

Effect of soil-spraying time on root-colonization ability of antagonistic *Streptomyces griseoviridis*

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The root-colonization ability of *Streptomyces griseoviridis* Anderson et al. was tested on turnip rape (*Brassica rapa* subsp. *oleifera* DC.) and carrot (*Daucus carota* L.) by the sand-tube method. Non-sterile sand was sprayed with a microbial suspension immediately or 7 days after the seed had been sown. Results expressed as population frequencies and densities indicated that *S. griseoviridis* effectively colonizes the rhizosphere when the microbe is applied immediately after sowing but less effectively when it is applied 7 days later. Detection values of *S. griseoviridis* were higher for turnip rape than for carrot. In sterile sand, *S. griseoviridis* invariably colonized the rhizosphere of turnip rape after each of the two applications. These findings indicate that *S. griseoviridis* can compete with indigenous soil microbes in the rhizosphere if it is sufficiently abundant in the soil before the seed emerges. If applied later, however, it competes rather poorly. In root-free nonsterile sand, *S. griseoviridis* dispersed and survived well.

Key words: actinomycetes, biological control, *Brassica rapa* ssp. *oleifera*, *Daucus carota*, plant growth-promoting rhizobacteria (PGPR), rhizosphere

Introduction

S. griseoviridis, a biocontrol agent used against some seed-borne and soil-borne plant pathogens (Tahvonen 1988), produces the auxin indole-3-

acetic acid (IAA). The concentration of IAA produced on solid media by *S. griseoviridis* is of the same magnitude as that reported to have a growth-promoting effect (Tuomi et al. 1994). Mycostop (Kemira Oy, Finland) is a biofungicide produced by fermentation of the spores and

mycelium of a *S. griseoviridis* strain isolated from peat by Tahvonen (1982).

Several isolates of *Streptomyces* spp., including the *S. griseoviridis* isolated from peat, produce polyene antibiotics. In contrast to nonsuppressive isolates most of the suppressive isolates produce a candicidin-type antibiotic (Raatikainen et al. 1993). Scanning electron microscopy studies show that *S. griseoviridis* is a hyperparasite of various plant pathogenic fungi (Tapio and Pohto-Lahdenperä 1991). Besides antibiosis and parasitism, competition is often mentioned as a mechanism of biocontrol. According to Sivan and Chet (1989), the inhibition of germination of chlamydospores might be due to competition between *Trichoderma harzianum* Rifai and *Fusarium oxysporum* Schlecht.: Fr. Rothrock and Gottlieb (1984), on the other hand, showed that the antagonism of *S. hygroscopicus* var. *geldanus* was due to the antibiotic production, not to competition for nutrients.

Microbes that colonize roots are ideal for use as biocontrol agents against soil-borne diseases (Weller 1988). Because soil spraying, like seed dressing is, an application method that has resulted in good biocontrol, our objective was to study how roots of turnip rape and carrot are colonized after soil-spraying treatment and to test whether application time has any effect on root colonization. We tested the root-colonization ability in both nonsterile and sterile sand to establish the effect of microbial competition in the rhizosphere on the colonization potential. As well as the growth of *S. griseoviridis* in the rhizosphere, we examined the dispersal of this antagonist in root-free sand.

Material and methods

Root colonization in nonsterile sand

The dispersal of *S. griseoviridis* in the rhizosphere was tested by the sand-tube method as described by Ahmad and Baker (1987) and Kor-

temaa et al. (1994). Seeds of turnip rape, *Brassica rapa* subsp. *oleifera* cv. Kulta, and carrot, *Daucus carota* cv. Nantes Fancy, were surface-sterilized with ethanol and sodium hypochlorite (NaOCl) as described by Kortemaa et al. (1994). PVC-plastic tubes 20 cm long and 3.2 cm in diameter were longitudinally sliced and fastened together with rubber bands. The tubes were blocked at the bottom with cotton wool and filled with nonsterile, sieved (0.5–1.2 mm) sand (pH 6.2). The sand, water and water-soluble fertilizer were mixed as described earlier, resulting in a water potential of –1 kPa (Kortemaa et al. 1994). One surface-sterilized seed was sown in each tube, and the tubes were placed vertically, five per each plastic pot containing the same sand mixture. A microbial suspension was prepared of Mycostop biofungicide as a 0.01% suspension in water. The average colony-forming unit (cfu) value of *S. griseoviridis* of the suspension detected on semi-selective water agar with glycerol (Kortemaa et al. 1994) was $8 \times 10^4 \text{ ml}^{-1}$; 5 ml of this microbial suspension was spread evenly with a pipette on the sand surface in each tube immediately after sowing (day 0) or was applied 7 days after sowing. The pots were covered with plastic bags, and no water was added after sowing. The pots were incubated for 4 weeks in a growth chamber (16 h light period, light intensity $150 \text{ mMols}^{-1}\text{m}^{-2}$ at 20°C and 8 h dark period at 18°C).

After 4 weeks, the tubes were opened, and the roots were cut into 2 cm segments. The sand adhering to the root segments was considered as rhizosphere soil. For population-density counts, the cfu values were determined by a dilution-plating method on water-agar plates, although this method did not permit population densities lower than 10^2 cfu g^{-1} soil to be detected. Sand-free root segments and above-ground portions of seedlings, i.e. stems and leaves, were placed on water-agar plates to isolate *S. griseoviridis*.

Root-colonization frequencies were counted for root segments and the rhizosphere, and the population density was counted as cfu g⁻¹ rhizosphere soil. Each experiment comprised 10 plants of turnip rape and carrot and the two application

times for the suspension. Five control plants were not treated. The experiment was conducted three times.

Root colonization in sterile sand

For this experiment, turnip rape was grown in sterile sand in a large glass pot containing four longitudinally sliced tubes. The sand was sterilized for 1 h and other material for 20 min at 121°C in an autoclave. A water suspension of *S. griseoviridis*, which had grown for 7 days on glucose-yeast-malt agar (GYM) (Kortemaa et al. 1994), was prepared for the spraying treatment instead of Mycostop suspension. The average population density of the suspension, which mainly consisted of spores of *S. griseoviridis*, was 6.5×10^6 cfu ml⁻¹. For these experiments, the sterile seed was sown, the sand-tubes were incubated, and *S. griseoviridis* was applied and isolated as described above. The only exception was that for population density counts the microbe was isolated in the same manner for one tube in each pot on water-agar and GYM-agar plates to ensure that the sand was not contaminated with other microbes. Each experiment contained one untreated control pot and three pots with the two application times of the *S. griseoviridis* suspension. The experiment was conducted twice.

Dispersal in nonsterile sand without plants

For this experiment a large plastic box (40 cm x 60 cm) and 32 plastic tubes were filled with the nonsterile sand-water-fertilizer mixture described above. The tubes were placed randomly in the box in a vertical position. Five millilitres of 0.01% Mycostop suspension was pipetted evenly onto the surface of each sand-tube; after this treatment, the tubes were not watered. The average population density of the suspension was 3×10^4 cfu ml⁻¹. The box with the sand tubes,

which was covered to avoid loss of moisture, was incubated in a growth chamber under the conditions described above for 4 weeks. Each week (7, 14, 21 and 28 days after treatment), eight tubes were randomly taken as a sample for measurements of population frequencies and densities at different depths of sand. The soil for these counts was sampled at every 2 cm between 0 and 12 cm from the top of the tube. The population density was counted by the dilution-plate method on water-agar plates. The experiment was conducted twice.

Statistical analysis

Cfu values were logarithmically transformed before analyses of variance [PROC GLM (SAS Institute Inc. 1988)]. Tukey's Studentized Range (HSD) Test was used to compare significantly different means.

Results

Root colonization in nonsterile and sterile sand

When Mycostop suspension was sprayed immediately (day 0) after sowing, the root-colonization frequencies on the roots and rhizosphere of turnip rape and carrot were higher than when the suspension was applied 7 days after sowing (Table 1). The frequency values were higher for turnip rape than carrot. The stems and leaves of turnip rape seedlings were colonized 100% after 0 and 73% after 7 days of treatment. For carrots, the corresponding values were 77% and 54%.

The differences in population density values between the two applications were significant ($P=0.05$) at all root depths in the rhizosphere soil (Table 2). At a distance of 0–2 cm from the seed, the differences were significant between both treatments and plant species.

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Table 1. Root-colonization frequency of *Streptomyces griseoviridis* on root segments and in rhizosphere of turnip rape (*Brassica rapa* subsp. *oleifera*) and carrot (*Daucus carota*) after two different spraying times of Mycostop suspension in nonsterile sand.

Depth (cm)	day 0 ^c	Root ^a	Root-colonization frequency (%)	
			day 7 ^d	day 0
			Rhizosphere ^b	
			day 7	
<i>Brassica</i>				
0–2	100 (30) ^e	100 (30)	100 (30)	100 (30)
2–4	100 (29)	47 (30)	100 (29)	47 (30)
4–6	100 (24)	10 (27)	100 (24)	10 (27)
6–8	83 (19)	18 (19)	83 (19)	23 (19)
8–10	85 (14)	0 (15)	85 (14)	0 (15)
<i>Daucus</i>				
0–2	97 (29)	51 (28)	97 (29)	61 (28)
2–4	83 (29)	4 (25)	83 (29)	7 (25)
4–6	82 (25)	0 (24)	82 (25)	0 (24)
6–8	65 (17)	0 (11)	64 (17)	0 (11)
8–1	23 (10)	0 (8)	22 (10)	0 (8)

^a *S. griseoviridis* isolated from root segments.

^b *S. griseoviridis* isolated from root segments and/or from rhizosphere soil.

^c Suspension sprayed on day 0 after sowing.

^d Suspension sprayed on day 7 after sowing.

^e Number of samples studied (n).

The rhizosphere of turnip rape was effectively colonized by *S. griseoviridis* in sterile sand. The results for the dilution series on GYM agar showed that the sand remained uncontaminated during the experiments. The root-colonization frequencies were 100%. Population densities

were high, and there were no significant differences between treatments (Table 3). The stems and leaves of turnip rape seedlings were colonized 66% and 75% after the 0 day and the 7-day treatments.

Table 2. Population densities (cfu values) of *Streptomyces griseoviridis* in rhizosphere of turnip rape (*Brassica rapa* subsp. *oleifera*) and carrot (*Daucus carota*) after two different spraying times of Mycostop suspension in nonsterile sand.

Treatment	Population density (cfu) 10 ² g ⁻¹ of soil				
	0–2	2–4	4–6	6–8	8–10
<i>Brassica</i>					
day 0	11 000 ^a	2 800 ^a	5 300 ^a	1 000 ^a	350
day 7	3 100 ^b	510 ^b	1 100 ^b	3 ^b	nd
<i>Daucus</i>					
day 0	530 ^c	< 1	270 ^a	< 1	< 1
day 7	220 ^d	< 1	nd	nd	nd

Means within a column followed by the same letter were not significantly different according to Tukey's Studentized Range (HSD) Test (P=0.05). nd: not detected

Table 3. Population densities (cfu values) of *Streptomyces griseoviridis* in rhizosphere of turnip rape (*Brassica rapa* subsp. *oleifera*) after two spraying times of spore suspension on sterile sand.

Treatment	Population density (cfu) 10 ² g ⁻¹ of soil				
	Depth (cm)				
	0–2	2–4	4–6	6–8	8–10
day 0	39 000	23 000	5 600	7 700	920
day 7	79 000	42 000	13 000	4 000	1 900

Means were not significantly different according to Tukey's Studentized Range (HSD) Test (P=0.05).

Dispersal in nonsterile sand without plants

S. griseoviridis dispersed well in root-free sand when 5 ml of Mycostop suspension was sprayed on the surface of the sand. The isolation frequencies of *S. griseoviridis* had already reached almost 100% at a depth of 0–6 cm from the top 7 days after inoculation (Fig. 1), and continued to accumulate over the next 2 weeks (14 and 21 days after treatment).

The population densities of *S. griseoviridis* isolated from different depths in the sand tube were highest at the top and decreased with depth (Fig. 2). The population of *S. griseoviridis* was stable during the experiment; differences in pop-

ulation densities between sampling days were not significant (P=0.05) for any depth category.

Discussion

Root colonization was more effective when *S. griseoviridis* was applied to nonsterile sand immediately after sowing than when it was applied 7 days later, probably because during those days the rhizosphere was colonized by other soil microbes with which *S. griseoviridis* was unable to compete. The results of sterile-sand experiments, in which the rhizosphere was 100% col-

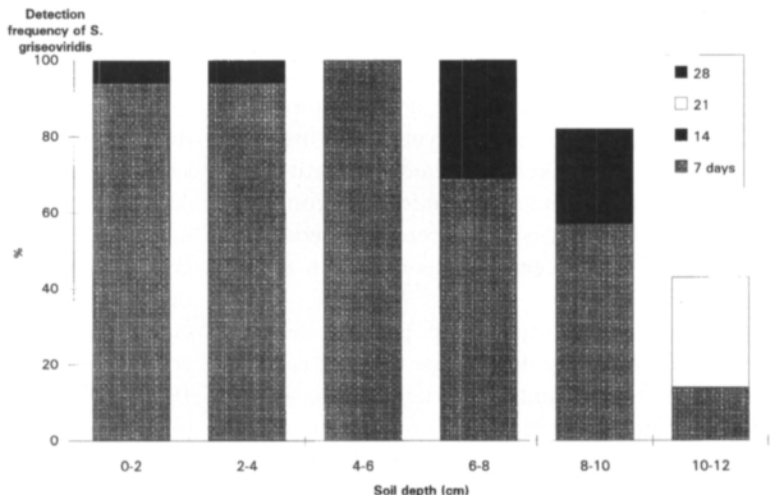


Fig. 1. Accumulation of isolation frequencies of *Streptomyces griseoviridis* at different soil depths during 28 days after spraying of Mycostop suspension on root-free sand.

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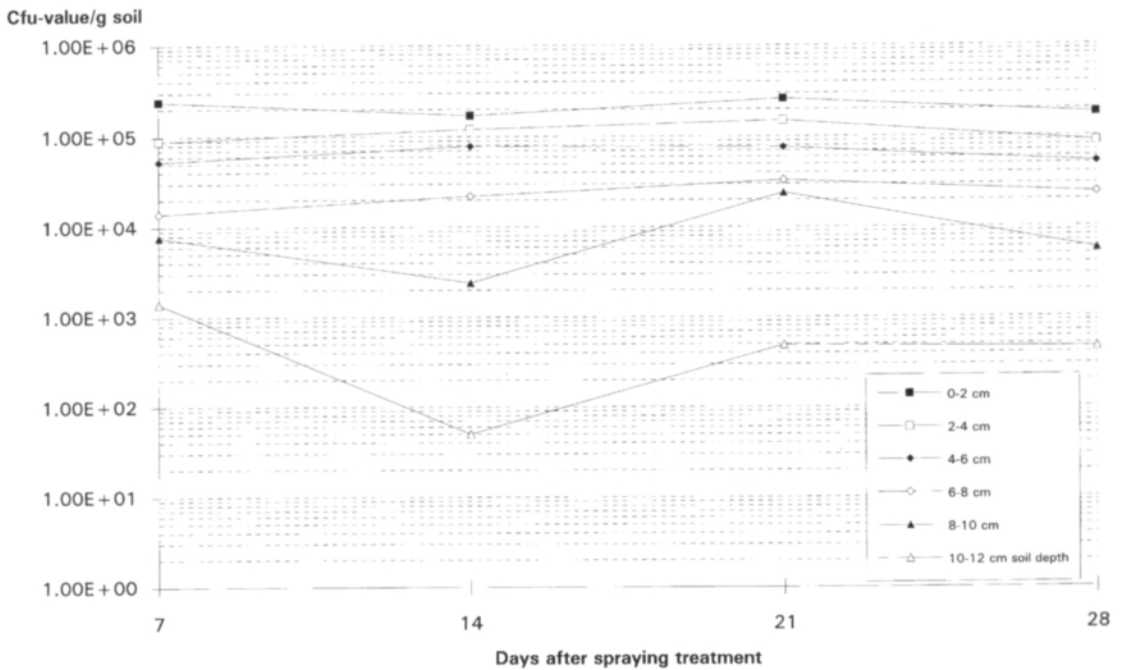


Fig. 2. Population densities (cfu values) of *Streptomyces griseoviridis* at different soil depths during 28 days after spraying of Mycostop suspension on root-free sand. Differences in population densities between sampling days were not significant ($P=0.05$) for any depth category.

onized by *S. griseoviridis* after both treatment times, support this contention. The higher population densities in sterile than in nonsterile sand are probably due to the higher inoculation densities and lack of competition with other microbes in sterile sand. The number of micro-organisms in nonsterile sand was probably rather low, and the absence of many of the microbes, both plant-growth-promoting and deleterious strains, to be found in soil with abundant organic material most likely affected competition. Scher et al. (1984) noted that microbial competition negatively affects the root-colonization capacity of fluorescent pseudomonads in nonsterile soil.

Isolation frequencies and population densities were greater for turnip rape than for carrot because the root exudates of turnip rape were probably more abundant or more available than those of carrot. The same results were obtained in our previous study (Kortemaa et al. 1994) us-

ing the plate test. Sterile plants of turnip rape and carrot inoculated with *S. griseoviridis* were grown on water-agar plates. The difference in root colonization between the two plant species was clear. Without the effect of other microbes, the difference between plant species was suggested to be of plant origin, i.e. due to root exudates or morphological differences.

In both plant species the upper part of the root was more frequently colonized by *S. griseoviridis* than the lower parts. The population densities in the rhizosphere of turnip rape were higher than in the root-free sand experiment but population densities were lowest in the carrot rhizosphere. The results are similar to those of our previous studies, which were done using other application methods (Kortemaa et al. 1994, 1997). All these differences were probably due to root exudates (Curl and Truelove 1986)

The soil-spraying treatment method used here resulted in better dispersal in sand than did seed

treatment (Kortemaa et al. 1994). Root-colonization was best when the *S. griseoviridis* suspension was mixed into the sand before sowing (Kortemaa et al. 1997). After seed treatment, the antagonist must actively colonize the rhizosphere with the aid of the root and seed exudates. After the soil-spraying treatment the antagonist dispersed well as shown by the experiment without plants, the antagonist being available all around the root. Seven days after seed sowing, the rhizosphere was mainly colonized by other soil microbes and *S. griseoviridis* was not able to colonize the rhizosphere effectively.

Our results suggest that *S. griseoviridis* can compete with indigenous soil microbes in the rhizosphere if it is well established in the sand before the seed emerges. If applied to the rhizosphere later, however, it competes rather poorly with other microbes. According to Lacey (1973), *Streptomyces* spp. colonize new substrates more slowly than do other bacteria and fungi. Our findings suggest that competition is probably not the main mode of action of antagonistic *S. griseoviridis*.

S. griseoviridis was isolated in great numbers in root-free sand with very little organic material. The antagonist survived well in root-free sand after the suspension had been mixed into the sand (Kortemaa et al. 1997). Hatzinger and Alexander (1994) showed that the bacteria which survived well in nonsterile soil were present in the highest population densities in the rhizosphere. Bahme and Schroth (1987) noted that the bacteria which survived in nonrhizosphere soil have good potential as biocontrol

agents because the bacteria can await the emerging seed in the soil. According to Wellington et al. (1990), populations of *S. lividans* and *S. violaceolatus* remain constant or decline in natural soil, and after a short mycelial growth phase, sporulation occurs and inoculants survive in the soil as spores.

Mohammadi and Lahdenperä (1994) found that seed dressing controls *Rhizoctonia solani* Kühn on cauliflower more effectively than does soil spraying or the mixing of Mycostop suspension into the growth substrate. On the other hand, soil-spraying treatment resulted in better cucumber seed emergence and a better gerbera flower yield than did other methods. According to El-Abyad et al. (1993) seed-coating treatment with antagonistic *Streptomyces* spp. was a more effective way of controlling various tomato pathogens than was soil inoculation. These results together with those of the present study support the idea that both timing and application method must be right if effective biocontrol is to be achieved with *S. griseoviridis*.

The dispersal of *S. griseoviridis* after soil-spraying treatment was effective in both the rhizosphere and root-free sand. The root-colonization ability of the antagonist depended on the application time. *S. griseoviridis* could not compete effectively with indigenous soil microbes, and the rhizosphere was effectively colonized only if the sand was treated immediately after sowing.

Acknowledgements. We thank Tuula Laine and Lahja Pesonen for their technical assistance.

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SELOSTUS

Kasvualustan käsittelyajan vaikutus *Streptomyces griseoviridis* -antagonistin juurten asutuskykyyn

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Mikrobit, jotka asuttavat tehokkaasti juuria, ovat luopuvia biotorjuntaeliöitä käytettäväksi maalevintäisten kasvitautien torjuntaan. Antagonistisen *Streptomyces griseoviridis* -sädebakteerin kykyä asuttaa rypsin ja porkkanan juuria testattiin hiekkaputkimenettämällä. Käsittelemätön hiekka kasteltiin mikrobiliuoksella joko välittömästi tai seitsemän vuorokauden kuluttua siemenen kylvöstä. Tulokset osoittivat, että *S. griseoviridis* asutti juurivyöhykkeen tehokkaasti, jos mikrobikäsitely tehtiin välittömästi kylvön jälkeen, mutta selvästi heikommin, kun käsittely tehtiin viikon kuluttua kylvöstä. Mikrobiitiheydet olivat rypsin juuressa suuremmat kuin porkkanan juuressa. Antagonisti eristettiin juuren yläosasta useammin

kuin alemmista osista. Steriilissä hiekassa *S. griseoviridis* asutti rypsin juuret tehokkaasti molempien käsittelyaikojen jälkeen. Nämä tulokset osoittavat, että *S. griseoviridis* pystyy kilpailemaan maassa luontaisesti esiintyvien mikrobien kanssa, jos antagonista on kasvualustassa runsaasti ennen siemenen itämistä. Jos antagonisti lisätään maahan myöhemmin, se pystyy suhteellisen huonosti kilpailemaan muiden mikrobien kanssa. Hyvän biotorjuntatuloksen saavuttaminen käytännön kasvintuotannossa edellyttää oikean käsittelymenetelmän ja -ajan tuntemista. *S. griseoviridis* levisi ja säilyi elävänä hyvin steriloimattomassa hiekassa, jossa ei kasvanut kasveja.