Brief report

Biocontrol strain *Pseudomonas fluorescens* WCS365 inhibits germination of *Fusarium oxysporum* spores in tomato root exudate as well as subsequent formation of new spores

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Summary

*Fusarium oxysporum* f.sp. *radicis-licopersici* (Forl) is a soilborne pathogenic fungus which can cause tomato foot and root rot (TFRR). Tomato root exudate is a good source of nutrients for both Forl and the TFRR-suppressing biocontrol bacterium *Pseudomonas fluorescens* strain WCS365. Incubation of Forl microconidia in tomato root exudate stimulates their germination. This phenomenon is observed, to a lesser extent, upon incubation in plant nutrient solution supplemented with citrate or glucose, the major organic acid and sugar components, respectively, of tomato root exudate. Here we show that induction of germination of microconidia is significantly reduced in the presence of *P. fluorescens* WCS365 in all tested media. Scanning electron microscopy revealed that *P. fluorescens* WCS365 colonizes developing hyphae. Efficient colonization correlates with low nutrient availability. Eventually, new microconidia are formed. The presence of *P. fluorescens* WCS365 reduces the number of newly formed microconidia. This reduction does not depend on physical contact between bacteria and hyphae. We discuss that the ability of *P. fluorescens* WCS365 to slow down the processes of microconidia germination and development of new microconidia of the phytopathogen, and therefore the ability to reduce fungal dissemination, is likely to contribute to the biocontrol efficacy of this strain.

Introduction

Biological control of soilborne plant pathogens is based on application of natural antagonists of the pathogen. Examples are (i) beneficial microorganisms which produce antimicrobial components, (ii) microbes which can induce systemic resistance by triggering the defence mechanism of the plant, and (iii) microbes which can out-compete pathogens because they are quicker in consuming nutrients secreted by the plants and faster in occupying of niches on the root surface (Bloemberg and Lugtenberg, 2004; Compant et al., 2005; Haas and Defago, 2005; Kamilova et al., 2005; Validov et al., 2007). Biological control of tomato foot and root rot (TFRR) caused by *Fusarium oxysporum* f.sp. *radicis-licopersici* (Forl) by various *Pseudomonas* strains has been a subject of several studies (Duffy and Défago, 1997; Chin-A-Woeng, 1998; 2000; Dekkers et al., 2000; Kamilova et al., 2005; Rezzonico et al., 2007; Validov et al., 2007). One of the best strains for stable biocontrol of TFRR in both soil and hydroponic systems is *Pseudomonas fluorescens* strain WCS365 (Dekkers et al., 2000; Kamilova et al., 2005; 2006a; Validov et al., 2007). This strain was isolated as a siderophores-producing antagonist (Geels and Schippers, 1983). It does not inhibit growth of *F. oxysporum* in media containing a sufficient amount of available ferric ions. It does not produce protease, lipase, chitinase, gluconase, HCN, N-acyl homoserine lactones or biosurfactants (F. Kamilova, unpubl. data), bacterial metabolites which represent biocontrol traits. Previously, it was shown that this strain is an excellent root colonizer of many plants, such as potato (Brand et al., 1990), tomato, wheat and radish (Dekkers et al., 1998). Because of its outstanding root colonization ability, WCS365 became a model strain for studying traits and genes involved in competitive root tip colonization after application on seeds (reviewed in Lugtenberg et al., 2001). Although strain WCS365 is highly competitive in root colonization, this trait does not play a major role in the biocontrol of TFRR, because colonization mutants of *P. fluorescens* WCS365
are not, or hardly, impaired in biological control of TFRR (Dekkers et al., 2000). These results exclude antagonism as well as competition for nutrients and niches as major mechanisms under the tested conditions. Indeed, proof that \( P. \) fluorescens WCS365 can induce systemic resistance in tomato plants was obtained by using a tomato split-root system, in which the biocontrol agent and phytopathogenic fungus Forl are spatially separated (Kamilova et al., 2005).

Confocal laser scanning microscopy revealed that under biocontrol conditions \( P. \) fluorescens WCS365 colonizes not only the tomato root surface but also hyphae of Forl (Bolwerk et al., 2003; De Weert et al., 2004). WCS365 cells are attracted to the root by tomato root exudate components such as major organic acids and amino acids, but not by sugars (De Weert et al., 2002). Similarly, the bacterial cells are attracted to Forl by fusaric acid (De Weert et al., 2004). However, the impact of the latter interactions on the fate of the fungus in rhizosphere is not clear.

Forl belongs to the group of fungi with asexual reproduction. It produces three types of spores: microconidia, macroconidia and clamydospores (Nelson, 1981). Microconidia are ellipsoidal and have no septa or only one septum. Macroconidia have three or four septa. Round chlamydospores have thick walls (Nelson et al., 1983). Spores are important in the disease cycle: macroconidia and microconidia are produced on the stem and on root surfaces of infected plants (Katan et al., 1997; Rekah et al., 2000; Rowe et al., 1977). They serve as secondary inoculants for spreading the fungus to neighbouring plants. Chlamydospores survive in soil for a long period of time and act as primary inocula upon planting of host plants (Couteaudier and Alabouvette, 1990).

Previously it was shown that tomato root exudate allows proliferation of Forl propagules (Kamilova et al., 2005). Steinikellner and colleagues (2005) also reported a stimulating effect of tomato root exudate on germination of microconidia of \( F. \) oxysporum f.sp. lycopersici (Fol) and of Forl. In addition, we have shown the excellent tomato root colonizers and biocontrol strains \( P. \) fluorescens PCL1751 (Kamilova et al., 2005), \( P. \) putida PCL1760 (Validov, 2007) and \( P. \) fluorescens WCS365 (Kamilova et al., 2006a) can successfully out-compete Forl for growth in tomato root exudate as well as in synthetic media with individual exudate components, such as citrate, succinate, glucose and fructose, as the sole carbon source. This is accompanied by inhibition of development of fungal propagules (Kamilova et al., 2006a). These findings stimulated us to investigate the effect of \( P. \) fluorescens WCS365 on the processes of germination of \( F. \) oxysporum microconidia and sporulation in the early stages of bacteria--fungal interactions in tomato root exudate. The results, as well as the possible implications of these interactions for the biocontrol of TFRR, are presented in this paper.

**Results and discussion**

**Effect of media and presence of \( P. \) fluorescens WCS365 on germination of Forl microconidia after 24 h**

After incubation of Forl microconidia in sterile tomato root exudate for 24 h, 68–76% of the spores had germinated (Table 1). A similar effect was observed in plant nutrient

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<th>Experiment 1</th>
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<tr>
<td>Tomato exudate</td>
<td>Tomato exudate</td>
<td>Citrate(^c)</td>
<td>Glucose(^d)</td>
</tr>
<tr>
<td>Forl(^h)</td>
<td>76 ± 5 (a)</td>
<td>68 ± 8 (a)</td>
<td>35 ± 2 (a)</td>
</tr>
<tr>
<td>Forl and WCS365 (10(^5) cfu ml(^{-1}))</td>
<td>45 ± 4 (b)</td>
<td>41 ± 5 (b)</td>
<td>nd</td>
</tr>
<tr>
<td>Forl and WCS365 (10(^6) cfu ml(^{-1}))</td>
<td>20 ± 5 (c)</td>
<td>16 ± 5 (c)</td>
<td>7 ± 3 (b)</td>
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\(^a\) Bacterial cells were grown overnight in 1/20 strength tryptic soy broth (TSB, Difco Laboratories) at 28°C, spun down at 10 000 r.p.m. for 3 min, washed three times in phosphate buffer saline (PBS) and used immediately. Microconidia were obtained after cultivation of Forl in Czapek-Dox (Difco Laboratories, MI, USA) liquid medium for 3–4 days, followed by filtration through three layers of sterile cheese cloth. The resulting spore suspension was spun down at 6000 r.p.m. for 10 min and washed three times in PBS and used immediately. Tomato (cultivar Carmello) root exudate was isolated as described by Kamilova and colleagues (2006b). Root exudate (pH 5.5) was sterilized by filtration through 0.22 \(\mu\)m filters and kept at 4°C until use. Only sterile batches were used. One milliliter amounts of each media were inoculated with microconidia and bacteria to final concentrations as indicated. The suspensions were incubated for 24 h at 28°C. The number of germinated microconidia and the total number of microconidia were counted in hematocytometer using light microscopy and the percentage of germination was calculated. The germination experiments were carried out in triplicate and were repeated twice.

\(^b\) Values in the columns indicate means of three replications with standard deviation. Letters after values indicate significant difference according to a Student’s \(t\)-test at 95% confidence level. Each replica contains in total at least 100 spores.

\(^c\) Citrate Na in PNS (0.3 mM; pH 5.5).

\(^d\) Glucose in PNS (0.3 mM; pH 5.5).

\(^e\) In all experiments Forl was added at concentration approximately 2–3 \(\times\) 10\(^8\) spore ml\(^{-1}\) and incubated at 28°C in the dark. Spore germination in control (PNS) did not exceed 6 ± 3%.

\(\text{nd, not determined.}\)
solution (PNS) supplemented with citrate or with glucose as the sole carbon source, although the percentages of germinated spores was significantly lower than in tomato root exudate and reached levels of 20–35% and 12–20% respectively. Citrate and glucose were used at concentrations similar to those found in tomato root exudate (Kamilova et al., 2006b).

Earlier, Bolwerk and colleagues (2005) observed that 25% of the Forl spores germinated upon cultivation in synthetic root exudate. Steinkellner and colleagues (2005) also reported a stimulating effect of tomato root exudate on germination of Forl, up to 30%. Results of our experiments indicate that the level of germinated microconidia in tomato root exudate can even reach values over 70% (Table 1). The differences in effects of tomato root exudate on the percentage of germinated spores in our experiments and in the experiments of Steinkellner and colleagues (2005) can be explained by differences in plant age, way of plant cultivation as well as in methods of root exudate isolation, all of which can have a dramatic effect on the composition and on the amount of exudate (Kamilova et al., 2006b).

Conidia of most filamentous fungi require the presence of low-molecular-mass nutrients such as sugars, amino acids or inorganic salts, for germination (Carille and Watkinson, 1994; Osherov and May, 2001). Organic acids, sugars and amino acids are present in tomato root exudate (Simons et al., 1997; Lugtenberg et al., 1999; Lugtenberg et al., 2001; Kamilova et al., 2006b), which is a suitable medium for the propagation of Forl (Kamilova et al., 2005; Validov, 2007). It is still unclear which component(s) of tomato root exudate induce germination of Forl microconidia. Ruan and colleagues (1995) showed that flavonoids from pea root exudate can induce spore germination of F. solani. However, this does not apply to F. oxysporum as the addition of polyvinylpyrrolidone, a binder of phenolic compounds such as flavonoids to tomato exudate, did not decrease – and even increased – the level of Fol and Forl germination (Steinkellner et al., 2005). Test of a wide group of different flavonoids in various concentrations for their influence on Fol spore germination revealed that only myrecetin and luteolin exhibited a low stimulating activity (Steinkellner and Mammerler, 2007). Our results on the effect of citric acid and glucose, individual major components of tomato root exudate (Table 1), together with the finding of Steinkellner and colleagues (2005) that root exudate of various plants, including non-host plants, do stimulate germination of Fol and Forl spores, indicating that exudate nutrients such as citrate and glucose are major inducers of germination of the Forl microconidia in tomato root exudate.

The addition of 10^6 P. fluorescens WCS365 cells ml⁻¹ to root exudate resulted in a decrease of the percentage of germinated spores from 68–75% to 41–45%. This inhibiting effect of the bacterium on the level of germination of microconidia became much more profound when the bacterial inoculum was 100 times higher (Table 1). Co-cultivation of bacteria with microconidia in PNS supplemented with either citrate or glucose as the sole carbon source had an even more drastic effect on the level of germination, which did not exceed that observed in control samples containing PNS only (Table 1).

Tomato root exudate, as well as several of its individual components, support the growth of P. fluorescens WCS365 (Kamilova et al., 2006a) and other excellent bacterial tomato root colonizers (Kamilova et al., 2005; Validov, 2007), which are fast in utilization of nutrients, thereby inhibiting fungus proliferation (Kamilova et al., 2006a; Validov, 2007). Our observations that P. fluorescens WCS365 inhibits germination of microconidia in tomato root exudate as well as in the tested citrate- and glucose-containing media, allows us to conclude that this effect is due to consumption of nutrients which act as the inducers of germination.

**Development of hyphae and microconidia after 72 h**

In order to evaluate the fate of Forl hyphae in tomato root exudate after a longer period of time, we applied tomato root exudate, spent tomato root exudate, PNS/citrate and PNS, all inoculated with Forl microconidia, to glass slides and visualized Forl by scanning electron microscopy. After 72 h of incubation, development of hyphae and new microconidia was observed in all tested media. In tomato root exudate, germination of fungal spores and subsequent development of hyphae, as observed after 24 h (data not shown) was followed by sporulation as observed after 72 h (Fig. 1A and B, Table 2). Newly developed spores represented microconidia, which were located either along hyphae or at close proximity to hyphal tips. We hardly observed the appearance of macroconidia and did not observe chlamydomspores. The highest numbers of formed microconidia were observed in tomato root exudate (Fig. 1A, Table 2) followed by PNS/citrate (Table 2), PNS (Fig. 1B, Table 2) and spent exudate (Table 2). In the latter two media there was no statistically significant difference in spore numbers (Table 2).

Spent tomato root exudate contains more than 10 times less organic acids and more then five times less sugars in comparison with tomato root exudate (Kamilova et al., 2006a). PNS/citrate is also a poorer medium: it contains at least twice less carbon than tomato root exudate (Kamilova et al., 2006b). PNS is completely devoid of carbon. So, these three media contain substantially less carbon, if any, than tomato root exudate, and in all these media we have recorded a significant reduction of the number of spores in comparison with tomato root exudate.

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Hyphae colonization and effect on sporulation by P. fluorescens WCS365

Co-cultivation of fungal spores together with P. fluorescens WCS365 bacteria revealed after 72 h that WCS365 cells colonized developing hyphae in all tested media (Fig. 2A–C). In tomato root exudate, bacterial cells attach to the hyphae mostly laterally and develop a single layer (Fig. 2A), whereas at some sites microcolonies appear (Fig. 2B). In PNS, the fungus is completely covered with bacteria, which are often attached by their poles (Fig. 2C), whereas in PNS/citrate the bacterial colonization level is in between that observed in tomato exudate and PNS (data not shown). Previously colonization of Forl hyphae by WCS365 in situ was reported by Bolwerk and colleagues (2003). De Weert and colleagues (2004) described in vitro colonization of growing hyphae under conditions which were not suitable for bacterial growth. In this study we observed that P. fluorescens WCS365 colonizes hyphae more aggressively under nutrient starvation conditions, such as in PNS (Fig. 2C), than under relatively rich conditions such as tomato root exudate (Fig. 2A). A similar behavior was visualized upon cocultivation of another biocontrol strain, Collimonas fungivorans Ter331, with Forl hyphae (Kamilova et al., 2007). The reason why hyphae are colonized by bacteria is likely to use nutrients excreted by fungus (De Boer et al., 2005; Kamilova et al., 2007).

Simultaneous inoculation of P. fluorescens WCS365 bacteria with Forl in tomato root exudate and in PNS/citrate significantly reduced the number of microconidia in comparison with the samples containing Forl only.

Table 2. Effect of P. fluorescens WCS365 and media on development of Forl sporesa.

<table>
<thead>
<tr>
<th>Media</th>
<th>absence</th>
<th>presence</th>
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<tbody>
<tr>
<td>Tomato root exudate</td>
<td>235 ± 33 (a)</td>
<td>55 ± 5 (a)*</td>
</tr>
<tr>
<td>Citrate</td>
<td>138 ± 22 (b)</td>
<td>15 ± 3 (b)*</td>
</tr>
<tr>
<td>Spent tomato root exudate</td>
<td>25 ± 4 (c)</td>
<td>nd</td>
</tr>
<tr>
<td>PNS</td>
<td>30 ± 5 (c)</td>
<td>14 ± 2 (b)*</td>
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a. One millilitre of each of the media were inoculated with fungal microconidia and bacteria (pre-grown as described in Table 1) to final concentrations of 10^6 microconidia ml^-1 and 10^6 cfu ml^-1 respectively. A volume of 200 μl of each microbial suspension was mounted on the surface of a sterile glass slide (1 x 1 cm). Glass slides were individually placed into the wells of a 12-wells plates (Cellstar, GreinerBio-one B.V., Alphen a/d Rijn, the Netherlands). Plates were covered with wet paper towels to prevent glass slides from drying. The glass slides were incubated for 72 h at 28°C. For standard fixation, the slides were fixed for 2 h at room temperature in a mixture of 2.5% (v/v) glutaraldehyde and 2% formaldehyde in PBS and rinsed with PBS. After fixation, samples were dehydrated by a graded series of acetone rinses (30–100%), and critical point dried. They were mounted on metal stubs with double-sided adhesive tape, coated with a thin layer of gold (SEM coating unit E5100, Polaron equipment Ltd, Watford, UK). The samples were directly examined in a JSM6400 scanning electron microscope (JEOL, Tokyo, Japan) operating at 5 kV. Each slide was observed thoroughly and the total number of microconidia on five randomly chosen fields (120 x 170 μm each) was calculated. Each treatment was performed in duplicate and the experiment was repeated three times. Differences between microconidia counts under various conditions were evaluated using a Student’s t-test at 95% confidence level.b. Values in the columns indicate means of two replications with standard deviation. One replica contains total counts of spores in five randomly chosen fields. c. Letters after values indicate significant difference according to a Student’s t-test at 95% confidence level. *Asterisk indicates significant difference between values in the same row according to a Student’s t-test at 95% confidence level. Spent tomato root exudate was obtained as described by Kamilova and colleagues (2006a).
There was no significant difference in the numbers of microconidia developed in PNS in the presence or absence of bacterial cells (Table 2). The reason why hyphae form microconidia likely is that the latter structures are responsible for initiating infection as well as fungal dissemination (Dahlberg and Van Etten, 1982; Rekah et al., 2000). The signal for starting this developmental process, as well as how the presence of bacterial cells interferes with the development of microconidia is unknown. As spent root exudate, isolated after growth of WCS365 in tomato root exudate, is reduced in its ability to induce development of new spores (Table 2), we conclude that physical contact between WCS365 and Forl is not required to inhibit microconidia formation.

**Implications for the mechanisms of biocontrol of *P. fluorescens* WCS365**

The conditions described here for the growth of *P. fluorescens* WCS365 together with Forl spores mimic those during the first weeks of biocontrol of TFRR in new stonewool. (i) New stonewool is practically sterile (Lugtenberg and Kamilova, 2008). (ii) Three weeks after seeding the tomato seeds the biocontrol bacterium *P. fluorescens* WCS365 is the dominant bacterium on the developing plant root where it represents even 90% of the cultivable microflora (S. Validov, F. Kamilova, B. Lugtenberg, 2007; unpubl. results). (iii) The medium is PNS and the only carbon source is tomato root exudate. Therefore, the observed effects of *P. fluorescens* WCS365 on germination and sporulation of Forl are relevant for biocontrol of TFRR in new stonewool. Without the presence of *P. fluorescens* WCS365, Forl spores would germinate near the root where the exudate concentration is highest. The excellent root colonizer *P. fluorescens* WCS365 will successfully compete for exudate components with Forl (Kamilova et al., 2006a) and delay germination of Forl spores (Table 1), thereby giving the biocontrol bacterium a better chance to proliferate on exudate. This would therefore enhance the biocontrol of TFRR, at least in the first stage, when plants are most vulnerable for infection by Forl. Furthermore, in areas where nutrients are available such as on the root and the stem, Forl hyphae will develop microspores (Table 2) which subsequently serve as secondary inocula to spread the fungus to neighbouring host plants (Rowe et al., 1977; Katan et al., 1997; Rekah et al., 2000). The presence of *P. fluorescens* WCS365 cells will inhibit this process (Table 2), resulting in less survival of Forl and therefore less spreading of the fungus during biocontrol.

There is no evidence to believe that the observed traits of *P. fluorescens* WCS365 are specific for this strain or for biocontrol strains in general. However, when *P. fluorescens* WCS365 is used as a biocontrol strain in new stonewool, the described effect will undoubtedly play a role in biocontrol. In later stages of plant growth, when other microbes have become dominant (Postma et al., 2000), the influence of *P. fluorescens* WCS365 on germination and sporulation of Forl will be limited. However, it is pos-
sible that other microbes have a similar effect. Anyhow, even under those conditions \( \text{P. fluorescens} \) WCS365 will still control TFRR based on the established mechanism of induction of systemic resistance (Kamilova et al., 2005).

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