1±180.9	13.42±1.19	56.6±5.7	13.77±1.95	29.0±3.1	369.3±81.7	0.30±0.03	45±1.7

[a] Fresh weight

[b] Dry matter content

Averages values±standard deviations of vegetative growth parameters from tomato plants colonised with the fungal endophytes DSE48, DSE49 and *L. orchidicola* are shown for two experiments (E and F). Statistical comparisons between colonised and non-colonised plants were carried out by one-way ANOVA according to LSD test (P=0.05; n=6). Average values of shoot fresh weights (FW) and dry matter contents (dmc%) ± standard deviations are shown

Values in bold denote significant differences between colonised and non-colonised control plants

The endophyte impact on plant–pathogen interaction

In order to analyse if DSE48, DSE49 and *L. orchidicola* could potentially be used as biocontrol agents, plants colonised or not by the three endophytes for 2 weeks were infected in four experiments with two concentrations (2×10^5 and 7×10^6 conidia mL^{-1}) of the fungal pathogen *V. dahliae*. Four or 5 weeks after pathogen inoculation, fresh and dry weights of shoots were evaluated, weight losses calculated (Table 4) and disease severity was assessed (Fig. 1). In experiment A, no disease symptoms could be observed and the pathogen showed no effect on plant growth within cultivation time. In experiment B, however, symptoms were obvious and weight losses were above 20% in control plants (plants without any endophyte infected with *V. dahliae*). Plants colonised by DSE48 behaved similar to controls. In contrast, leaves of plants colonised by DSE49 or by *L. orchidicola* showed 30% less disease symptoms (Fig. 1) and weight losses were significantly reduced to 14% and 4% for DSE49- and *L. orchidicola* treatment, respectively (Table 4). In the experiments C and D with the higher pathogen dosage, disease index in controls was similar as before, but weight losses were even higher (between 25% and 30%). Reduction of the symptoms and the weight losses were again observable in plants pre-inoculated with DSE49 and *L. orchidicola*, but differences were only significant for the decrease in the disease index with DSE49 in one experiment (Fig. 1, experiment D).

The endophyte impact on fruit yield and quality

Fruits from plants inoculated with DSE48, DSE49 and *L. orchidicola* as well as the control plants were harvested between 15 and 21 weeks after inoculation (six plants per treatment), grouped and three groups were analysed concerning number, total FW and DW of tomatoes (Table 5). Two and 3 weeks after inoculation, root endophytic colonisation was visualised by microscopy. In one experiment, fruit number from plants colonised by *L. orchidicola* was twice as much as those from controls during the first harvest period and biomasses (FW and DW) consequently were similarly increased (Table 5, experiment E, harvest I). Any significant differences were not evident for the other endophytes and also not for the later two harvest periods.

Table 4 Weight loss percentages of tomato plants after *V. dahliae* infection

	2×10^5 conidia mL^{-1} (n=18)		7×10^6 conidia mL^{-1} (n=12)	
	A	B	C	D
	Fresh weight loss percentages			
Control	9.6	22.2	30.2	25.1
DSE48	11.0	23.4	29.0	22.5
DSE49	12.7	**14.4**	25.1	19.1
L. orchidicola	7.9	**4.5**	28.9	17.1

Two weeks after inoculation with the endophytic fungi DSE 48, DSE 49 and *L. orchidicola*, plants were infected with 30 ml of a 2×10^5 or 7×10^6 conidia mL^{-1} pathogen suspension. Fresh weights were measured and fresh weight losses were calculated as the ratio of the values from pathogen-infected to non-infected plants. Average values of fresh weight losses are shown. Statistical comparisons between treatments were performed by one-way ANOVA according to LSD test (P=0.05)

Values in bold denote statistically significant differences between endophyte-colonised and non-colonised plants (control)

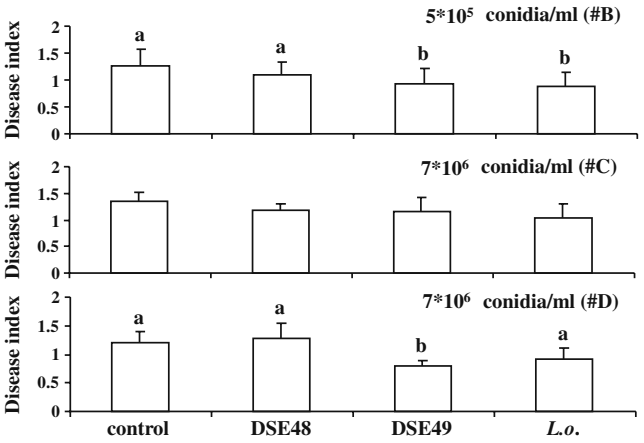

Fig. 1 Bioprotectice effects of fungal endophytes. Disease indices of leaf symptoms caused by *V. dahliae* infection in tomato control plants and plants colonised by the endophytes DSE48, DSE49 or *L. orchidicola* were estimated based on a 0–4 scale 4 weeks after inoculation with 30 ml of a 2×10^5 conidia mL^{-1}-pathogen suspension (experiment B; $n=18$) or 5 weeks after inoculation with 30 ml of a 7×10^6 conidia mL^{-1}-pathogen suspension (experiment C and D; $n=12$). Averages values are shown with their ±standard deviations. Significant differences of the endophyte-colonised plants to the respective controls are indicated by different letters above the columns. Statistical comparisons between treatments were performed by Kruskal–Wallis test ($P=0.05$). No disease symptoms were observed in experiment A

This increase could, however, not be observed in the second experiment, where numbers and biomasses were similar in all treatments at the three harvest periods (Table 5, experiment F). If marketable fruits were analysed separated from fruits with blossom-end rot, results showed significantly higher DW of healthy fruits at the beginning of the harvest (Table 5, experiment E, harvest I) and they were similar for blossom-end rot fruits during the harvest periods in both experiments (data not shown) indicating that none of the endophytes had any influence on this type of disease. All treatments of both experiments except control plants showed colonisation by the inoculated fungus in roots after 3 weeks of inoculation. Some root surface contamination by fungi and bacteria was observed in all treatments including controls (data not shown). In order to find a reason for the difference between the two experiments, root colonisation by the endophyte *L. orchidicola* was quantified. This showed no significant difference between the experiments concerning the infection frequency (68% and 44%) but total colonisation intensity was significantly higher in root of experiment E (16.7%) than in experiment F (3.4%). Colonisation was not quantified for the other two fungi, because they showed no significant impact on fruit yield and quality.

Fruit quality analysis was carried out with mixed samples of red-ripe tomatoes from two dates of the harvest (12 fruits from ten plants per treatment). Amount of titratable acids, reducing sugars and carotenoids (lycopene

Table 5 Influence of the endophytes on yield

Parameter	Har-vest time	E				F			
		Control	DSE48	DSE49	*L. orchidicola*	Control	DSE48	DSE49	*L. orchidicola*
Total fruit number (per plant)	I	3.7±1.6	4.5±1.6	5.0±2.4	**7.3±2.4**	4.5±2.5	5.3±2.5	2.7±2.2	4.8±3.7
	II	12.5±5.1	12.7±3.7	13.0±3.0	15.7±3.7	16.7±1.9	15.2±2.9	15.0±3.6	15.5±4.0
	III	33.3±6.9	33.0±4.2	32.2±1.9	31.5±5.1	39.5±1.6	41.7±7.4	37.0±10.2	37.0±4.9
Fruit FW (g per plant)	I	247.8±103.4	336.4±123.3	350.9±140.4	**503.9±105.6**	283.4±135.8	327.9±137.7	161.7±134.92	291.4±197.3
	II	709.6±261.7	764.0±199.6	770.4±179.7	930.8±142.6	854.1±87.6	775.9±149.4	785.7±219.7	861.8±118.2
	III	1859.7±281.3	1962.0±324.6	1920.8±116.7	1851.7±247.3	1940.5±451.8	2144.2±600.8	1878.2±598.2	2014.1±253.9
Fruit DW (g per plant)	I	13.9±5.4	18.8±7.3	20.2±8.8	**29.4±6.8**	15.5±7.3	17.9±7.7	8.6±7.2	16.5±11.3
	II	40.8±15.0	42.9±10.9	43.8±11.3	53.0±8.4	50.4±3.8	45.1±9.5	44.9±13.74	50.7±7.8
	III	110.9±19.3	115.7±18.1	112.9±5.8	109.9±13.7	124.4±12.2	129.5±32.5	113.3±37.9	122.1±14.7
healthy fruit DW (g per plant)	I	12.3±7.2	16.8±7.4	16.7±6.1	**25.8±9.7**	15.1±7.1	17.9±7.7	8.6±7.2	16.3±11.0
	II	34.1±18.1	31.9±13.5	34.0±11.5	46.4±7.4	45.4±5.2	40.2±5.6	39.1±15.1	42.2±4.9
	III	95.4±13.3	95.8±24.1	92.6±11.8	98.2±13.1	103.8±5.7	101.1±28.1	93.1±31.0	103.1±10.0

Fruit number, fresh and dry weights (FW and DW) and marketable fruit dry weight per plant were estimated in two parallel experiments (E and F). Harvests were carried out between weeks 15 and 22 after endophyte inoculation and fruits were grouped into I (week 1–2), II (week 3–4) and III (week 5–7). Averages values are shown with their ±standard errors. Statistical comparisons between treatments were performed by one-way ANOVA according to LSD test ($P=0.05$; $n=6$)

Value in bold denotes significant differences between the colonised and non-colonised control plants (control)

and ß-carotene) were measured (Table 6). Significant differences, however, could not be detected among the treatments respect to the control plants, except at the first harvest, where glucose levels were 17% higher in fruits from plants colonised by *L. orchidicola* than in those from control plants.

Discussion

DSEs are in some cases able to form mutualistic interactions with plants (Jumpponen 2001). We therefore analysed the impact of three newly isolated DSEs on their natural host tomato concerning (1) vegetative growth, (2) interaction with a pathogen and (3) fruit yield and quality.

When plants were harvested 6 weeks after inoculation, one of the dark septate endophytes (DSE49) showed a reproducible impact on shoot development of tomato plants, in both experiments. The effects of the other two fungi (DSE48 and *L. orchidicola*) on young plants seem to depend on the environmental conditions, since these were only obvious in one of the two experiments. The effects on the young plants, however, disappeared after 22 weeks of cultivation. These differences in vegetative plant growth promotion were probably not a result of inoculation and growth conditions, but depend very likely more on plant growth stage of harvest. This indicates that the beneficial effect of the endophytes seems to be only transient up to the stage around inflorescence emergence (about 7 weeks) and can be compensated in controls at later times of development. Most studies on DSE–plant interactions could not show positive effects (Jumpponen 2001). Plant growth promotion could be, however, observed in some investigations, but either plants were relatively young (Wu et al. 2010) and/or effects were dependent on the host (Fernando and Currah 1996) or on environmental conditions like elevated CO_2 concentrations (Alberton et al. 2010) or N fertilisation (Jumpponen et al. 1998; Upson et al. 2009). Another factor influencing the outcome of the interaction was the experimental design. Results were different under sterile conditions compared to those obtained in open pot cultures (reviewed in Jumpponen and Trappe 1998). Also in the present study, plants were grown in open pots and showed additional microorganisms on their root surfaces. This could have affected the interaction between the three endophytes and tomato plants, but the conditions are more close to the current horticultural practice than sterile cultures. No studies on the influence of DSE on plant performance at different stages have been conducted, but a similar phenomenon was observed for the endophyte *P. indica*; colonised barley plants showed significantly higher shoot lengths at 9 and 12 weeks after inoculation, but shoots were not taller at the end of the vegetation period (Achatz et al. 2010). Also, experiments in tomato with *P. indica* showed that endophyte-inoculated plants increased shoot fresh weights 5–8 weeks after inoculation (Fakhro et al. 2010), but such differences disappeared at later harvests (Andrade-Linares et al. 2010).

Acceleration of early processes of plant development can be caused by several characteristics of root endophytic fungi. It has been shown that such organisms directly produce auxin-like substances (Sirrenberg et al. 2007; Vinale et al. 2008) or indirectly influence hormone signal transduction and hormone-regulated gene expression (Barazani et al. 2007; Vadassery et al. 2008; Schäfer et al. 2009). The growth of younger plants might be more sensitive for such changes and also the observed modifications in root architecture in the older plants could be caused by phytohormones. Root length was reduced in one experiment by DSE48 and root diameter enhanced in both experiments by DSE49 (Table 3). As auxin inhibitors increase root length and reduce root diameter (Zhao and Hasenstein 2009), it will be interesting to analyse if these DSEs also produce auxin-like substances like other fungal root endophytes do (see above).

Table 6 Metabolite contents in tomato fruits

	19 weeks after inoculation				21 weeks after inoculation			
Metabolites in 100 g FW	C	DSE48	DSE49	*L. orchidicola*	C	DSE48	DSE49	*L. orchidicola*
Acids [mg]	386±22.6	395.3±7.41	415.1±11.84	398.7±8.62	368.2±0.70	360.7±10.35	372.8±2.06	349.8±13.97
Glucose [g]	1.07±0.01	1.13±0.038	1.15±0.004	**1.26±0.044**	1.57±0.035	1.52±0.053	1.55±0.043	1.6±0.081
Fructose [g]	1.22±0.041	1.34±0.043	1.33±0.012	1.39±0.077	1.75±0.007	1.71±0.018	1.73±0.038	1.78±0.068
Lycopene [mg]	5.5±0.295	5.34±0.016	4.79±0.025	4.66±0.196	7.62±0.054	6.57±0.519	7.34±0.406	6.60±0.152
ß-Carotene [mg]	0.32±0.04	0,31±0.042	0.36±0.017	0.32±0.000	0.30±0.025	0.27±0.023	0.29±0.035	0.27±0.003

Marketable fruits with the same red intensity were sampled from plants 19 and 21 weeks after inoculation with the fungal endophytes DSE48, DSE49 and *L. orchidicola* and analysed. Averages values are shown with their ±standard errors. Statistical comparisons between treatments were performed by one-way ANOVA according to LSD test (P=0.05; n=6)

Value in bold denotes significant differences between the colonised and non-colonised control plants (C)

Isolates of DSEs have been shown to reduce disease symptoms in Chinese cabbage and eggplant after challenge with *Verticillium* pathogens (Narisawa et al. 2002; 2004). It was therefore analysed in four experiments (A–D), if the three DSEs are able to reduce the symptoms in tomato caused by *V. dahliae*. At low concentrations (10^5 conidia mL^{-1}) of the pathogen, weight loss percentage and yellowing of the leaves were only observed in experiment B by two of the endophytes (DSE49 and *L. orchidicola*; Table 4 and Fig. 1). At higher pathogen concentrations (10^7 conidia mL^{-1}) symptoms were in the same range, but only DSE49 could reduce the disease index in one out of two experiments. These differences could be based on the variations in the conditions in the four experiments.

Disease symptoms were developed when inoculum density was high (experiment C and D) or at reduced density only, if humidity was low enough (experiment B in Table 1). Stem resistance of water flow is increased in infected plants because of the vessels occluded by the pathogen and it is reflected in lower relative water content in wilted leaves (Pegg and Brady 2002). This phenomenon could be enhanced by the decreased humidity in experiment B, although inoculum density was also low (Table 1). Another factor could be the high radiation in experiment A compared to the other three experiments (Table 1). Leaf photosynthesis is greatly impaired by *V. dahliae* infection, with lower net assimilation rates (Pegg and Brady 2002) and at the high radiation in experiment A might had compensated this effect. If disease symptoms were detectable, reduction of these symptoms by the two DSEs was only clear in one out of three experiments. One might argue that this is due to the higher inoculum density in experiment C and D. The concentration of the pathogen in the plant must not be correlated with the severity of the symptoms (Veronese et al. 2003) but it could have affected the plant protection ability of DSE49 and *L. orchidicola*.

Another variation between experiments B on the one site and C or D on the other is the use of pure substrate versus substrate mixed with sand. In spite of the same mineral nutrient solution in all experiments, the amount of organic compounds is higher in the pure substrate. This could be important for the plant–DSE interaction and DSE ability to induce resistance. DSEs can facilitate the uptake of organic nitrogen, phosphate, sulphur compounds and of carbon (reviewed in Mandyam and Jumpponen 2005) and positive growth promotion by different DSEs was only observed when plants were grown in substrate amended with organic nitrogen (Upson et al. 2009). It is interesting to note that plant growth promotion at early stages was much lower in experiment C and D than in A and B (data not shown) also indicating the role of the substrate for the functioning of the DSEs. The mechanism by which DSE49 and *L. orchidicola* protect the tomato plants and whether this involves plant tolerance or resistance remains to be studied. Antagonistic activity against *V. dahliae* was evaluated for these isolates, but no growth inhibition or mycoparasitism were observed (data not shown). This might indicate that the induction of plant defence called priming (Conrath et al. 2006) could be the mechanism behind the observed phenomenon. Priming is based on the activity of particular plant hormones like jasmonate, ethylene or salicylic acid (Gutjahr and Paszkowski 2009; Shoresh et al. 2010). If the level of phytohormones is affected by the DSEs remains to be shown.

Two weeks after first flowering, fruit numbers were increased in experiment E by a factor of 3.5 in plants colonised by *L. orchidicola* and 1.75 and 2.2 for DSE48- and DSE49-inoculated plants. All these differences could not be observed at later dates of harvest (Table 5). As already discussed for the vegetative growth, it seems that inoculated plants reach earlier the generative phase of development and therefore show higher numbers of flowers and fruits in the beginning. At later phases, control plants catch up and develop at the end similar numbers of generative organs. This phenomenon seemed to be related to the intensity of endophytic colonisation which was lower in experiment B where no effect on fruit biomasses was observable. Low temperature of (18°C/15°C day/night) at the beginning of the experiment was monitored in the first 5 weeks after inoculation in the climate chamber for experiment F. This might have directly affected fungal growth rates or indirectly the colonisation process due to influences on plant physiology at low temperatures mirrored by the increased dry matter of the plants compared to those in experiment E (Table 3). Other differences of plants growing at lower temperature are higher starch accumulation and lower rate of net photosynthesis (Venema et al. 1999) or increased abcisic acid contents (Daie and Campbell 1981) which all might influence the colonisation capacity of the fungal endophytes.

Quality analyses were carried out with fruits mixed from both experiments. This mode of sampling was possible, because deviations among the values obtained were low (Table 6). Measuring the contents of titratable acids, reducing sugars and carotenoids (lycopene and ß-carotene) showed only one significant effect by endophytic colonisation: glucose concentrations were increased in tomatoes from plants where the roots were colonised by *L. orchidicola* (Table 6). As influence on vegetative growth and on the development of generative organs, this could be however only observed at the early date. Glucose amounts depend among others on enzymatic activity of the acid invertase (e.g. Johnson et al. 1988) and this could be also regulated by phytohormones. High levels of gibberellic acid, auxins and abscisic acid induce the expression of the corresponding genes (Roitsch et al. 2003). Hence, differences between inoculated and control plants could be

controlled by plant hormones which play an integral role not only in controlling growth and development but also in regulating the sink strength (Roitsch et al. 2003). One could speculate that glucose levels in fruits are enhanced during certain stages in plants colonised by *L. orchidicola* due to systemic induction of biosynthesis of these phytohormones.

Conclusion

Tomato plants grown under horticultural conditions and colonised by the dark septate endophytes DSE48, DSE49 and *L. orchidicola* showed some positive responses. The ability of DSEs to confer benefits to the plants seems, however, to be restricted to the early stages of vegetative or generative development. This does not lead to an overall increased yield or fruit quality but yield of the first harvest was increased by *L. orchidicola*. Also, a plant-protective effect could be only observed under particular conditions. Therefore, the effects of the fungal endophytes depended on the cultivation management employed in the present experiments. Under natural conditions, these slight differences in rate of development and disease resistance might, however, give DSE-colonised plants an advantage which finally leads to a better ecological performance. If this could be used in horticulture under detrimental conditions like drought, low plant-available nutrients or natural occurring pathogens has to be further investigated.

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