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ORIGINAL ARTICLE

Paenibacillus polymyxa antagonizes oomycete plant pathogens *Phytophthora palmivora* and *Pythium aphanidermatum*

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Abstract

Aim: To find sustainable alternatives to the application of synthetic chemicals for oomycete pathogen suppression.
Methods and Results: Here, we present experiments on an *Arabidopsis thaliana* model system in which we studied the antagonistic properties of rhizobacterium *Paenibacillus polymyxa* strains towards the oomycete plant pathogens *Phytophthora palmivora* and *Pythium aphanidermatum*. We carried out studies on agar plates, in liquid media and in soil. Our results indicate that *P. polymyxa* strains significantly reduced *P. aphanidermatum* and *P. palmivora* colonization in liquid assays. Most plants that had been treated with *P. polymyxa* survived the *P. aphanidermatum* inoculations in soil assays.
Conclusions: The antagonistic abilities of both systems correlated well with mycoidal substance production and not with the production of antagonistic substances from the biocontrol bacteria.
Significance and Impact of the Study: Our experiments highlight the need to take biofilm formation and niche exclusion mechanisms into consideration for biocontrol assays performed under natural conditions.

Introduction

Oomycetic pathogens cause one of most devastating groups of diseases. They are present in practically all cultivated soils and attack root systems, particularly under warm and humid conditions. Almost all plants are susceptible to root rot, and the disease is difficult to control once the rot has begun. *Pythium* attacks juvenile tissues such as the root tip. The fungus enters the root and causes a rapid, black rot of the entire primary root. The plantlets wilt or collapse and die. *Pythium* species form several types of spores e.g. zoospores, oospores and chlamydospores. They can all serve as resting structures and can undergo a long period of dormancy.

Several strategies have been used to fight these pathogens using fungicides. As most of them pose an environmental risk, there is considerable interest in replacing these chemicals with environmentally friendly biocontrol agents. Despite decades of research on the biological control of oomycetic soil borne plant diseases (Van Luijik 1938), there still are no commercially successful examples against diseases caused by *Pythium* and *Phytophthora*. There are a number of reasons for the lack of grower adoption; among the most important is insufficient knowledge on the mechanism of action of pathogens, as well as biocontrol agents.

We earlier observed a significant yet inconsistent reduction of *Pythium* root rot under natural conditions when the plants were preinoculated by *Paenibacillus polymyxa* biocontrol strains. *Paenibacillus polymyxa*, a common soil bacterium, belongs to the group of plant growth promoting rhizobacteria. Activities that have been found to be associated with *P. polymyxa*-treatment of plants in field experiments include nitrogen fixation, soil phosphorus solubilization, the production of antibiotics, auxin, cytokinin, chitinase and hydrolytic enzymes as well as the

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promotion of increased soil porosity (Timmusk and Wagner 1999; Timmusk *et al.* 1999). All these activities might be of importance for plant growth promotion. However, the mechanism by which *P. polymyxa* exerts its beneficial effect should be understood to ensure its reproducible performance in agro-ecological systems. A biocontrol bacterium can affect plant growth by various mechanisms (Glick *et al.* 1999; Timmusk *et al.* 1999, 2005; Weller *et al.* 2002; Timmusk 2003; Perneel *et al.* 2007; Rezzonico *et al.* 2007; Tran *et al.* 2007). Plant root exudates and root electrical signals selectively influence bacterial colonization and biofilm formation (van West *et al.* 2002; Bergsma-Vlami *et al.* 2005; Kiely *et al.* 2006; van Loon 2007). The colonization rarely occurs as individual cells. Complex multicellular communities such as biofilms and fruiting bodies are commonly coexisting forms in nature (Davey and O'Toole 2000; Palkova 2004; Ngo Thi and Naumann 2007).

Oomycetic pathogens, which form the group of the most devastating pathogens in agriculture as well as several other root pathogens, infect pronominally juvenile root tissues such as root tips. As PGPRs are known to colonize various regions on plant roots, effort has been made to modify biocontrol agents to colonize plant root tips. In certain PGPRs, efficient root colonization is linked to their ability to secrete a site-specific recombinase. Transfer of the site-specific recombinase gene from a rhizosphere-competent *Pseudomonas fluorescens* into a rhizosphere-incompetent *Pseudomonas* strain enhanced its ability to colonize root tips (Dekkers *et al.* 2000). We showed that genetically unmodified *P. polymyxa* stains colonize plant root tips as a biofilm, and we have characterized the pattern of biofilm formation (Timmusk and Wagner 1999; Timmusk 2003; Timmusk *et al.* 2005). We also confirmed that the *Arabidopsis* model faithfully reflects *P. polymyxa* colonization in soil (Timmusk *et al.* 2005). Biofilms consist of cells and an extracellular matrix, which provides an almost infinite range of macromolecules produced by the bacteria. The persistent structures of biofilms could act as a sink for nutrients in the rhizosphere, therefore reducing the availability of root exudate nutritional elements for pathogen stimulation or subsequent colonization on the root. The mechanism initially reported by Thomashow's group (Weller and Thomashow 1994) has gained less attention, most likely due to difficulties in studying natural systems. However, biofilms could have the potential to be successful in fighting the similar root colonizing pathogens under natural conditions.

In this report, we focused on *P. polymyxa B2*, *B5* and *B6* antagonistic mechanisms against the well-characterized model oomycetic pathogens *Phytophthora palmivora* and *Pythium aphanidermatum*.

The first stage of the pathogen infection is colonization: the establishment of the pathogen at the appropriate site to encyst and infect plant root. *Arabidopsis thaliana* is not the host for *P. palmivora* as it does not carry out zoospore encystment. However, *P. palmivora* zoospores colonize around *A. thaliana* root tips. Hence, we chose the earlier-characterized *A. thaliana* model system (Timmusk *et al.* 2005) to study oomycete colonization patterns and niche exclusion as the mechanism of antagonism.

Materials and Methods

Antagonism studies *in vitro*

Inhibitory studies between the bacterium and fungi were conducted on V8 plates. The bacterial strains were streaked on to the plate after inoculation with fungal plugs. *Phytophthora palmivora* cultures were grown on 20% (v/v) V8 medium containing 0·3% $CaCO_3$, 1·5% nutrient agar (Oxoid, Basingstoke, UK) and 0·003% (w/v) cholesterol in Petri dishes at 28°C for 48 h in the dark, and subsequently for an additional 48 h under illumination (8 W, Gro-Lux fluorescent tubes). *Pythium aphanidermatum* cultures were grown on 10% V8 media containing 2·5% (w/v) nutrient agar and incubated at 25°C in the dark. The experiments were repeated on 20% (v/v) V8 medium, rye agar medium, oat meal agar and potato dextrose agar. Three Petri dishes were used for each treatment.

Preparation of zoospores

Phytophthora palmivora strain P6390 (van West *et al.* 1999) was originally provided by M.D. Coffey. Cultures were grown on 20% (v/v) V8 medium containing 0·3% $CaCO_3$, 1·5% nutrient agar (Oxoid) and 0·003% (w/v) cholesterol in Petri dishes at 28°C for 48 h in the dark, and subsequently for an additional 48 h under illumination (8 W Gro-Lux fluorescent tubes). The zoospores were harvested as follows: 10 ml of 2 mmol l^{-1} sodium phosphate buffer (pH 7·2) was added to each Petri dish. The surface of the colony was gently scrapped with a glass rod allowing the sporangia to be dislodged. The suspension of sporangia was poured into a 50-ml beaker, which was placed at –20°C for 2 min. The suspension typically contained $\approx 10^6$ zoospores per ml. *Pythium aphanidermatum* (Appiah *et al.* 2005) was originally provided by J.W. Deacon (University of Edinburgh). Cultures were grown on 10% V8 media containing 2·5% (w/v) nutrient agar and incubated at 25°C in the dark. Strips of the *Pythium* mat were cut from mature colonies and immersed for 4–6 h in 2 mmol l^{-1} sodium phosphate buffer (pH 7·2)

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at 22°C. Using this method, ≈10^5 zoospores ml^{-1} were obtained.

Plant material and growth conditions

Seeds of *A. thaliana* ecotype C24 were surface-sterilized by incubation in saturated and filtered aqueous calcium chlorate solution for 30 min, followed by repeated washing in sterile distilled water. Seeds were sown on solid MS-2 medium (Murashige and Skoog 1962). Plants were replanted after germination and subsequently grown on solid MS-2 medium for 2 weeks in a growth chamber at 22°C with a 16-h light regime (150–200 $\mu E\ s^{-1}\ m^2$).

Plant inoculation by *Paenibacillus polymyxa*

The *P. polymyxa* strain B2 was isolated from wheat rhizospheres (Lindberg and Granhall 1984). *P. polymyxa* B5 and B6 were isolated from peanut rhizospheres (Haggag and Timmusk 2007). The bacteria were grown overnight in L broth (Miller 1972) at 30°C. After 2 weeks of growth on MS-2 medium, plants were inoculated by soaking their roots in 30 ml diluted overnight cultures of *P. polymyxa* (≈ 10^6 bacteria ml^{-1}) for 2 h. Control plants were soaked in 10× diluted L broth.

Infection of seedlings with fungal zoospores

Plant seedlings were soaked for 2 h in *P. polymyxa* B2, B5 and B6 overnight culture (≈ 10^6 bacteria ml^{-1}) or LB medium. The liquid around the plant roots was removed by pipetting and replaced by 1ml of zoospore suspension. The infection process in the suspension was followed directly under a reversed microscope (Olympus IX-70) using seven independent *P. polymyxa* and zoospore treatment. Images were acquired using Nikon D2X camera**.** For quantification, the zoospore infected roots were removed and placed to an Eppendorf tube containing 1ml sterile PBS. After vorterxing and Petroff–Hausser chamber counts, the zoospore density was calculated from seven independent samples. For the soil experiments, 2-week-old *A. thaliana* plantlets grown in MS medium were preinoculated with *P. polymyxa* and zoospores as described above and planted in greenhouse soil (Weibulls' K-soil, Svalov Weibull AB, Sweden). Control plants were soaked in 10× diluted L broth. Plant survival was recorded 7 days after planting (Table 1). To analyse the fungal infection sites, entire roots and relevant segments thereof were subjected to staining by fuchsin, a dye that is known to stain cytoplasm (Fig. 1). Each treatment using twelve plants was performed in three replicates. The experiment was carried out three times.

Table 1 Inhibitory effect of *Paenibacillus polymyxa* B2, B5 and B6 on *Pythium aphanidermatum* and *Phytophthora palmivora* in liquid and soil assays

Treatment	Zoospores around the root in liquid assays (per ml)	Surviving plants in the soil assay (of 12 total)
P. aphanidermatum	$10^4 \pm 0{\cdot}7 \times 10^3$a*	3 ± 1·15a
P. polymyxa B2	ND	11 ± 0·58b
P. polymyxa B5	ND	12b
P. polymyxa B6	ND	12b
P. polymyxa B2 + *P. aphanidermatum*	20 ± 1·9b	10 ± 0·58b
P. polymyxa B5 + *P. aphanidermatum*	20 ± 1·9b	11 ± 0·58b
P. polymyxa B6 + *P. aphanidermatum*	$10^3 \pm 0{\cdot}7 \times 10^2$c	5 ± 0·58c
P. palmivora	$10^4 \pm 0{\cdot}7 \times 10^3$a	
P. polymyxa B2 + *P. palmivora*	30 ± 1·9b	ND
P. polymyxa B5 + *P. palmivora*	30 ± 1·5b	ND
P. polymyxa B6 + *P. palmivora*	$2 \times 10^3 \pm 0{\cdot}1 \times 10^3$c	ND

ND, not determined.

*Values (mean value ± SE) followed by the same letter are not significantly different ($P < 0{\cdot}05$) according to Fisher's least significant difference test.

Microtitre plate assay for biofilm formation

Bacteria grown overnight on NA plates were resuspended in NB medium and diluted to a final optical density at 600 nm (OD_{600}) of 0·002. Cultures were transferred to standing culture vessels. Polystyrene 96-well microtitre plates were filled with 150 μl of culture per well. The cultures were allowed to stand at 30°C for the specified times. The extent of biofilm formation was assayed by staining with crystal violet. After the incubation period, cultures were removed, and microtitre plate wells were gently washed three times with 150 μl of sterile water to remove loosely associated bacteria, then dried at 30°C for 30 min. Samples were stained by the addition of 1% crystal violet solution to each well above the initial inoculation level and incubated for 20 min. The vessels were then washed. The intensity of crystal violet staining was measured after the addition of dimethyl sulfoxide to each dry well. The samples were incubated for 20 min, after which the OD_{590} values were measured on a plate reader. All samples were tested in seven independent wells.

Statistical analysis

Zoospore colonization data in liquid assays and plant survival data in soil assays were submitted to ANOVA and to

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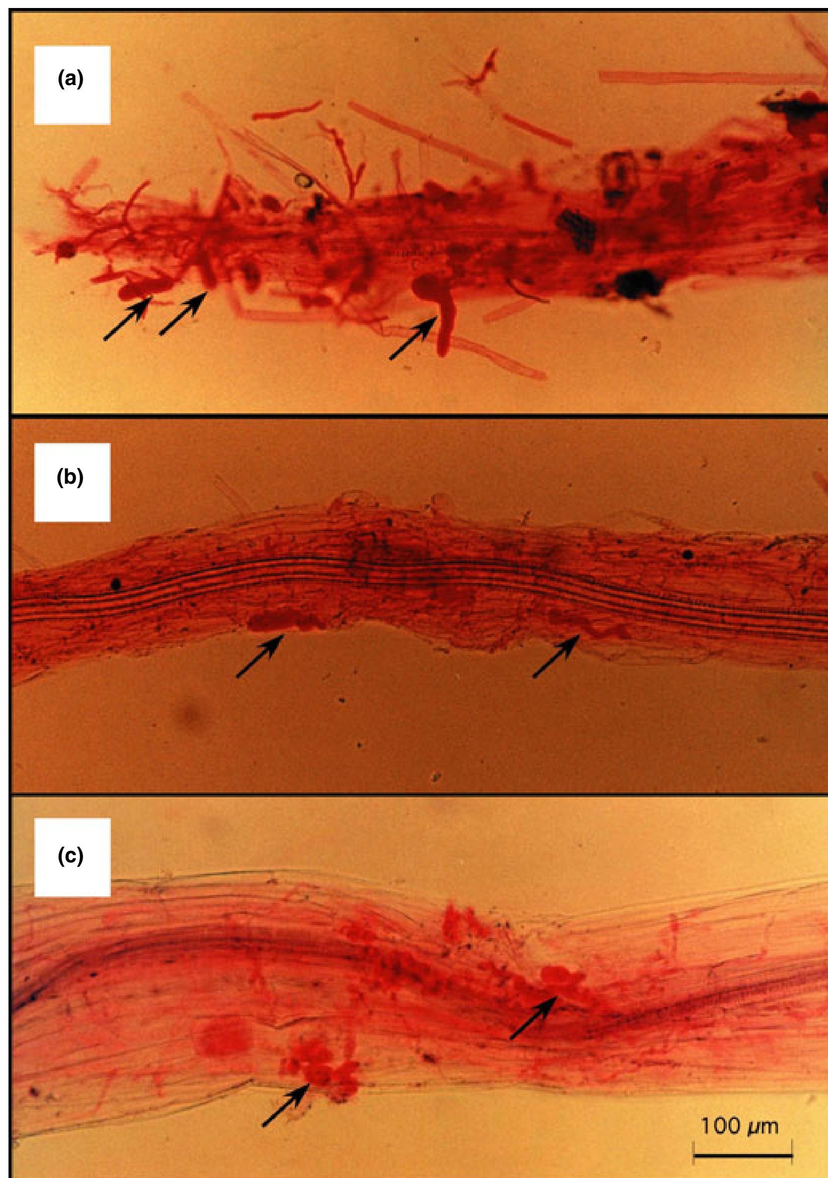

Figure 1 Analysis of oomycete infection sites on *Arabidopsis thaliana* roots. *Arabidopsis* seedlings were grown, treated and prepared for microscopy as described in Materials and Methods. (a) Most of *Pythium aphanidermatum* hyphae were detected in and around plant root tip. (a)Roots from pathogen-infected plants contained remarkably more *P. aphanidermatum* hyphae than (b) roots from pathogen-infected plants, pretreated with *Paenibacillus polymyxa* strains. (c) Relatively small number *P. aphanidermatum* hyphae were detected in an area close to the root neck. Arrows indicate *P. aphanidermatum* hyphae.

Fisher's least significance test ($P < 0{\cdot}05$) using the STATVIEW (San Francisco, CA) statistics package.

Results

Antagonism *in vitro*

Paenibacillus polymyxa is known to produce several antagonistic substances. Here, we studied the antagonistic properties of *P. polymyxa* B2, B5 and B6 against *P. palmivora* or *P. aphanidermatum* on agar plates. Pieces of agar containing *P. palmivora* or *P. aphanidermatum* were placed in the centre of the plate, and the bacteria were streaked as shown in Fig. 2. Upon incubation, the radial growth of *P. aphanidermatum* was not affected by any of *P. polymyxa* strains used in the experiment and resulted in a full spreading of the oomycete and overgrowth on the entire plate (Fig. 2a). The radial growth of *P. palmivora* was severely inhibited by similar rate of all the stains. A typical example is shown in Fig. 2b.

Pythium aphanidermatum and *P. palmivora* colonization and *P. polymyxa* antagonism in liquid assays

The studies show that *P. aphanidermatum* and *P. palmivora* zoospores predominantly colonize the root tip and the adjacent region of the roots of 2-week-old *Arabidopsis* plants (Fig. 3a, b). To investigate this, we

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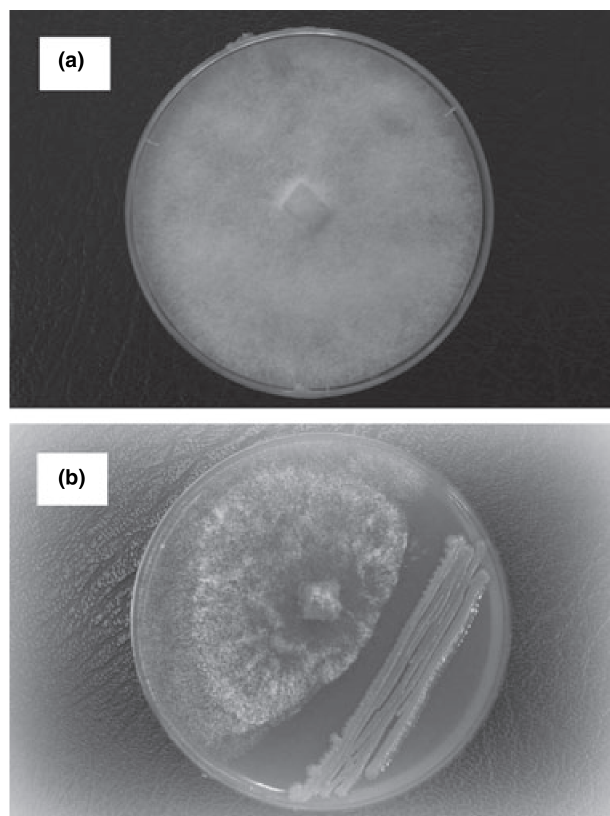

Figure 2 Inhibitory effect of *P. polymyxa* B2, B5 and B6 on *Pythium aphanidermatum* and *Phytophthora palmivora* (plate assay). (a) *Paenibacillus polymyxa* strains did not antagonize *P. aphanidermatum* (typical example shown). (b) *P. polymyxa* strains antagonize *P. palmivora*.

treated *A. thaliana* roots with *P. polymyxa* B2, B5 and B6 prior to infection by oomycete zoospores. In control experiments, the bacteria were omitted. Figure 3 shows micrographs of plant root tips. When roots were not pretreated with the bacterial isolates, *P. aphanidermatum* zoospores were abundantly present around the tip of the main root and the adjacent root segment (Fig. 3a). $10^4 \pm 0{\cdot}7 \times 10^3$ zoospores ml^{-1} sterile PBS were counted surrounding the root, then no bacterial pretreatment was applied. When plant roots had been preinoculated by *P. polymyxa* B2, only 0·2% of the zoospores was attached to this region of the root (Table 1). A similar pattern was observed in case of *P. polymyxa* B5 pretreatment. A typical example for the B2 and B5 strains is given in Fig. 3e. Note that the *P. polymyxa* biofilms which formed on plant roots (Timmusk *et al.* 2005) are not visible using the magnification for reversed microscope direct visualization in liquids. When plant roots were pretreated with *P. polymyxa* B6, 10% of the zoospores compared to the plain *P. aphanidermatum* treatment attached to the root. This is significantly more than the pretreatment with the other bacterial strains *P. polymyxa* B2 and B5 (Fig. 3c, Table 1).

Figure 3 shows a similar analysis of antagonism between *P. polymyxa* and *P. palmivora*. Figure 3b illustrates the tip of a lateral root with zoospores of *P. palmivora* associated ($10^4 \pm 0{\cdot}7 \times 10^3$ oomycetic zoospores ml^{-1} PBS, Table 1). In Fig. 3f, where the roots were pretreated with *P. polymyxa* B2 and B5, zoospores were again occluded. In comparison to plain pathogen treatment (see above), only 0·3% of zoospores were counted around to the root regions where *P. polymyxa* is known to colonize. In agreement with the *P. aphanidermatum* experiment (Fig. 3c), the strain B6 again showed less ability in antagonizing oomycetic zoospores (Table 1). Interestingly, although the plate assay only showed an antagonistic effect against *P. palmivora*, the root attachment experiments in liquid suggest antagonism against both oomycetes.

Antagonism in soil assays

The previous experiments do not necessarily reflect a biologically relevant setting. Therefore, we tested the effect of *P. polymyxa* B2, B5 and B6 treatment in soil assays (Table 1). As *A. thaliana* is not a host for *P. palmivora*, soil experiments were only performed with *P. aphanidermatum*. Survival rates of *Arabidopsis* plants infected by *P. aphanidermatum* were determined after 7 days of growth. One half of the plants had previously been inoculated by *P. polymyxa*, the other half served as a control. Survival rates are given in Table 1. These rates show that *P. polymyxa* B2 and B5 inoculation caused significant protection; 83% of *Arabidopsis* plants survived when they were pretreated with the bacterial strains. Compared to *P. polymyxa* B2 and B5 pretreatment, it was observed that *P. polymyxa* B6 preinoculation caused a significantly lower (42%) protection against the oomycetic pathogen. Inoculation with *P. polymyxa*, without subsequent *Pythium* infection, did not result in a significant loss of viability (Table 1).

Analysis of fungal infection sites on plant roots

To assess the results of the soil assay in more detail, a microscope investigation of plant roots was performed (Fig. 1). Here, the treated plants were analysed after 3 days of growth in soil. At this point of time, stress symptoms are already visible on plants infected with *P. aphanidermatum*, but they are still viable. Entire roots, and relevant segments thereof, were subjected to staining by fuchsin to stain the cytoplasm. In both *P. polymyxa* B2, B5, B6 pretreated and control plants, hyphae and sporangia of *P. aphanidermatum* were observed. A typical example is shown in Fig. 1a–c. Figure 1a shows a root tip

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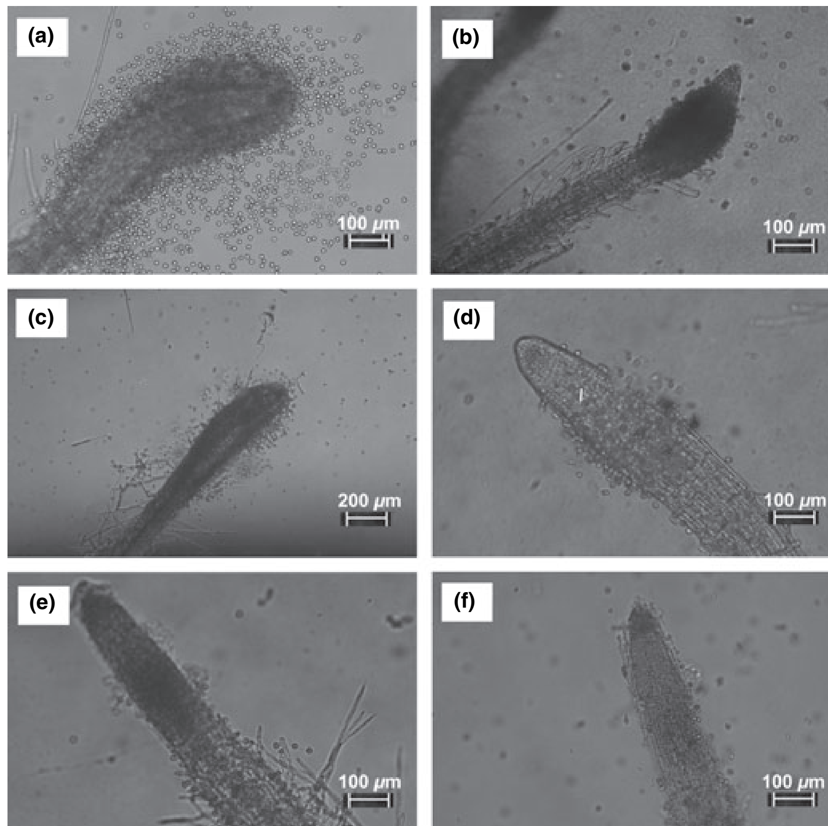

Figure 3 Inhibitory effect of *Paenibacillus polymyxa* B2, B5 and B6 on *Pythium aphanidermatum* and *Phytophthora palmivora* root colonization (liquid assays). Axenic seedlings of *Arabidopsis thaliana* C24 were grown and inoculated with the *P. polymyxa* and pathogens as described in Materials and Methods. The pattern of (a) *P. aphanidermatum* and (b) *P. palmivora* zoospore colonization on *A. thaliana* root is affected by *P. polymyxa* preinoculation (c–f). *P. polymyxa* B5 pretreatment caused somewhat reduced *P. aphanidermatum* (c) *and P. palmivora* (d) zoospore colonization. *P. polymyxa* B2 and B6 pretreated sample showed (e) significantly less *P. aphanidermatum* (typical example shown) and (f) *P. palmivora* zoospore colonization.

and adjacent region of a plant only infected by the pathogen. As expected, many hyphae were observed in this region (arrows). In *P. polymyxa*-pretreated plants, the hyphal material was located around the root tip and the adjacent zone was significantly smaller (Fig. 1b). Upon inspection of other root regions, it was noted that the upper part of the root close to the root neck was also slightly infected when the plants were not pretreated with *P. polymyxa* (Fig. 1c). When pretreated, this region was free from fungus (data not shown).

Microtitre plate assay for biofilm formation

An assay for biofilm formation on solid surfaces was performed on microtitre plates. Figure 4 shows the time course of biofilm formation as assayed by crystal violet staining and gentle washing of standing cultures grown at 30°C.

During the first 8 h, all *P. polymyxa* strains initiated biofilm formation in a similar manner. After approximately 4 h, the B6 strain showed a significant decrease in staining, suggesting a remarkably reduced ability to form biofilms than strains B2 and B5. In contrast, biofilm from strains B2 and B5 enlarged throughout the 25 h by similar rate.

Discussion

Biocontrol agents do not generally perform well enough under uncontrolled conditions in soil to compete with chemical fungicides. To be able to apply the biocontrol strains effectively and reproducibly, we have more to learn about their mechanism of action.

In this study, we showed that *P. polymyxa* inoculation is effective in protecting the *A. thaliana* model system against zoospore colonization (Figs 2 and 3). This antagonistic effect was further confirmed in soil experiments (Table1, Fig. 1). We also demonstrated the pattern of *P. aphanidermatum* and *P. palmivora* zoospore colonization on *A. thaliana* model system, which we had developed (Timmusk *et al.* 2005). Generally, it is known that *Arabidopsis* is not a host for *P. palmivora*. In our experiments, the *P. palmivora* zoospores were shown to colonize plant roots in liquid assays (Fig. 3b, d and f). The role of the zoospore is to find an appropriate site to colonize, and if successful, colonization cysts will form on the surface of the host (van West *et al.* 2002). Assuming that zoospores remain motile for 10 h, and taking 160 $\mu m\ s^{-1}$ as their maximum swimming velocity, they might traverse a distance of at most 5·8 m (Carlie 1986). This rate of movement depends on soil water content, porosity and the presence of encystment agents.

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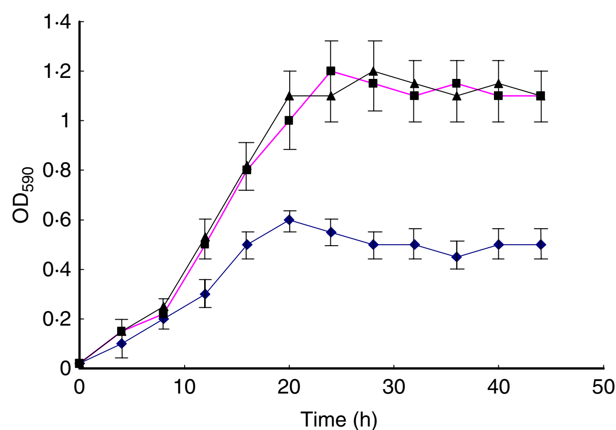

Figure 4 Solid surface assay of *Paenibacillus polymyxa* B2 (–▲–), B5 (–■–) and B6 (–◆–) biofilm formation. The crystal violet assay was used to measure the solid surface biofilm formation at 30°C. Preparation and analysis were as described in Materials and Methods.

Moreover, frequent collisions with solid objects might decrease this distance. The duration of zoospore activity is dependent on the energy supply and environmental conditions but in, for example the *Phytophthora* species, this does not exceed 24–48 h, even under ideal conditions (Erwin and Ribeiro 1996). It would not be surprising therefore if zoospores attempt to settle on, and infect, any plant they encounter in the limited time available, rather than to colonize selectively only particular host species (Gow *et al.* 1999).

The oomycete zoospore accumulation around *Arabidopsis* model system roots mainly follows the pattern of the other plants, e.g. *Lolium perenne* and *Triticum sativum* (van West *et al.* 2002). Hence, even though the widely used model plant *A. thaliana* is not generally known as its host, it can be successfully employed to study *P. palmivora* zoospore colonization and biocontrol agents that prevent colonization prior to encystment and infection.

The oomycete pathogen colonization pattern appears to be similar to the *P. polymyxa* colonization pattern we described earlier (Timmusk *et al.* 2005). Consequently, it was of interest to study whether *A. thaliana* root pretreatment with *P polymyxa* could interfere with the colonization process of oomycete zoospores. *P. polymyxa* is known to be a biocontrol bacterium due to its widely reported production of antibiotic compounds (Timmusk 2003; He *et al.* 2007) Here, we show that *P. polymyxa* B2, B5 and B6 strains are able to antagonize the *P. aphanidermatum* and *P. palmivora* zoospores around the *Arabidopsis* root. However, the strains showed no antagonism on different *P. aphanidermatum* plates against *P. aphanidermatum* (Fig. 2a). In contrast, a clear antagonism which differed from strain to strain yet coincided with the antagonism in liquid assays was observed in soil assays (Fig. 3c, e; Table1). Stains B5 and B6 have been shown to produce similar amounts of antibiotic substances (Haggag and Timmusk 2007). In the case of *P. palmivora*, the zone of inhibition on agar plates was similar for all strains on the various media plates used (a typical example is given in Fig. 3b). However, the ability of *P. polymyxa* B2, B5 and B6 to antagonize *P. palmivora* varied under biological settings i.e. on the microscope liquid assays (Fig. 3d, f). We have not ruled out the possibility that antibiotic production differs with root vicinity. Indeed a real-time monitoring over the growth period of various parameters including antibiotic and possible biosurfactante production would be needed to estimate the role of different factors. However, the results here clearly indicate the correlation with the *P. polymyxa* strain mycoidal substrate production and ability to form biofilms (Fig. 3, Table 1). Strains B2 and B5 were shown to produce the highest amounts of mycoidal substances (Fig. 4). Both *P. aphanidermatum* and *P. palmivora* zoospore colonization in liquid assays was reduced (Fig. 3e, f; Table 1), and *P. aphanidermatum* infected plant survival rates in soil assays were higher when the seedlings were pretreated with B2 and B5 strains (Table 1). A total of 83% of the plants survived following that treatment, while B6 treatment resulted in only 42% survivors (Table1). Hence, the effectiveness of the *P. polymyxa* B2, B5 and B6 isolates in the liquid assays correlated well with the soil experiment data and with the ability of the bacterial isolate to produce mycoidal substances (Fig. 3, Table 1, Fig. 4). Earlier, we reported on the ability of the *P. polymyxa* strains B5 or B6 to antagonize the crown root rot caused by *A. niger* under field conditions (Haggag and Timmusk 2007). The incidence of the pathogen infection was reduced in peanut plant rhizospheres inoculated with both *P. polymyxa* strains. The strains B5 and B6 produced similar amounts of antagonistic substances, but B5 was superior in mycoidal substance and biofilm production. The strain also showed significantly greater antagonistic abilities against the pathogen, and niche exclusion was suggested as being involved in the observed biocontrol effect (Haggag and Timmusk 2007).

Our results highlight the need to consider the importance of bacterial biofilm establishment on plant roots as a biocontrol mechanism. It is most likely that plants employ different abilities for pathogen control at various times. It is possible that besides the other mechanisms, *P. polymyxa* can excrete its biofilm formation in the oomycete pathogen infection region, i.e. a niche exclusion is of critical importance at host plant juvenile growth stages. The mechanism has garnered less attention compared to other *P. polymyxa* biocontrol mechanisms reported. Microbial biofilms are comprised of both cells

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and extracellular matrix. The extracellular matrix may form up to 98% of the biofilm (Sutherland 2001; Kolter 2005). Here, we have used assays on solid surfaces (Fig. 4) to show the difference in extracellular matrix production of *P. polymyxa* B2, B5 and B6 strains. Plant growth promoting bacteria often utilize quorum sensing to modulate and coordinate their interactions with plants, including tissue maceration, antibiotic production, toxin release and horizontal gene transfer. A high population density provides us with a chance to perform certain processes that single cells cannot carry out efficiently (Danhorn and Fuqua 2007). Hence, biocontrol efficiency could correlate with the ability of the bacterial strain to produce quorum sensing signalling molecules. In Gram-positive bacteria, oligopeptides are known to function as signalling molecules (Danhorn and Fuqua 2007).

Fourier transform infrared microspectroscopy assays from wild-type as well as biofilm formation mutants are under investigation in our laboratory for the detection and characterization of antibiotics as well as for the development of major biofilm forming metabolites produced by the wild-type bacterial strains (Ngo Thi and Naumann 2007). Furthermore, atomic force microscopy assays are being performed, as biofilm persistence and its physical properties are important for agricultural applications.

Once its pathways to biofilm development are more fully understood, the management of *P. polymyxa* biofilm formation in resident populations of the cropping system could become possible (Battin *et al.* 2007). This will be a step towards ensuring their reproducible performance in natural environments.

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