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ORIGINAL ARTICLE

Microconidia germination of the tomato pathogen *Fusarium oxysporum* in the presence of root exudates

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Abstract

In this study we assessed microconidia germination of the tomato pathogens *F. oxysporum* f. sp. *lycopersici* (*Fol*) and *F. oxysporum* f. sp. *radicis-lycopersici* (*Forl*) in the presence of root exudates. Tomato root exudates stimulated microconidia germination and the level of stimulation was affected by plant age. Treatment of root exudates with insoluble polyvinylpolypyrrolidone, which binds phenolic compounds, indicated that tomato root exudates contain phenolic compounds inhibitory to *F. oxysporum* microconidia germination. Our study indicates that tomato root exudates similarly stimulate microconidia germination of both *Fol* and *Forl*. However, individual *F. oxysporum* strains differ in the degree of germination response to the root exudates. Furthermore, root exudates from non-host plants also contain compounds that stimulate microconidia germination of *Fol*. In general, the effects of root exudates from non-host plants did not differ considerably from those of tomato. The ability of phenolic compounds to inhibit germination of *Fol* seems not to be plant-specific.

Keywords: Fusarium oxysporum *f. sp.* lycopersici, *root exudates, tomato, spore germination, phenolic compounds*

Introduction

The majority of plant-pathogen interactions in the soil rely on the release of soluble or volatile components from seeds and/or roots which stimulate the germination of soil-borne propagules. Background information about the activation of quiescent fungal propagules by such exudates is fundamental in understanding the initiation and control of root infections by soil-borne pathogens (Nelson 1991).

The quality and quantity of substances released by the root into the rhizosphere are conditioned by the plant species, the growth conditions, the developmental stage, environmental factors, mechanical or disease injuries as well as microbial activities (Curl & Truelove 1986, Hale et al. 1978). Most substances exuded by plant roots, such as sugars, amino acids, organic acids, phenolic compounds, flavonoids, enzymes, fatty acids, growth regulators, nucleotides, tannins, carbohydrates, steroids, terpenoids, alkaloids, polyacetylenes and vitamins, are involved in plant metabolic processes (Bertin et al. 2003), but as reviewed by Nelson (1991) little is known about the specific exudate molecules initiating vegetative growth of fungal propagules in the rhizosphere.

Fusarium oxysporum Schlecht. is an extremely common soil fungus that occurs in the rhizosphere of many plant species. Most *F. oxysporum* strains in soil live saprophytically on organic substrates. However, some soil-borne *F. oxysporum* strains can cause plant diseases, especially root rots and wilt disease. Other *F. oxysporum* strains are effective as biocontrol agents. *F. oxysporum* is a very complex group, divided into formae speciales and physiological races depending on the pathogenicity toward particular plant species or cultivars (Armstrong & Armstrong 1981). On tomato two diseases are known, caused by *F. oxysporum*. *Fusarium* wilt of tomato is caused by *Fusarium oxysporum* f. sp. *lycopersici*. This pathogen penetrates the roots mainly through wounds and proceeds into and throughout the vascular system, leading to functional collapse, systemic wilting and often the death of the infected plant. Whereas *Fusarium* crown and root rot on tomatoes is caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. This fungus invades susceptible plants through wounds and natural openings created by newly emerging roots. Infected plants may either totally wilt and die, or persist in a weakened state,

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producing a reduced number of inferior fruits (Jarvis & Shoemaker 1978).

F. oxysporum produces chlamydospores, macroconidia and microconidia. All stages are present in infected tissues or soil (Nelson 1981). Details of the colonization process of *F. oxysporum* within and outside the vascular system have been studied by several authors (Brammall & Higgins 1988, Charest et al. 1984, Lagopodi et al. 2002, Olivain & Alabouvette 1997, 1999, Rodriguez-Gálvez & Mendgen 1995) and the mycelial development in the vicinity of plant roots has been investigated by Steinberg et al. (1999), but little is known about the germination of *F. oxysporum* propagules, a key step in plant-pathogen interactions (Nelson 1991). The purpose of our study was to assess the effect of root exudates of host (tomato) and non-host plants on spore germination of *Fusarium oxysporum* pathogenic on tomato. We report specifically the impact of phenolic compounds produced by host and non-host plants and the effect on different fungal strains.

Materials and methods

Plant material and extraction of root exudates

Seeds of tomato (*Lycopersicon esculentum* Mill. cv. Micro Tom), barley (*Hordeum vulgare* L. cv. Astrid), cucumber (*Cucumis sativus* L. cv. Hoffmanns Giganta), tobacco (*Nicotiana tabacum* L. cv. Samsun), sweet pepper (*Capsicum annuum* L. cv. WE 880 Block) and bean (*Phaseolus vulgaris* L. cv. Taylor's Horticulture) were surface-sterilized with household bleach (3.8% NaOCl) for 5 min, rinsed repeatedly in sterile double-distilled water and sown in a seedling tray. After 20 days of cultivation the plants were transplanted in 12 cm plastic pots (volume 630 ml). All plants were cultivated in sterilized perlite (Granuperl S 3-6, Knauf Perlite GmbH, Vienna, Austria). According to their moisture requirements the plants were watered with full strength nutrient solution throughout the experiments. The nutrient solution consisted of 472.30 mg $Ca(NO_3)_2$, 261.40 mg K_2SO_4,136.00 mg KH_2PO_4, 369.72 mg $MgSO_4$, 8.004 mg NH_4NO_3, 50.000 mg $Fe_6H_5O_7 \times 3$ H_2O,1.30 mg $Na_2Bo_4O_7 \times 4$ H_2O, 1.500 mg $MnSo_4 \times 4$ H_2O, 0.60 mg $ZnSO_4 \times 7$ H_2O, 0.450 mg $CuSO_4 \times 5$ H_2O, 0.028 mg $Al_2(SO_4)_3$, 0.028 mg $NiSO_4 \times 7$ H_2O, 0.028 mg $Co(NO_3)_2 \times 6$ H_2O, 0.028 mg TiO_2, 0.014 mg $LiCl_2$, 0.014 mg $SnCl_2$, 0.014 mg KJ, 0.014 mg KBr and 0.070 mg MoO_3 (Steineck 1951, modified). All experiments were performed in a plant growth chamber (York International) at 24°C with a photoperiod of 16 h light/ 8 h dark (light intensity 296 μmol m^{-1} s^{-1}) and done twice. After various periods of cultivation (10–100 days) the plants were harvested non-destructively by gently washing the perlite from the roots with tap water. The plants were inserted in a beaker containing sterile distilled water, such that the roots were completely submerged, and placed in a plant growth chamber for 24 h at 24°C. Thereafter, the plants were removed from the beaker and the resulting exudate solution was adjusted with sterile distilled water to 1 g root fresh weight per 20 ml exudate solution (w/v) (Stevenson et al. 1995, Pinior et al. 1999). In order to get additional information on the plant material besides the root fresh weight also the fresh weight of the shoot, the phenological growth stage (according to Anonymous 1997) and the pH value of the root exudate were determined. The exudates were passed through 0.22 μm sterilfilters (Steriflip, Millipore, Bedford, U.S.A.) and stored at −20°C up to further investigation.

Fungal cultures

F. oxysporum f. sp. *lycopersici* and *F. oxysporum* f. sp. *radicis-lycopersici* were stored as microconidial suspension in 30% glycerol at −80°C, fortnightly transferred to Czapek Dox Agar (Dhingra & Sinclair 1995) and grown at 24°C in darkness. The *F. oxysporum* f. sp. *lycopersici* isolate Fol 004 (race 1), isolate Fol 007 (race 2) and isolate Fol 029 (race 3) were kindly provided by B.J. Cornelissen, Institute for Molecular Cell Biology, Amsterdam. *F. oxysporum* f. sp. *radicis-lycopersici* isolate Forl 154 and isolate Forl 155 was provided by Laboratory N. Benhamou, Université Laval, Québec. *F. oxysporum* f. sp. *radicis-lycopersici* isolate Forl 873.95 was purchased from Centraalbureau voor Schimmelkulturen (Utrecht, NL). Under sterile conditions fungal culture plates were flooded with sterilized water and the resulting conidia suspension was filtered through three layers of filter paper (Vliesscheiben für Kannenfilter, Laporte Ges.m.b.H., Wels, Austria) to separate the microconidia from the mycelium. The microconidia suspension was concentrated by centrifugation at 3000 g for 10 min and adjusted to 1.0×10^7 microconidia/ml water using a haemocytometer.

Germination experiments

The germination assay was performed in sterile culture plates (24 wells, Greiner bio-one, Nr. 662160, Frickenhausen, Germany). To determine the effect of phenolic compounds subsamples of the sterilfiltered exudates were treated with water insoluble PVPP (polyvinylpoly-pyrrolidone). PVPP is extremely hydrophilic and known as a substance which binds very efficiently phenolics, including a wide variety of flavonoids (Loomis & Battaile 1966, Doner et al. 1993). Therefore the sterilfiltered exudates were mixed with 0.08% PVPP (P-6755, Sigma-Aldrich, St Louis, USA) and shaken at 24°C for 2 h. The PVPP was removed by filtration and this PVPP-treatment was repeated with the filtrate.

Aliquots of 500 μl of non-treated and PVPP-treated root exudate respectively were mixed with

100 μl of spore suspension (three wells per sample) and incubated at 24°C in the dark while being shaken at 200 rpm. Microconidia germination was determined microscopically after 24 h. Two-hundred spores in each well were randomly examined for presence of germ tubes. A microconidium was considered germinated if the germ tube length was at least as long as the spore (see Figure 1). The experiments were performed three times. Sterilized water and Czapek Dox solution were included in the germination experiments as a control. An additional germination experiment was conducted with Czapek Dox solution (with and without sucrose) and sucrose solution (30 g/l H_2O). Preliminary experiments indicated that PVPP-treatment of Czapek Dox solution and water did not affect microconidia germination.

Statistical analysis

Analysis of variance was done after a variance check by Levene's test. Mean values were compared using Fisher's least significant difference ($LSD_{\alpha\ 0.05}$). These analyses were performed using appropriate standard statistical methods (procedure GLM of SAS statistical software package).

Results

The germination of *F. oxysporum* f. sp. *lycopersici* isolate Fol 007 in the presence of tomato root exudates is shown in Figure 2A. Plant age had a significant effect on microconidia germination of *F. oxysporum* f. sp. *lycopersici* in tomato root exudates. The germination rate in the presence of root exudates showed a slight increase from the earliest growth stages (4–5%) up to 40-day-old plants (15.7%) followed by a drastic decrease in the presence of root exudates from 50- and 60-day-old tomatoes (2.0–3.5%). With root exudates from 70-, 80- and 90-day-old tomatoes a significantly higher germination rate (17.8–25.4%) was observed; whereas root exudates from 100-day-old tomatoes resulted again in a lower germination (4.0%) of microconidia. 3.2% germination was observed in water alone. The germination rate in the presence of root exudates was significantly lower than in Czapek Dox medium (83.5%), an optimal medium for cultivation of *F. oxysporum* f. sp. *lycopersici.* The results for untreated and PVPP-treated root exudates are represented separately, as the multi-factorial statistical analyses have shown significant treatment by plant age interactions (F value = 7.00, $p < 0.0001$).

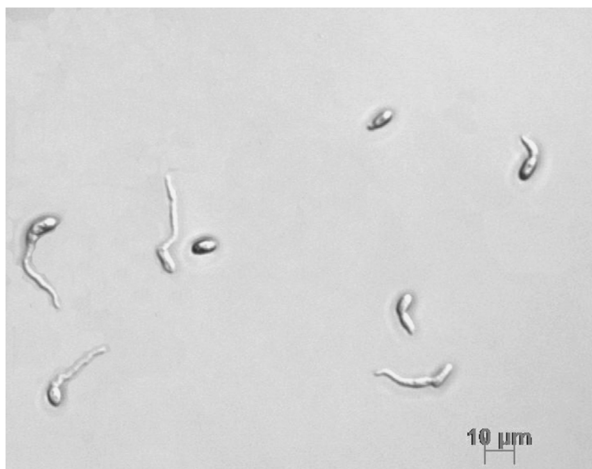

Figure 1. Germinating microconida of *Fusarium oxysporum* f. sp. *lycopersici* (Fol 007) incubated at 24°C for 24 h. Bar = 10 μm.

Treatment of root exudates with PVPP resulted in a germination rate 17.0–55.2% higher than with untreated exudates (Figure 2B). The lowest germination rate (35.5%) was observed with root exudates from 20-day-old tomatoes, the highest germination rate (57.3%) with root exudates from 60-day-old tomatoes. The stimulatory effect of PVPP-treated root exudates was significantly higher than that of water and significantly lower than that of Czapek Dox medium. PVPP-treatment of Czapek Dox medium and water had been tested in preliminary tests and had shown no effect on microconidia germination (data not shown).

The respective phenological growth stages and more detailed information on plant material are presented in Table I. Within the first 40 days of plant cultivation the greatest relative tomato root weight gain was determined. The mean root weight of tomato plants after 40 days of cultivation was between about one and a half times (bean) and 62 times (tobacco) higher than the root weight of the investigated non host plants. The pH-values of the investigated root exudates were in the range of 6.22–7.52.

The germination rate of *F. oxysporum* f. sp. *lycopersici* isolate Fol 007 in the presence of root exudates from tomato (host-plant) and non-host plants (plant age 40 days) is shown in Table II. These root exudates have been selected, as the experiments of the time series mentioned above, have shown high effects for both, untreated and treated root exudates of this plant age. Microconidia germination in the presence of root exudates was significantly higher than in the presence of water. On average the stimulatory effect of tomato root exudates (61.8%) was significantly higher than of the non-host plants bean (52.4%), barley (49.0%), tobacco (47.8%) and cucumber (43.3%). Sweet pepper root exudates (57.8%) did not differ significantly from tomato root exudates. The treatment of the root exudates with PVPP resulted in a significantly increase of spore germination ($p < 0.0001$). The germination rate in PVPP-treated root exudates was between 1.75 times (tomato) and 2.45 times (cucumber) higher than in untreated root exudates.

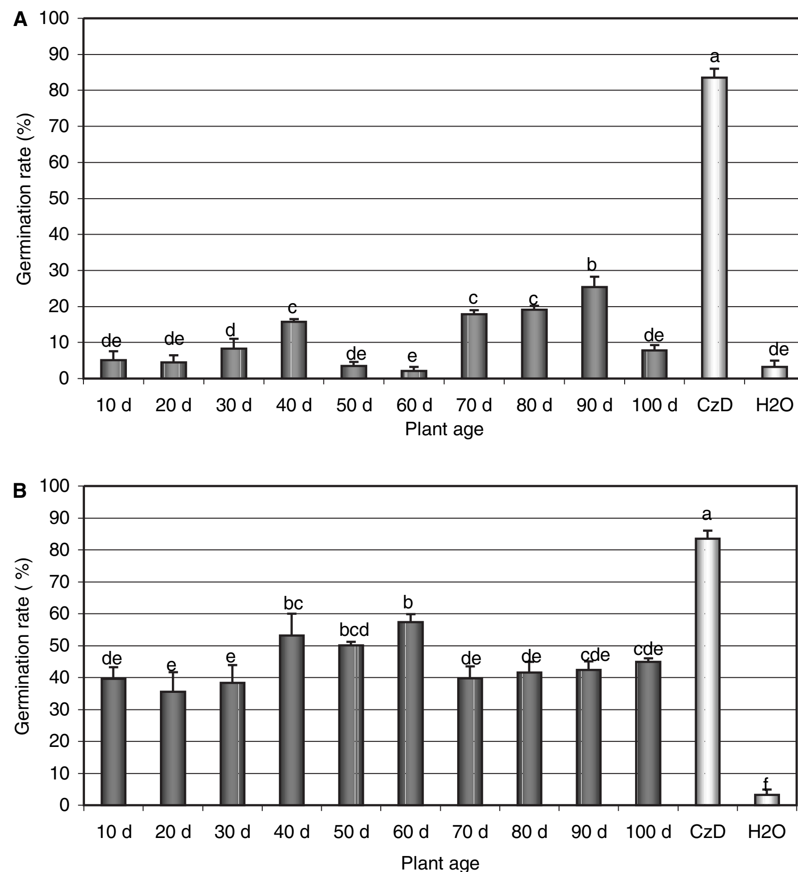

Figure 2. Germination rate (%) of *Fusarium oxysporum* f. sp. *lycopersici* (Fol 007) in root exudates collected from tomatoes between 10 and 100 days of plant growth. (A) Root exudates without PVPP-treatment; (B) Root exudates after treatment with PVPP. Error bars represent standard error of the mean. Bars with the same letter are not significantly different – (A) $LSD_{\alpha\ 0.05} = 5.36$; (B) $LSD_{\alpha\ 0.05} = 10.86$.

The germination rate of individual *Fusarium* strains was also studied in the presence of root exudates from 40-days-old tomato plants (Table III). Microconidia germination was highest for *F. oxysporum* f. sp. *lycopersici* isolate Fol 007 (39.1%), followed by isolate Fol 029 (34.9%), and was lowest

Table I. Phenological growth stages, plant weight and pH-value of root exudates.

Plant age	Phenological growth stage[1]	Mean root weight	Mean shoot weight	pH-value[2]
Tomato				
10 days	BBCH 10	0.007	0.018	7.52
20 days	BBCH 11	0.020	0.107	6.98
30 days	BBCH 12	0.186	1.249	7.14
40 days	BBCH 17	1.069	3.184	6.22
50 days	BBCH 20	1.593	4.081	6.70
60 days	BBCH 51–61	1.950	10.490	6.72
70 days	BBCH 51–63	2.275	15.748	6.90
80 days	BBCH 63–72	2.847	20.433	6.79
90 days	BBCH 64–73	2.030	22.013	6.88
100 days	BBCH 68–73	2.770	27.803	6.93
Barley				
40 days	BBCH 13–15	0.219	0.518	6.67
Cucumber				
40 days	BBCH 11–16	0.186	1.573	6.99
Tobacco				
40 days	BBCH 11–14	0.017	0.297	7.41
Sweet pepper				
40 days	BBCH 16	0.171	0.795	7.50
Bean				
40 days	BBCH 71–75	0.656	3.549	7.36

[1]BBCH – Growth stages of plants (Anonymous 1997); [2]pH-value of root exudates (1g root/20 ml exudate).

Table II. Germination rate (%) of *Fusarium oxysporum* f. sp. *lycopersici* (Fol 007) in root exudates from tomato (host plant) and non-host plants.

	Germination rate (%)		
Plant	Untreated	PVPP-treated	Mean*
Tomato	44.9	78.7	61.8 a
Sweet pepper	35.8	79.8	57.8 ab
Bean	35.2	69.6	52.4 bc
Barley	32.6	65.4	49.0 cd
Tobacco	30.2	65.3	47.8 cd
Cucumber	25.0	61.6	43.3 d
H_2O	10.6		10.6 e

Source	DF	Mean Square	F Value	Pr > F
Treatment	1	35190.28	448.41	< 0.0001
Plant	6	8423.99	13.92	< 0.0001
Treatment*plant	5	373.68	0.74	0.5946
Error	104	10492.39		

* Means with the same letter are not significantly different ($LSD_{\alpha\ 0.05} = 7.10$).

for isolate Fol 004 (10.1%). Spore germination of *F. oxysporum* f. sp. *radicis-lycopersici*-strains was in the range of 15.2% (isolate Forl 155) to 30.6% (isolate Forl 873.95). Treatment of the root exudates with PVPP resulted in a 2.3–27.6% higher spore germination. The highest level was observed with *F. oxysporum* f. sp. *lycopersici* isolate Fol 007 (66.7%) and the lowest with *F. oxysporum* f. sp. *lycopersici* isolate Fol 004 (13.9%). Statistical evaluation by LSD-test indicates a significant difference between Fol 007 and most of the other strains ($p <$ 0.0001). PVPP-treated root exudates have shown a significant higher germination rate than untreated root exudates ($p = 0.0002$). The increase lay between 2.3% (Forl 873.95) and 27.6% (Fol 007).

In a fourth experiment the effect of sucrose on spore germination was tested. In comparison to Czapek Dox medium including sucrose (85.0%), the stimulatory effect of Czapek Dox medium without sucrose reached about one third (23.5%), sucrose alone reached about the half (44.5%).

Discussion

The stimulation of plant pathogens by root exudates is an integral part of the concept of inoculum potential (Lockwood 1986). Propagules of several plant pathogenic fungi have been shown to germinate in the presence of root exudates (Nelson 1991), but little is known yet on the microconidia germination of *F. oxysporum* in the presence of root exudates. Microconidia are efficient fungal propagules of *F. oxysporum* for the infection of tomato plants and have primarily been used in a number of studies (e.g. Huertas-Gonzáles et al. 1999, Olivain & Alabouvette 1997, Lagopodi et al. 2002).

In our study, we showed that tomato root exudates stimulate microconidia germination of *F. oxysporum* f. sp. *lycopersici* only moderately, the specific stimulation level depending on plant age as well as on the fungal strain. This plant age-dependent effect can be attributed to quantitative and qualitative differences in exudated compounds due to physiological changes during plant development. Plant age plays

Table III. Germination rate (%) of *Fusarium oxysporum* f. sp. *lycopersici* strains (Fol) and *Fusarium oxysporum* f. sp. *radicis-lycopersici* strains (Forl) in root exudates collected from 40 days old tomato plants.

	Germination rate (%)		
Strain	Untreated	PVPP-treated	Mean*
Fol 007	39.1	66.7	52.9 a
Fol 029	34.9	46.9	40.9 ab
Forl 154	25.9	47.1	36.5 b
Forl 873.95	30.6	32.9	31.7 b
Forl 155	15.2	40.1	27.7 b
Fol 004	10.1	13.9	12.0 b

Source	DF	Mean Square	F Value	Pr > F
Treatment	1	6317.37	14.50	0.0002
Strain	5	3388.18	7.78	< 0.0001
Treatment*strain	5	529.01	1.21	0.3083
Error	96	435.64		

* Means with the same letter are not significantly different ($LSD_{\alpha\ 0.05} = 13.81$).

a decisive role in root exudation, similarly to plant species, cultivars, temperature or stress factors (Brammall & Higgins 1988, Hale et al. 1978, Short & Lacy 1976). Compounds, such as root exudates sugars and organic acids, were shown to vary during individual growth stages of tomato (Kravchenko et al. 2003, Lugtenberg et al. 1999). Sugars are known to be stimulatory to germination of *Fusarium* spp. (Nelson 1991).

We also found that the stimulatory effect of the Czapek Dox medium we used could be attributed predominantly to sucrose. The sugar content in the root exudates has not been evaluated. However, in the light of PVPP-treatment in our study, different sugar levels in the root exudates during the time course experiment seemed not to be responsible for the varying effect of the exudates on microconidia germination. Our results lead to the conclusion that tomato root exudates, among stimulating molecules e.g., sugars do contain a major amount of compounds which are inhibitory to microconidia germination of *F. oxysporum*. We found that germination was clearly enhanced after the treatment of root exudates with water-insoluble polyvinylpolypyrrolidone (PVPP). This substance facilitates the trapping of flavonoids and other phenolic compounds which plants exude through their roots (Doner et al. 1993, Ruan et al. 1995). Our results indicate that tomato root exudates do contain such constitutively produced phenolic compounds inhibitory to *F. oxysporum* f. sp. *lycopersici* microconidia germination and apparently the levels of these compounds vary with the plant age. Interestingly, El Khatib et al. (1974) reported that the accumulation of phenolics in tomato plants is subjected to the growth stage. The functions of these substances are largely unknown. Besides other functional roles flavonoids and other phenolic compounds are reported for their accumulation in general defence responses in plants (Beckmann 2000, Ferraris et al. 1987, Kosuge 1969, Morrissey & Osbourne 1999).

Ruan et al. (1995) reported that treatment of pea root exudate with PVPP results in a reduced germination of macroconidia of *F. solani* f. sp. *pisi* indicating the presence of stimulatory phenolic compounds in the rinsate. They found that a large variety of flavonoids released by legume roots exhibits a stimulatory effect on macroconidia germination. In this context a high level of phenolic compounds in the exudates seems to trigger favourably an important step of the plant-*F. solani* interaction. Our data point towards a completely different concept. Occurrence of phenolic compounds in the tomato exudates seems to affect microconidia germination of *F. oxysporum* negatively in most cases. This would mean that reduced levels of phenolic compounds are favourable for the germination. This seems in contrast to the observation reported by Ruan et al. (1995) about the stimulatory effect on germination of phenolic compounds in root exudates, however, it has to be kept in mind that the effect on macroconidia (Ruan et al. 1995) could differ from the effect on microconidia we used in our study and moreover, legume root exudates (Ruan et al. 1995) might differ in their composition from tomato root exudates. While a number of soil microbes do respond to flavonoids produced by legumes (Straney et al. 2002), the effect of related compounds produced by tomatoes is widely unknown. Flavonoids produced by tomato, such as kaempferol, naringenin, quercetin and rutin, are primarily documented in fruits (Hermann 1979, Muir et al. 2001, Stewart et al. 2000). These flavonoids have been tested with *F. solani* pathogenic on pea or bean (Ruan et al. 1995). While naringenin was highly stimulatory to macroconidia germination, kaempferol and quercetin showed no effect. Unfortunately, no data are available on the effect of these compounds on *F. oxysporum* f. sp. *lycopersici*.

Our data indicate that tomato root exudates similarly stimulate microconidia germination of both *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *radicis-lycopersici*. However, individual *F. oxysporum* strains differ in their disposition to germinate. Looking at the mycelial development of *F. oxysporum* in the vicinity of tomato roots Steinberg et al. (1999) also reported strain specificity. Thus, our tested *F. oxysporum* strains seemed to be physiologically different and therefore responding variously to exudate compounds. In the light of the variability among different fungal strains, the possibility of strain-specific responses to root exudates cannot be ruled out entirely.

Although *F. oxysporum* f. sp. *lycopersici* is known as highly specialized on tomatoes, we could show that the fungus also responds to root-released signals from non-host plants. Root exudates from non-host plants exhibited a stimulatory effect on microconidia germination of *F. oxysporum* f. sp. *lycopersici* similar to the effect of root exudates from the host plant tomato. These observations are in line with data reported by Steinberg et al. (1999) who found no differences in mycelial development of *F. oxysporum* in the vicinity of tomato and wheat roots. It is known that non-host plants can function as symptomless carriers of wilt fusaria (Armstrong & Armstrong 1948, Katan 1971). A stimulatory effect on the germination of chlamydospores in the vicinity of non-host plants has also been documented (Schippers & van Eck 1981). Thus, host recognition by this pathogen seems to not be a major factor in microconidia germination. Our results seem in contrast to the host selectivity of germination of *F. solani* f.sp. *pisi* reported by Straney et al. (2002). The exudation of compounds inhibitory to the microconidia germination of *F. oxysporum* f. sp. *lycopersici* seems not to be limited to the host (tomato) of the tested fungus. Root exudates of the tested non-host plants, cucum-

ber, bean, barley, tobacco and sweet pepper, seemed to contain PVPP-binding compounds which showed a similar inhibitory effect on microconidia germination as root exudates of the host plant tomato. Thus, the ability of these preformed antifungal phenolics to inhibit germination of *F. oxysporum* f. sp. *lycopersici* appears not to be plant specific.

To summarize, our results suggest that phenolic compounds are of major importance in initial processes of the tomato-*F. oxysporum* interaction. Further studies are needed to identify the compounds involved and to clarify their role as regulatory signals.

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