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Pseudomonas fluorescens DR54 Reduces Sclerotia Formation, Biomass Development, and Disease Incidence of *Rhizoctonia solani* Causing Damping-Off in Sugar Beet

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Abstract

Effects of the biocontrol strain, *Pseudomonas fluorescens* DR54, on growth and disease development by *Rhizoctonia solani* causing damping-off in sugar beet were studied in soil microcosms and in pot experiments with natural, clay-type soil. In pot experiments with *P. fluorescens* DR54-treated seeds, significantly fewer *Rhizoctonia*-challenged seedlings showed damping-off symptoms than when not inoculated with the biocontrol agent. In the rhizosphere of *P. fluorescens* DR54 inoculated seeds, the bacterial inoculant was present in high numbers as shown by dilution plating and immunoblotting. By the ELISA antibody technique and direct microscopy of the fungal pathogen grown in soil microcosms, it was shown that the presence of *P. fluorescens* DR54 on the inoculated seeds had a strong inhibitory effect on development of both mycelium biomass and sclerotia formation by *R. solani.* In the field experiment, plant emergence was increased by treatment with *P. fluorescens* DR54 and the inoculant was found to be the dominating rhizosphere colonizing pseudomonad immediately after seedling emergence.

Introduction

In Danish sugar beet production, the important root pathogenic microfungi are the oomycetes *Aphanomyces cochleoides* and *Pythium ultimum* and the basidiomycete *Rhizoctonia solani* (H. C. Pedersen, Danisco Seed, personal communication). Numerous antagonistic bacteria producing different antifungal subtances have shown a potential for biological control of the pathogen *P. ultimum* in sugar beet production [e.g., 5]. In our laboratory, *P. fluorescens* DR54 was isolated as an antifungal agent toward plant pathogens causing damping-off in sugar beet and was previously shown to reduce *Pythium ultimum* infections in pot experiments [1]. The antifungal activity of *P. fluorescens* DR54 toward both *P. ultimum* and *R. solani* was primarily determined by production of the cyclic lipopeptide viscosinamide, as demonstrated by *in vitro* experiments on laboratory media [2, 3]. Viscosinamide was also produced in the spermosphere and rhizosphere of sugar beet when the seedlings were treated with

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P. fluorescens DR54 and grown in soil microcosms [4]. In these experiments, *P. fluorescens* DR54 was further shown to colonize the roots of the sugar beet seedlings and to reduce mycelial density, radial growth, and oospore formation of the *P. ultimum* pathogen in adhering rhizosphere soil [4].

The first aim of this work was to study whether there was a correlation between the biocontrol effects evaluated by plant health and the putative inhibitory effect of *P. fluorescens* DR54 measured directly on the target fungus, *R. solani,* in soil microcosms. The specific, inhibitory effects of *P. fluorescens* DR54 on *R. solani* biomass development and growth pattern were studied using an immunological method (ELISA) and direct microscopy. Finally, it was studied whether *P. fluorescens* DR54 had a significant biocontrol on *R. solani* in pot experiments and on damping-off-causing fungi in the field.

Materials and Methods

Soil, Plants, and Microorganisms

The field experiment was performed at Danisco Seed, Holeby, Lolland, representing an area with intensive sugar beet cropping in Denmark. Both the field soil and test soil collected for pot and microcosm experiments were a sandy loam with pH 7.5 and a high clay content (16 to 19%). For pot and microcosm experiments, the collected soil was first sieved (4-mm mesh) and wetted to 56% of WHC (13% water content, w/v) using a perfusion-spray technique. The soil was kept at 4°C for 24 h before use. Sugar beet (cv. "Madison," Danisco Seed A/S, Holeby, Denmark) was used as test plant. For pot experiments, noncoated seeds were used, whereas commercially coated seeds without fungicide (Thiram) were used in the field experiments.

Pseudomonas fluorescens DR54 was isolated from sugar beet rhizosphere [1]. The strain was stored at −80°C in glycerol (1:1 w/v) and maintained for short periods on L-agar (1% Bacto Tryptone, Difco Laboratories, Detroit), 0.5% yeast extract (Difco), 1% NaCl, 0.01% glucose, 2% agar (Difco); pH 7.2). Bacteria for use as inocula were cultivated in L-broth for 16–24 h. A Tn5::gfp mutant of *P. fluorescens* DR54, BN-14 [6], was used for some experiments involving root colonization studied by fluorescence microscopy. The mutant was maintained on LB agar containing 10 ppm kanamycin. The pathogenic microfungus, *Rhizoctonia solani* AG4 (Danisco 92009), was isolated from sugar beet rhizosphere and was maintained on PDA.

Field Experiment

At the Danisco Seed field station at Holeby, Denmark, a field experiment with sugar beet was carried out in May 2000. This period was relatively dry and soil water content was approx. 40% of water holding capacity (WHC) at the time of sowing. Three rows of seeds without the standard fungicide Thiram, but including the *P. fluorescens* DR54 inoculant, and three rows without inoculant (controls) were sown in the field. One hundred seeds were sown in each row. The seeds were inoculated in the field immediately before sowing using *P. fluorescens* DR54 culture grown to stationary phase (16–18 h) in L-broth. A suspension of 50 µl (10^8 cfu/ml) culture was pipetted onto each seed, which was then immediately sown and covered with 5 cm soil.

Six plants from each of two rows (day 18) and of three rows (day 27) were harvested and rhizosphere populations of *Pseudomonas* spp. were enumerated as described for pot experiments (see below). At day 18 plants in only two of the rows had emerged. Field emergence of sugar beet seedlings was recorded 24 days after sowing.

Pot Experiments

Seeds were inoculated by gentle agitation for 30 min in 30 ml inoculum (4×10^9 CFU/ml) of *P. fluorescens* DR54. Control seeds were agitated in 0.9% NaCl solution rather than spent medium, because the latter would contain viscosinamide and possibly other antifungal compounds [2]. To determine the inoculum density on the seeds (approx. 10^8 CFU per seed) before sowing, cells were extracted from the seeds for dilution plating on Gould's S1 agar [7] and 1/10 strength of L-agar. Three seeds were pooled and extracted in 1 ml 0.9% NaCl by vortexing (30 s) and sonication in a water bath (30 s). Dilutions were made from three replicates of each three seeds.

Pathogen-infested seeds were prepared by placing 20 nongerminated sugar beet seeds 1 cm from the edge of a petri dish containing water agar. *Rhizoctonia* inoculum from a 3- to 5-day-old PDA culture was placed in the center and the plates were incubated for 1 week at 15°C. One infested seed, subsequently used as fungal inoculum, was placed in the center of each soil-filled pot (9 cm diam., 9 cm deep) containing approx. 400 g soil. Preliminary experiments had shown consistently that a lower fungal inoculum representing only one-half of a seed resulted in less disease (50–80% healthy seedlings) than the standard fungal inoculum representing a whole seed (30–60% healthy seedlings).

Two individual pot experiments including 4 or 6 replicate pots were carried out. Six noninfested seeds (with or without *P. fluorescens* DR54 inoculant) were sown at 1 cm depth in each pot, forming a circle of seeds 3.5 cm away from the pathogen-infested seed in the center. Pots were placed in a growth chamber at 15°C for 7 days with a 16-hour light period covered in transparent plastic bags to preserve the soil moisture. At the end of incubation, seedling emergence was recorded and the plants were harvested to evaluate the disease symptoms on the roots (discoloration or rotting).

Total populations of *Pseudomonas* spp. colonizing the sugar beet rhizosphere were enumerated using Gould's S1 selective medium [7] according to Thrane et al. [4]. In the present study, all seedlings from each pot were pooled into one sample. Bacteria associated with the rhizosphere soil were extracted in 0.9% NaCl (1 ml per root) by vortexing (30 s) and sonicating in a water bath (30 s). After a new vortexing (10 s), the extract was left 5 min for sedimentation. Identification of the *P. fluorescens* DR54 strain

among the developed *Pseudomonas* colonies on Goulds S1 plates [7] was carried out using immuno-staining. The colony blotting with specific antibody targeting *P. fluorescens* DR54 colonies on the plates was carried out according to Kandel et al. [8]. Preparation of polyclonal antibody, including protocol for immunization, determination of specificity, and removal of weak cross reactions, was as described by Hansen et al. [9].

Microcosm Experiments

The soil microcosm used in this study was essentially the same as described in our previous study [4]. It consisted of a petri dish into which two prewetted (tap water) glass-fiber filters (42.5 mm, GF/C, Whatman International) were placed in the bottom. On top were placed a sugar beet seed (with or without bacterial inoculation) and fungal inoculum (one *Rhizoctonia* infested seed). Noninfested seeds (with or without *P. fluorescens* DR54) were made as described in the pot experiments. The noninfested seeds were placed 3.5 cm away from the pathogen-infested seed as in the pot experiments described above. Soil was placed on top of the glass-fiber filter with seeds. Microcosms with fungal inoculum but without bacterial inoculant were prepared to compare the growth of the fungus with and without the germinating seed. The microcosms were incubated in plastic bags at 15°C for 6 days in the dark. Each experiment had 4–8 replicates of each treatment consisting of petri dishes with two filters. Data are shown as the average of results from two different experiments. Separate microcosms were used for microscopy and quantitative antibody analysis (ELISA) (see below) since both of these methods were destructive.

Biomass determination of *R. solani* was determined by ELISA in extracts from the filters of the microcosms. Each analysis was performed on one half of a filter. The part of the filter that had been placed opposite to the infested seed was used for the extraction, and one sample (replicate) consisted of two half-filters from the same petri dish. Each sample was ground in a mortar with 8 ml extraction buffer provided with the kit (see below) and the suspension was vortexed (30 s) and sonicated in a water bath (30 s) before 50–100 µl was used in the assay. In each experiment 3 replicates were extracted and analysed by ELISA with the Agriscreen Kit 11-003 (Adgen, UK). The ELISA protocol for determination of *Rhizoctonia* biomass was carried out according to the manufacturer using a standard curve obtained from freeze-dried *Rhizoctonia* mycelium.

Total populations of *Pseudomonas* spp. were enumerated using Gould's S1 selective medium. In the microcosms, a primary aim was to determine the biocontrol effects by *P. fluorescens* DR54 on *R. solani.* Changes in fungal growth pattern, sclerotia formation, and activity of *R. solani* were studied using vital fluorescence staining and microscopy. The fungal mycelium grew well within the glass fiber filters as visualized after application of Calcofluor White (Sigma Fluorescent Brightner, Sigma F-3397) and Nile Red (Sigma 3013) stains directly to the filter. The stains spread easily within the filters resulting in nearly homogenous staining, and spatial disturbance of the mycelium was minimal during stain application. Stock solutions of the stains were 1 mg ml^{-1} in water (Calcofluor White) or in DMSO (Nile Red). The stains were applied in a water solution at a final concentration of 5 µl/ml. Three aliquots of 100 µl stain solution were applied to each filter in the petri dishes using a pipette tip to penetrate the soil compartment. For microscopy, a Nikon Eclipse TE 300 inverted microscope equipped with a high pressure Hg 100 W lamp was used. UV (330–380 nm excitation filter and 420 nm barrier filter), FITC and rhodamine filter sets were used. Microscopic examination was carried out in a 15-mm-wide area close to the seed [4]. Hyphal lengths were recorded according to Hansen et al. [10] and an average of the recorded lengths was determined by counting the intersections in three areas of 1.0 mm^2. The area of sclerotia formed was determined within the area around the seed by using a counting net with 10×10 squares each of 0.0008 mm^2 areas. Photographs were taken with 200 or 400 ISO Fujicolor films.

Statistical Methods

The data shown are averages of each treatment including standard errors, *t*-tests, or both.

Results

Biocontrol of R. solani *by* P. fluorescens *DR54 in Field and Pot Experiments*

In field experiments, the number of seedlings emerging (day 24) was higher in rows containing plants inoculated with *P. fluorescens* DR54 (64.8 ± 1.5) than in control rows (51.0 ± 1.7), as shown in Fig. 1A. The results also demonstrated that the number of rhizosphere-colonizing *Pseudomonas* spp. (day 18) was 60-fold higher in rows with *P. fluorescens* DR54 inoculum (8.2×10^5) than in control rows (4.8×10^5) (Fig. 1B). The inoculant strain comprised approx. 99% among the CFUs. Thus, a large number of this population was apparently colonizing the emerging roots as 5×10^6 CFU of the inoculant was applied to each seed. As emergence increased and the seedlings developed further, the number of *P. fluorescens* DR54 decreased by a factor of 90 on the roots of inoculated seedlings to $5.4 \times 10^3 \pm 0.7 \times 10^3$ (day 27). Throughout the experiment a significant population of *P. fluorescens* DR54 had, however, colonized the seed coats (10^5–10^7 CFU per seed).

Both of the two pot experiments supported the inhibition of *R. solani* by *P. fluorescens* DR54 inoculated on the sugar beet seeds. Representing one of the experiments, Fig. 2A thus demonstrates a significant biocontrol effect by *P. fluorescens* DR54, resulting in both a higher emergence and better health of the seedlings. In the pot experiments, seedling emergence was approx. 100% for all treatments. However, examination of roots showed large differences in plant

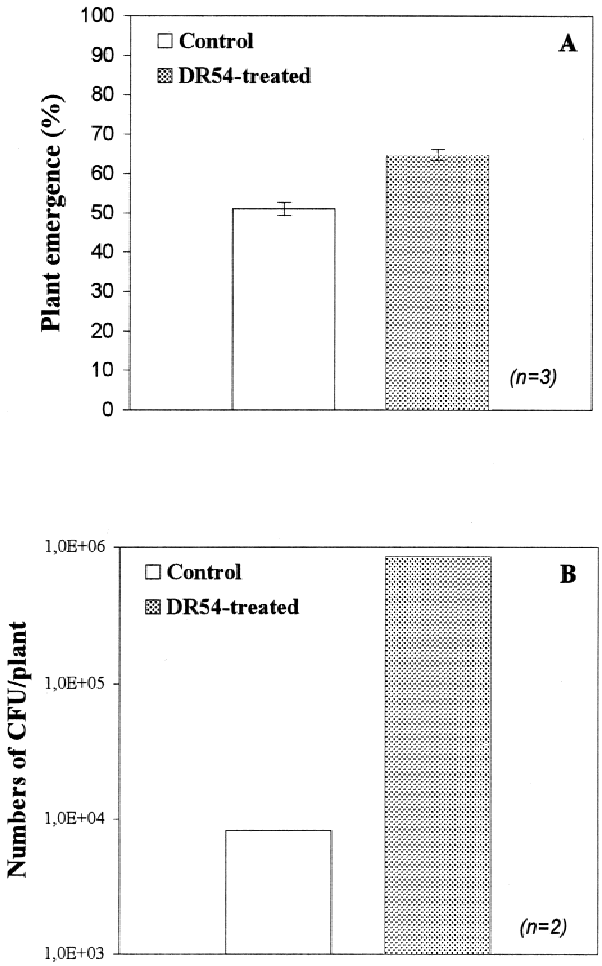

Fig. 1. Field experiment at Danisco Field Station at Holeby, Denmark. (A) In plots where seeds were inoculated with *Pseudomonas fluorescens* DR54, seedling emergence was increased 24 days after sowing ($n = 3$). Each row (replicate) had 100 seeds. Standard error is shown. (B) The number of *Pseudomonas* spp. was significantly higher on roots in the *P. fluorescens* DR54 inoculated plots 18 days after sowing ($n = 2$). The inoculant was shown to constitute up to 99% of the CFU formed on Gould's S1.

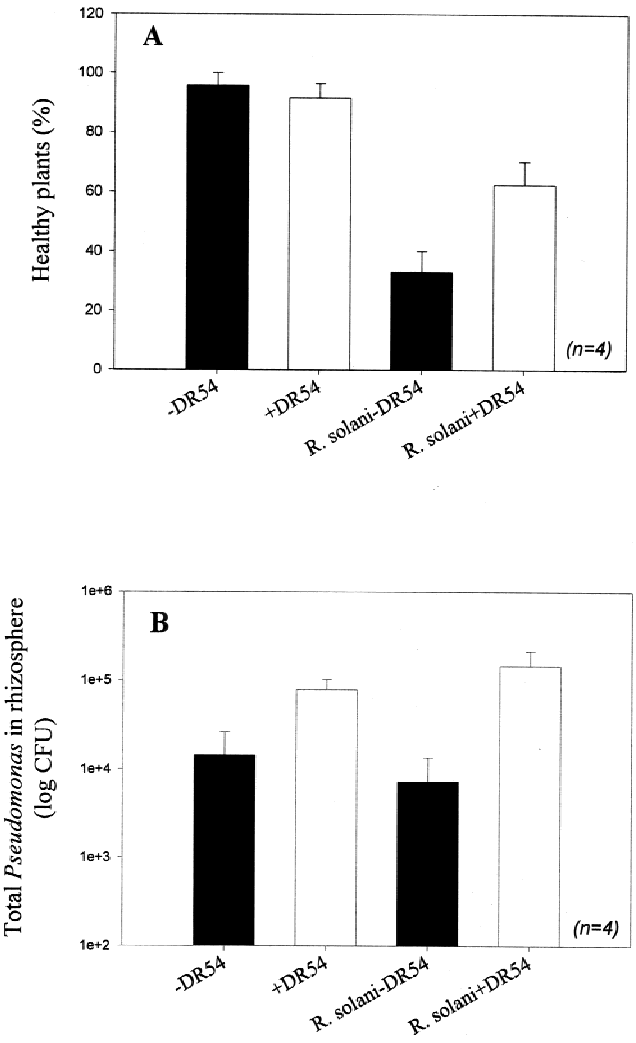

Fig. 2. Pot experiment showing biocontrol of *Rhizoctonia solani* challenged sugar beet by *Pseudomonas fluorescens* DR54. (A) When no *R. solani* inoculum was added to pots, there was no difference in seedling emergence and health ($p > 0.2$, *t*-test). When seedlings were challenged with *R. solani, P. fluorescns* DR54 had a significant biocontrol effect ($p < 0.02$, *t*-test). (B) Rhizosphere colonization of total *Pseudomonas* spp. When seeds had been inoculated with *P. fluorescens* DR54, rhizosphere *Pseudomonas* populations were significantly larger ($p < 0.05$, *t*-test), and approx. 98–100% of the *Pseudomonas* spp. colonies formed from rhizosphere extracts from the bacteria-inoculated seeds were *P. fluorescens* DR54. The figure shows data from one pot experiments ($n = 4$). Error bars represent standard error.

health between treatments. In noninfested pots (control), practically all seeds germinated and all seedlings were healthy (92–96%), independent of the presence or absence of *P. fluorescens* DR 54 (Fig. 2A). In these experiments there was thus no negative effect of the bacterial inoculant on seed germination and root development ($p>0.2$, *t*-test). It could also be deduced that natural disease pressure from pathogens in the soil must have been low under the conditions in the pot experiment. When the seeds were infested with *R. solani* in the pot experiments, however, the biocontrol effect of *P. fluorescens* DR54 was significant ($p < 0.02$, *t*-test), since the number of healthy seedlings increased from 33% without the inoculant to 63% with the inoculant (Fig. 2A). In a similar pot experiment with 6 replicates, biocontrol was also significant ($p < 0.005$).

Based on bacterial enumerations and colony blotting after extraction of bacteria from seedling rhizosphere (day 7), the pot experiments also documented that the inoculant *P. fluo-*

rescens DR54 had colonized the emerged roots and was present only on inoculated seedlings. The results in Fig. 2B thus showed that inoculated seedling roots had a significantly higher ($p < 0.05$, *t*-test) population density of total *Pseudomonas* spp. (7.8–14.5 × 10^4 CFU per root) than the noninoculated ones (7.3–14.4 × 10^3 CFU per root). This colonization and activity of the inoculant in the rhizosphere most likely was a prerequisite for the biological control (Fig. 2A). Most important, however, was that the immunoblot assay confirmed a large majority (98–100%) of the *Pseudomonas* spp. colonies developing in samples from inoculated seedling roots were indeed *P. fluorescens* DR54.

Effect of P. fluorescens *DR54 on* R. solani *Growth Pattern in Microcosm Experiment*

The photomicrographs in Fig. 3 illustrate that one impact of *P. fluorescens* DR54 on *R. solani* previously observed on agar plates [3] was a large increase in hyphal branching. In the microcosms (day 4) the hyphae challenged with *P. fluorescens* DR54 thus became highly branched (Fig. 3B) compared to the unchallenged ones (Fig. 3A). A second impact on fungal growth was a reduction in radial extension of the mycelium (day 4), since the hyphae grew a much shorter distance from the infestation site in the microcosms with *P. fluorescens* DR54 inoculum (data not shown). As shown in Fig. 4A, the impact on fungal growth further resulted in reduced density of the *R. solani* mycelium, as expressed by mycelium biomass developed per unit area of the filters. The presence of *P. fluorescens* DR54 thus decreased the recorded biomass from approx. 9 μg per filter to approx. 2 μg per filter ($p < 0.05$, *t*-test), which was just as low as the value recorded (approx. 2 μg per filter) in the absence of both the seed and the inoculant. Figure 4B shows that the fourth impact on mycelial growth was a very strong inhibition of sclerotia development in the mycelium ($p < 0.03$, *t*-test), when *P. fluorescens* DR54 was present in the microcosms. The insignificant development of sclerotia in inoculated microcosms was similar to that observed when neither seeds nor bacterial inoculant were present. It was clear from these results that seed germination and seedling root development promoted fungal growth including sclerotia formation, but this was effectively antagonized by the *P. fluorescens* DR54 when the latter was present in the microcosms.

P. fluorescens *DR54 Colonization and Cell Wall Effects on* R. solani *Mycelium*

A presumed mechanism of the *R. solani* antagonism by *P. fluorescens* DR54 was the direct action of metabolites such as antibiotic (viscosinamide) or hydrolytic enzyme production (chitinase) [1, 2]. If such metabolites were released from the inoculant in the relatively dry soil systems, where diffusion is often limited in contrast to *in vitro* systems, only direct colonization of the hyphae might ensure a close interaction between the biocontrol inoculant and the fungal cell wall. The use of Gfp-marked *P. fluorescens* DR54 inoculum in selected microcosm experiments showed that the introduced bacteria colonized and caused changes in the *R. solani* cell wall. Figure 5 is a photomicrograph showing the Gfp-fluorescing cells of the inoculant, both occurring as single cells and as small microcolonies of several cells on the hyphal

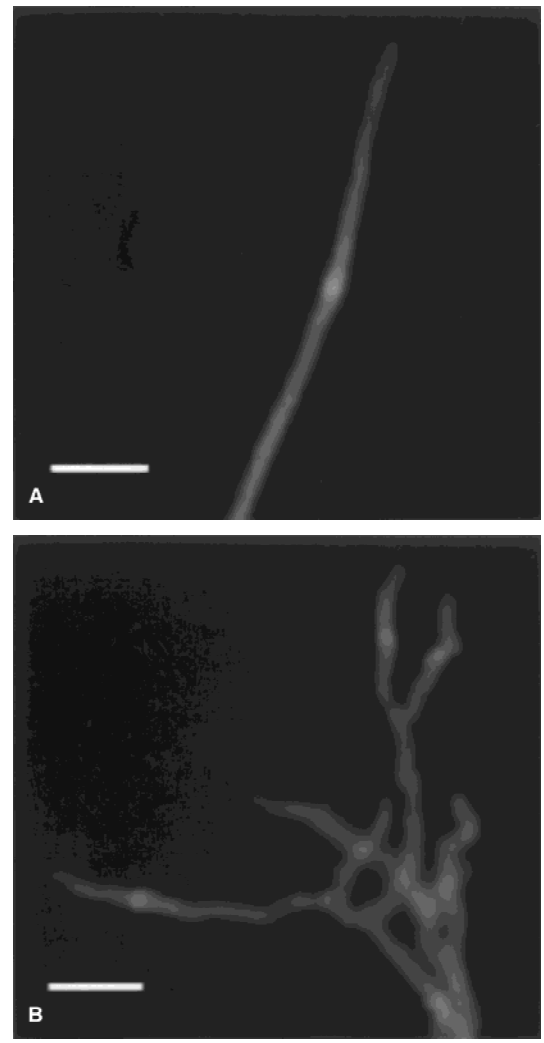

Fig. 3. Micrographs showing *R. solani* growing in microcosms. Comparison of *Rhizoctonia solani* control hyphae (A) and hyphae challenged with *P. fluorescens* DR54 (B). When no *P. fluorescens* DR54 was present, hyphae were straight (A), whereas the biocontrol bacterium induced branching of the hyphal tips. Structures were stained with Calcofluor White. Bars = 50 μm.

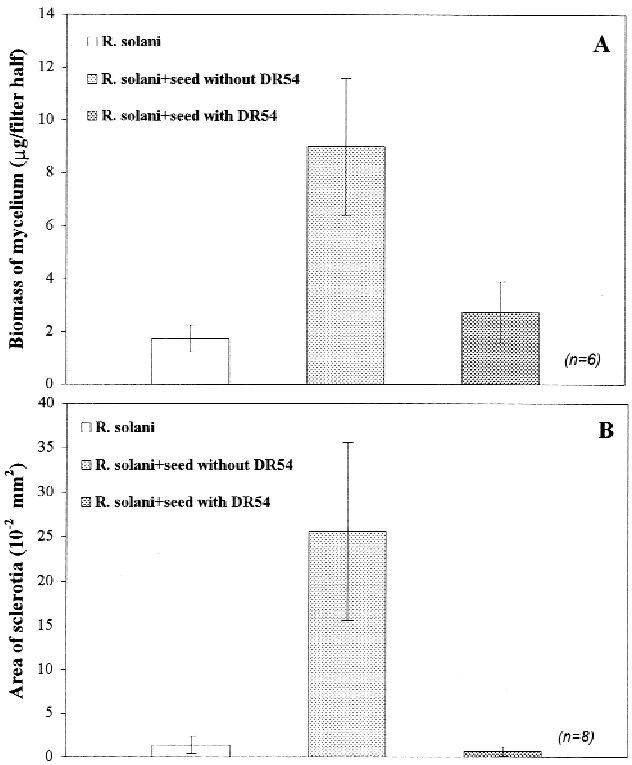

Fig. 4. Quantification of sclerotia formation and fungal biomass of *Rhizoctonia solani* in soil microcosms. (A) Biomass of *R. solani* growing in the filter half including the germinating seed was measured using Elisa-antibody technique with a *Rhizoctonia* specific antibody ($n = 6$). *P. fluorescens* DR54 significantly reduced the biomass of *R. solani* ($p < 0.05$, *t*-test). (B) In microcosms the areas covered by sclerotia were measured by direct microscopy within the 15-mm-wide area around the seed. In the presence of a seed, sclerotia formation was strongly stimulated ($p < 0.03$, *t*-test). In the presence of *P. fluorescens* DR54 on the seeds, sclerotia either were not detected or were only detected in small numbers ($n = 8$). Bars are shown with standard error.

surface (Fig. 5, thin arrows). Interestingly, the hyphae showed a relatively strong autofluorescence at locations where the inoculant was colonizing most intensively. This could be seen on local parts of the hyphae (Fig. 5, thick arrows) where the *P. fluorescens* DR54 cells could be identified by the GFP marker. Occasionally, other parts of the hyphae also showed locally strong autofluorescence (data not shown), which was possibly due to a comparable effect of stressful metabolites from native soil bacteria.

Discussion

Rhizosphere Colonization and Biocontrol Effects of P. fluorescens *DR54*

In field experiments, the inoculant *P. fluorescens* DR54 was predominant within the total *Pseudomonas* spp. population

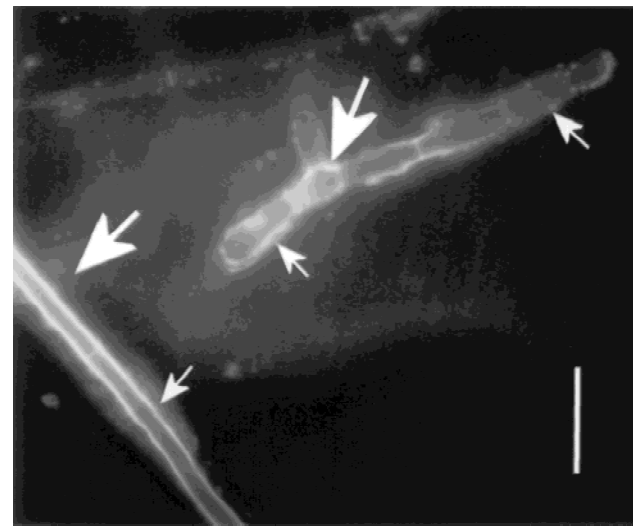

Fig. 5. Micrograph of *R. solani* and *P. fluorescens* DR54 (GFP-marked strain) growing in a soil microcosm. *R. solani* cell walls were frequently showing autofluorescence (thick arrow) when growing in soil microcosms, and the fungus could be visualized without staining. Small colonies and diffuse areas of *P. fluorescens* DR54 gfp-1 (thin arrows) colonizing hyphae of *R. solani* are seen. Bar = 50 µm.

during seed germination and early seedling emergence. In May 2000, the weather was unusually dry and warm in Denmark. It was thus promising that survival of *P. fluorescens* DR54 in the relatively dry soil (26–35% WHC at harvest) was high enough to support a significant rhizosphere population throughout the early phase of seed germination and seedling emergence (approx. 2 weeks). Soon after, inoculant population decreased significantly. The *P. fluorescens* DR54 population furthermore seemed to exert a significant biocontrol under field conditions in the early seedling emergence, resulting in clearly improved field emergence of the sugar beet seedlings. It was likely that this effect was due to inhibition by *P. fluorescens* DR54 of the indigenous, fungal pathogens in the soil.

Although the actual pathogens were unknown in the field experiment, we know that most root diseases in Danish sugar beet production are caused by *Pythium ultimum* and *R. solani. P. fluorescens* DR54 was originally selected for its *in vitro* antagonism toward both fungi [1], and we have recently demonstrated significant biocontrol effects of the strain against *P. ultimum* in soil microcosms under near-field conditions [4]. The present study support these investigations, documenting a biocontrol effect of *P. fluorescens* DR54 against the other important pathogen, *R. solani,* in pot experiments and soil microcosms. Pathogenicity of the ac-

tual *R. solani* isolate was documented in the pot experiments with two different levels of pathogen infestation. The results showed a direct correlation between infestation level and plant pathogenesis, as indicated by seedling emergence and health; Henis and Ben-Yephet [11] similarly found a positive correlation between *R. solani* infestation and severity of disease development in bean seedlings.

Our pot and microcosm experiments supported the field observations, demonstrating a clearly improved emergence of healthy sugar beet seedlings in presence of the *P. fluorescens* DR54 inoculant, which established in the rhizosphere surrounding the seedling roots. A rhizosphere population of culturable *P. fluorescens* DR54 could thus be demonstrated during the early phase of seedling root development when the plants were presumably most susceptible to attack by the *R. solani* pathogen. However, a strong decline in the *P. fluorescens* DR54 population at the time when many seedlings had emerged was noted in field experiments. Furthermore, the laboratory experiments also verified the significant biocontrol effect of seed-inoculated *P. fluorescens* DR54, resulting in a high percentage of healthy seedlings when the seeds were infested with *R. solani.* Despite the low numbers of inoculant present on the seedling roots, this population was apparently responsible for the significant increase in plant health under field and growth chamber conditions. It is possible that *P. fluorescens* DR54 exerts the largest biocontrol effect on the germinating seeds, similar to that of fungicide-treated seeds. This hypothesis is supported by the large inhibitory impact on *R. solani* observed in microcosms close to the seed due to the high number of inoculant present on seeds and, as indicated in a previous study, the large quantities of viscosinamide present on *P. fluorescens* DR54 inoculated seeds [4].

Mechanism of P. fluorescens *DR54 Antagonism against* R. solani

The studies with germinating sugar beet seedlings in small soil microcosms gave us a unique opportunity to study the interactions between seedling roots and microorganisms in detail. In preparations without bacterial inoculant, *R. solani* was first shown to grow poorly in bulk soil but very well in close proximity to the germinating seed and developing root. During the early steps of germination, compounds from the seed have indeed been proposed to support the initial development of *R. solani* mycelium, before infection of the seedling can take place [12]. If this hypothesis of root infection is correct, the biocontrol strain *P. fluorescens* DR54 may inhibit the initial development of mycelium, and fungal biomass may thus remain inadequate for infection. The hypothesis presumptions agree well with the observation that low disease level in presence of *P. fluorescens* DR54 concurred with reduced mycelial biomass and sclerotia formation by *R. solani* close to the seed or seedling root surfaces as measured in microcosms. It should be noticed that the strong development of mycelial biomass and sclerotia formation in microcosms without *P. fluorescens* DR54 were tightly coupled and the sclerotial formation may simply reflect the rapid extension, maturation, and aging of hyphae, growing under optimal conditions in the proximity of seeds or seedling roots. Naiki and Ui [13] and Van Bruggen and Arneson [14] also found a positive correlation between disease severity and sclerotia formation on sugar beet, which may possibly be explained by the stronger mycelium development on infested roots.

According to the hypothesis of biocontrol by *P. fluorescens* DR54 of *R. solani* outlined above, diseased roots may only occur if the development of mycelium density or biomass reaches a critical threshold for infection. It is therefore conceivable that the early inhibition of *R. solani* growth, i.e., hyphal extensions toward the seed or seedling root surfaces, is important for biological control by the *P. fluorescens* DR54 inoculant. One advantage is that the inhibition of *R. solani* growth may be exerted by at least two antagonistic mechanisms in *P. fluorescens* DR54, production of antibiotic (viscosinamide) [2] and hydrolytic, cell wall-degrading enzyme (chitinase) [1]. In an earlier study [4], where *R. solani* mycelium was challenged by purified viscosinamide under *in vitro* conditions, we could demonstrate a number of growth modifications at the hyphal tips, e.g., increased branching, swelling, and septation, which led to inhibited radial growth in the inhibition zone. Whether a direct inhibition of pathogen growth by the viscosinamide antibiotic also took place in the soil systems investigated was ascertained in a previous study, as the viscosinamide compound may actually be detected in rhizosphere of *P. fluorescens* DR54 inoculated sugar beet seedlings under laboratory conditions [4]. It was interesting in this context that we could observe direct surface attachment and microcolony growth of *P. fluorescens* DR54 inoculant cells on the surface of *R. solani* hyphae, using Gfp-labeled inoculant. Using simpler agar systems Fridlender et al. [15] showed lysis and damage of soil-borne plant pathogens by an antagonistic *P. cepacia* strain, but our study in soil microcosms is the first documentation of a very close association between the cells of a biocontrol agent and its target pathogen in soil. Further, there were indications of

molecular interactions between the microorganisms, although their chemical nature obviously remained obscure. Modifications of the cell wall resulting in strong autofluorescence is well known from plant cells, where polyaromatic compounds may accumulate in response to external stress [16].

From studies in the field, pots, and microcosms of effects of *P. fluorescens* DR54 on disease development and growth of the pathogen, it is apparent that the inoculant has biocontrol potential against *R. solani* and other fungi causing damping-off disease. During the time of seed germination and during early plant development, the inoculant is active and present in high numbers. Further, it is suggested that *P. fluorescens* DR54 has long-term effects on the *R. solani* population in soil because of the negative impact of the inoculant on fungal biomass development and survival structure formation, and finally by colonization of *R. solani* hyphae remaining in the soil.

Acknowledgments

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