APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1998, p. 2304–2307 Vol. 64, No. 6
0099-2240/98/$04.00+0
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Impact on Arbuscular Mycorrhiza Formation of *Pseudomonas* Strains Used as Inoculants for Biocontrol of Soil-Borne Fungal Plant Pathogens

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Received 19 September 1996/Accepted 25 February 1998

The arbuscular mycorrhizal symbiosis, a key component of agroecosystems, was assayed as a rhizosphere biosensor for evaluation of the impact of certain antifungal *Pseudomonas* inoculants used to control soil-borne plant pathogens. The following three *Pseudomonas* strains were tested: wild-type strain F113, which produces the antifungal compound 2,4-diacetylphloroglucinol (DAPG); strain F113G22, a DAPG-negative mutant of F113; and strain F113(pCU203), a DAPG overproducer. Wild-type strain F113 and mutant strain F113G22 stimulated both mycelial development from *Glomus mosseae* spores germinating in soil and tomato root colonization. Strain F113(pCU203) did not adversely affect *G. mosseae* performance. Mycelial development, but not spore germination, is sensitive to 10 μM DAPG, a concentration that might be present in the rhizosphere. The results of scanning electron and confocal microscopy demonstrated that strain F113 and its derivatives adhered to *G. mosseae* spores independent of the ability to produce DAPG.

An increasing demand for low-input agriculture has resulted in greater interest in soil microorganisms that increase soil fertility or improve plant nutrition and health. However, in addition to testing the ability of microbial inoculants to improve plant performance (12), it also is critical to assess the impact of these inoculants on other key rhizosphere processes. Because of the importance of arbuscular mycorrhizal (AM) associations (5), biological control agents must be compatible with the formation and functioning of AM associations (6). In this context, it has been found that certain *Pseudomonas* strains which produce antimicrobial metabolites (25) and fungal biocontrol agents, such as *Trichoderma* sp. (9) and *Gliocladium* sp. (26), do not exhibit inhibitory effects on AM fungi.

The aim of the present study was to explore the impact of biocontrol *Pseudomonas* strains which do and do not produce the antifungal metabolite 2,4-diacetylphloroglucinol (DAPG) on the formation of AM associations by *Glomus mosseae* (Nicol. and Gerd.) Gerdemann and Trappe, a representative AM fungal species from temperature ecosystems (29). Phloroglucinol antibiotics are phenolic metabolites with antimicrobial properties (8). In particular, DAPG is involved in the biocontrol activity of the plant-growth-promoting rhizobacteria (10, 11, 13, 17). The parameters measured included AM fungal spore germination (both in vitro and in soil), the amount of plant-independent mycelial growth (in vitro), and the degree of establishment of AM associations on the developing root systems of tomato plants (in soil). In addition, the interaction between *Pseudomonas* strains and *G. mosseae* was examined by scanning electron and confocal microscopy to determine whether direct cell-to-cell contact or production of DAPG is a factor in the interaction between the fungus and the bacteria.

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Microbial strains. The isolate of *G. mosseae* used in this study was obtained originally from Rothamsted, United Kingdom. Wild-type *Pseudomonas* sp. strain F113 was isolated from the rhizosphere of mature sugar beets (13). The following two genetically modified derivatives of strain F113 also were tested: F113G22, a DAPG-negative mutant of F113, which was constructed by using Tn*5*::*lacZY* (28); and *Pseudomonas* sp. strain F113(pCU203), a DAPG overproducer (13). The antibiotic resistance characteristics of these *Pseudomonas* strains are as follows: F113, 100 μg of rifampin per ml; F113G22, 50 μg of kanamycin per ml; and F113(pCU203), 200 μg of chloramphenicol per ml. The latter two strains are not resistant to rifampin.

Mycorrhizal fungal spore germination and mycelial growth in vitro. The experiments to examine mycorrhizal fungal spore germination and mycelial growth in vitro were conducted as described by Azcón-Aguilar et al. (4). Sporocarps of *G. mosseae* were obtained from rhizospheres of onion (*Allium cepa* L.) plants grown in pot cultures. The rhizosphere samples were kept in polyethylene bags at 4°C, and after collection the sporocarps were stored on damp filter paper at 4°C. Resting spores freshly excised from the sporocarps were surface sterilized in a solution containing 20 g of chloramine T per liter, 200 mg of streptomycin per liter, and 1 drop of Tween 80 per liter (24) for 20 min and were then washed five times in sterile water. The *Pseudomonas* biocontrol strains F113 and F113 (pCU203) (antifungal strains) and the mutant strain F113G22 (with impaired biocontrol ability) were grown at 28°C for 24 h on Luria-Bertani (LB) medium (10 g of tryptone per liter, 5 g of yeast extract per liter, 5 g of NaCl per liter, 15 g of Difco agar per liter), centrifuged, and then washed three times in 0.25× Ringer's solution (Oxoid) prior to use.

Pseudomonas suspensions were adjusted to an optical density at 650 nm of 0.4, which corresponded to a concentration of 10^8 CFU ml^{-1}, and 50-μl portions were spread onto the agar surfaces in petri dishes (diameter, 9 cm) containing water agar

TABLE 1. Effects of *Pseudomonas* strains on *G. mosseae* spore germination in vitro and in soil and on mycelial growth in vitro

Pseudomonas strain	% Spore germination		Mycelial development (mm/germinated spore)
	In vitro	In soil	
None (control)	70 a[a]	60 a	10 a
F113 (DAPG$^+$)	69 a	62 a	33 b
F113G22 (DAPG$^-$)	72 a	67 a	47 c
F113(pCU203) (DAPG over-producer)	71 a	64 a	15 a

[a] Values are the means of values for 30 replicates for spore germination in vitro, 100 replicates for spore germination in soil, and 5 replicates for mycelial development in vitro. Values in a column not followed by the same letter differ significantly at $P = 0.05$.

(0.8% Bacto Agar [Difco]) buffered with 10 mM MES [2-(*N*-morpholino)ethanesulfonic acid] (9). The final pH after sterilization at 120°C for 20 min was 7.0. Six surface-sterilized spores of *G. mosseae* were transferred individually to each *Pseudomonas*-inoculated petri dish; these six spores were located at the vertices of an imaginary hexagon with sides approximately 3.5 cm long. Incubation was at 25°C in the dark, and the plates were sealed with Parafilm. Each treatment consisted of five replicate plates and five control plates containing *G. mosseae* spores growing axenically. Spore germination was evaluated after 28 days. A spore was considered germinated if a germ tube was clearly visible. Hyphal growth from germinated resting spores also was assessed by light microscopy. Mycelial growth was estimated by a gridline intersect method (4).

In another experiment, the effect of the antimicrobial metabolite DAPG on AM fungal spore germination and mycelial growth was tested after purified DAPG was added to axenic cultures of *G. mosseae* spores. Pure DAPG was obtained from the Chemistry Department, University College, Cork, Ireland. A stock DAPG solution (100 mg ml^{-1} in methanol) was prepared and filter sterilized (Millipore). Appropriate dilutions were mixed with melted water agar to obtain concentrations ranging from 1 to 1,000 μM.

To examine the effects of the *Pseudomonas* strains on AM fungal spore germination in soil, the following experiments, based on previous studies, were carried out (18, 25). Each experimental unit consisted of a slide frame that held two membrane filters (diameter, 45 mm; pore size, 0.45 μm; type HT Tuffryn). Twenty unsterilized *G. mosseae* spores were introduced between the two membranes. Twenty-five grams of soil was placed in a 9-cm-diameter petri dish, and the sandwich units were then laid onto this soil layer. An agricultural soil collected from Granada Province (Spain) was used. The characteristics of this test soil, a Cambisol, were as follows: pH (H_2O), 6.8; concentration of available ($NaHCO_3$-extractable) P, 15 mg liter^{-1}; total N concentration, 2,600 mg liter^{-1}; organic C concentration, 0.8%. The soil consisted of 58.7% sand, 26.4% silt, and 14.9% clay. This soil was collected from the upper 20 cm of the soil profile, and the soil was sieved through a 2-mm mesh prior to use. One milliliter of a bacterial suspension containing 10^8 CFU ml^{-1} (as determined by optical density) or 1 ml of a DAPG dilution (range of concentrations used, 1 to 1,000 μM) was applied to the membranes. Another 25 g of soil was added to cover the sandwich units. The soil was moistened with distilled water to field capacity, and the petri dishes were sealed with Parafilm. The dishes (five replicates for each treatment) were then incubated at 25°C for 2 weeks. Upon removal from each dish the sandwiches were opened, and the germ tubes were stained with trypan blue (27). The percentage of germinated spores was calculated for each treatment. All experiments were conducted twice, and the data for the level of germination for each treatment, including both in vitro and soil tests, were subjected to a hypothesis test against the corresponding control data. The z statistic for germination tests was calculated from the experimental data and compared with the z statistic tabulated at the 5% significance level. Mycelial growth data were processed by the analysis of variance method and Duncan's multirange test ($P \leq 0.05$).

Results summarized in Table 1 showed that none of the *Pseudomonas* strains tested inhibited germination or mycelial development of *G. mosseae* in vitro or in soil. Moreover, strains F113 and F113G22 had a significant stimulatory effect on mycelial growth.

The effects of purified DAPG on spore germination and on the subsequent development of the fungal mycelium are shown in Table 2. These results indicate that only the highest concentration of the antifungal compound assayed (1,000 μM) inhibited spore germination. Development of fungal mycelia was more sensitive, and significant inhibition was observed in the presence of 10 μM DAPG.

Attachment of bacteria to spores. Sporocarps of *G. mosseae* were surface sterilized by treating them with a solution containing 4% chloramine T and 300 μg of streptomycin per ml for 30 min and were rinsed five times over a 1-h period with sterile distilled water. A portion of each sporocarp was manipulated with thin pointed forceps to remove the finely branched and interconnected hyphae surrounding the spores. The single spores were then sterilized as described above and were cleaned by subjecting them to four 30-s sonication pulses followed by four rinses with sterile distilled water.

The *Pseudomonas* strains were grown separately in LB liquid medium overnight at 28°C with gentle shaking. After centrifugation at 3,000 × *g* for 20 min, each supernatant was discarded, and the pellet was resuspended in 15 ml of 50 mM phosphate buffer (pH 7.2). The concentration of bacterial cells in each suspension was between 10^7 and 10^8 CFU ml^{-1} and was adjusted by using optical density measurements.

For the attachment assay, spores were prepared and examined by scanning electron and confocal microscopy as previously described by Bianciotto et al. (7).

When the three *Pseudomonas* strains were incubated with sporocarps and isolated spores of *G. mosseae*, they all displayed the same attachment behavior with these fungal structures. Spores treated with *P. fluorescens* F113, F113G22, or F113(pCU203) had only a few bacterial cells on their cell walls. When the sporocarps were incubated with strain F113, a few

TABLE 2. Effects of the antifungal compound DAPG on *G. mosseae* spore germination in vitro and in soil and on mycelial growth in vitro

DAPG concn (μM)	% Spore germination		Mycelial development (mm/germinated spore)
	In vitro	In soil	
0	75 a[a]	62 a	12 a
1	77 a	60 a	15 a
10	76 a	65 a	5 b
100	65 a	64 a	1 c
1,000	10 b	38 b	0.2 c

[a] Values are the means of values for 30 replicates for spore germination in vitro, 100 replicates for spore germination in soil, and 5 replicates for mycelial development in vitro. Values in a column not followed by the same letter differ significantly at $P = 0.05$.

bacterial cells were found among the hyphal network that covers the spores. Identical results were obtained with strain F113G22. To rule out the possibility that the fungal surfaces were contaminated previously by other bacteria, some spores of *G. mosseae* were incubated with no bacteria, and the fungal surfaces were always free of bacterial cells. These results are consistent with those reported previously by Bianciotto et al. (7), who found that bacterial strains always adhere to *Gigaspora margarita*, another AM fungus.

Mycorrhiza formation. Five-day-old seedlings of tomato (*Lycopersicon esculentum* L.) obtained from surface-sterilized (50% [vol/vol] commercial bleach solution, 10 min) seeds germinated on wet filter paper in petri dishes were transplanted into pots (capacity, 1 liter) containing Granada agricultural soil (described above) that had been sieved through a 4-mm-mesh screen.

The mycorrhizal fungal inoculum was obtained in a pot culture (1) with *A. cepa* L. (onion) as the host plant and was added to produce a rhizosphere soil containing five sporocarps per g (with an average of six mature spores per sporocarp) together with some single spores, mycelium, and mycorrhizal root fragments. Fifty grams of this mycorrhizal inoculum per pot was thoroughly mixed with the soil. The *Pseudomonas* cultures tested were grown on LB medium, washed three times in 0.25× Ringer's solution (Oxoid), and then adjusted to an optical density at 650 nm of 0.4. Two milliliters per seedling (one seedling per pot) was applied to the roots at the time of transplantation.

The test plants (five replicates per treatment) were grown in a greenhouse with a day-night cycle consisting of 16 h of light at 21°C and 8 h of darkness at 15°C at a relative humidity of 50% and a photosynthetic photon flux density of 600 to 700 mmol m^{-2} s^{-1}. During the assay, the plants were fertilized (10 ml/week/pot) with Long Ashton nutrient solution (19) lacking P. Throughout the experiment, the pots were weighed every day, and the water loss was compensated for by top watering (with tap water) to reach field capacity. The pot bioassay was carried out twice with two different growth periods (6 and 8 weeks). When the plants were harvested, plant shoot and root dry weights were recorded after drying at 70°C. Representative root samples were stained for mycorrhiza examination (30). The percentage of the total root length that became mycorrhizal was calculated by a gridline intersect technique (16).

At the end of the experiment and before biomass and AM quantifications were performed, representative root samples from seedlings in each pot were suspended in 9 ml of Ringer's solution and vortexed for 7 min, and serial dilutions were inoculated onto LB medium containing the appropriate antibiotics to measure colonization of the tomato rhizosphere by the *Pseudomonas* inoculants. Data from the pot experiments were processed by the analysis of variance method and Duncan's test ($P \leq 0.05$). In the case of the percentage of mycorrhizal root length, the data were subjected to arcsine square root transformation. To evaluate bacterial population density, the data were transformed to a log scale prior to statistic analyses.

Table 3 summarizes the impact of the *Pseudomonas* strains on the formation of AM associations. *Pseudomonas* inoculation increased the percentage of root length that became mycorrhizal (significantly for F113 and F113G22) and improved shoot growth in the mycorrhizal plants by the second harvest time tested (8 weeks). Log-transformed data showed that there were no statistically significant differences ($P = 0.05$) in the level of establishment in the tomato rhizosphere among the three strains. The log values were 7.55, 7.75, and 7.44 CFU g of dry roots^{-1} for strains F113, F113G22, and F113(pCU 203), respectively. These population data were obtained after 6 weeks of plant growth.

TABLE 3. Effects of *Pseudomonas* strains on mycorrhiza formation and biomass production by tomato plants growing for either 6 or 8 weeks in soil microcosms in the greenhouse

Pseudomonas strain	Biomass production (g [dry wt])				Mycorrhizal root length (%) after:	
	Shoots after:		Roots after:			
	6 weeks	8 weeks	6 weeks	8 weeks	6 weeks	8 weeks
None (control)	0.69 a[a]	0.85 a	0.25 a	0.31 a	35 a	43 a
F113 (DAPG$^+$)	0.60 a	1.15 b	0.23 a	0.43 ab	52 b	68 b
F113G22 (DAPG$^-$)	0.63 a	1.23 b	0.24 a	0.42 ab	58 b	71 b
F113(pCU203) (DAPG overproducer)	0.75 a	1.18 b	0.30 a	0.50 b	44 ab	57 ab

[a] Values are the means of values for five replicates. Values in a column not followed by the same letter differ significantly at $P = 0.05$.

Discussion and conclusions. Data presented in this paper indicate that certain *Pseudomonas* strains that produce DAPG and that are used as biocontrol agents (11) do not exhibit detrimental effects on the AM fungus *G. mosseae*. This general conclusion is based on the following two facts: (i) no bacterial treatment adversely affected mycorrhizal colonization (Table 3), and (ii) even with the DAPG overproducer F113(pCU203) the mycelial development (as determined by germination tests) was not significantly less than the mycelial development when no bacteria were introduced (Table 1).

In spite of the fact that the rhizosphere is nutritionally enriched compared to bulk soil, which may affect synthesis of DAPG (28), the data from the germination test performed in relatively nutrient-poor bulk soil and the data for the development of AM associations in the rhizosphere revealed similar tendencies, with the wild-type strains F113 and F113G22 stimulating both mycelial development and colonization of roots and the DAPG overproducer strain F113(pCU203) producing effects similar to those of the control. It is likely that F113 (pCU203) produced the antifungal compound during the experiment but at doses which do not have an adverse effect compared to the control but are rather inhibitory compared to the other two strains. The concentration of the antifungal compound DAPG required to produce negative effects on the germination and development of AM fungal spores (10 μM) might be present in the rhizosphere of *Pseudomonas*-inoculated plants (8).

Water agar is a nutrient-poor medium, and this may have affected DAPG synthesis by the strains in this study. The data in Table 1, which show that F113(pCU203), a DAPG overproducer, inhibited mycelial development compared to the other two strains, suggest that DAPG production could have occurred. However, we do not support using antagonism on water agar as a method for looking at DAPG production.

Previously, other authors (20, 23) have described the resistance of various crop plants and microorganisms to DAPG. The results indicate that the levels of DAPG required to inhibit fungal pathogens in vitro may also result in phytotoxic effects (31). A comparison of the levels of DAPG which result in 50% inhibition of fungal pathogens (23) with the levels that result in significant inhibition of *G. mosseae* suggested that AM fungi are at least as sensitive as pathogenic fungi. Therefore, it appears that intrinsically greater resistance to DAPG may not account for the observed lack of a negative effect of biocontrol strains on *G. mosseae* (in soil).

Another interesting finding is that F113 and F113G22 are able to improve the formation of AM associations, as previously shown for other rhizosphere microorganisms (3). The precise mechanism(s) that accounts for such microbial stimulation, however, has not been clearly identified yet. Most current evidence indicates that many microorganisms develop functions in the rhizosphere which may affect not only the plants but also other microbial members of the soil community (21, 22). This is an effect exhibited by the so-called "mycorrhiza helper bacteria," which have a positive influence on formation of ectomycorrhizal (14, 15) and AM (3) associations. Specialized activities, such as the production of vitamins, amino acids, hormones, etc., may be operating in microbe-microbe interactions involving AM fungi and *Pseudomonas* strains (2) and may account for the stimulatory effects found in this study. On the basis of these observations and due to the importance of AM associations in agroecosystems, the release of microbial inoculants that produce antifungal metabolites deserves a detailed analysis to determine the possible effects on the performance of AM fungi (6).

In summary, it is significant that the biocontrol organism *Pseudomonas* sp. strain F113 did not exhibit antifungal activity against *G. mosseae*, a representative mycorrhizal fungus, and that, in addition, this strain had a significant stimulatory effect on mycelial development from *G. mosseae* spores and on the overall processes involved in the formation of AM associations in soil. This stimulation did not appear to be related to DAPG production, as strain F113G22, which is deficient in DAPG production, also had a such stimulatory effect.

The lack of any inhibitory activity by the biocontrol agent *Pseudomonas* sp. strain F113 against a beneficial fungal symbiont (*G. mosseae*) and the lack of inhibitory activity by other *Pseudomonas* strains (25) have implications concerning the evaluation of biocontrol strains not only with regard to target fungal pathogens but also with regard to the ecological impact of biocontrol strains on beneficial resident soil microbial populations.

This study was supported by the EU Biotechnology Programme (IMPACT projects BIO2-CT93-0053 and BIO4-CT96-0027).

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