Effect of Pathogen Inoculum, Antagonist Density, and Plant Species on Biological Control of Phytophthora and Pythium Damping-off by *Bacillus subtilis* Cot1 in High-Humidity Fogging Glasshouses

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ABSTRACT

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Bacillus subtilis Cot1 prevented Phytophthora and Pythium damping-off of *Astilbe*, *Photinia*, and *Hemerocallis* microplants and conventional *Brassica* seedlings under high-humidity conditions in fogging glasshouses. With *Photinia*, biocontrol activity was similar to that of the commercial fungicide metalaxyl when the antagonist concentration applied to roots was $\geq 3 \times 10^5$ CFU/g root fresh weight (RFW) and fungal inoculum was $\leq 10^2$ oospores per g of peat. *B. subtilis* Cot1 colonized the developing root system of *Photinia* microplants and *Brassica* seedlings growing in peat substrate during the 28-day in vivo acclimatization period in the fogging glasshouse. With inocula of 4×10^6 and 3×10^5 CFU/g RFW, spore numbers remained between 10^5 and 10^6 CFU/g RFW in the older sections of the root system and between 10^4 and 10^5 CFU on root-tip sections. *B. subtilis* Cot1 application slightly reduced damping-off on *Daphne* plants. Poor persistence on *Daphne* roots and suppression of *B. subtilis* Cot1 by spent media of *Daphne* tissue cultures suggests that poor biocontrol activity was due to the release of inhibitory compounds by *Daphne* roots.

Additional keywords: micropropagation, plant tissue culture.

ZUSAMMENFASSUNG

Eine Wurzelapplication mit *Bacillus subtilis* Cot1 verhinderte Phytophthora und Pythium Auflaufkrankheiten in gewebekulturvermehrten *Astilbe*, *Photinia*, und *Hemerocallis* Jungpflanzen und konventionellen *Brassica* Sämlingen unter hohen Luftfeuchtigkeitsbedingungen in Nebeltreibhäusern. Bei *Photinia* war Aktivität des Antagonisten vergleichbar mit der des kommerziellen Fungizides Metalaxyl wenn die Antagonistenkonzentration auf $\geq 3 \times 10^5$ CFU/g Wurzelfrischgewicht (RFW) und die *Pythium ultimum* Konzentration auf $\leq 10^2$ oospores per g von Torf Substrat eingestellt worden war. *B. subtilis* Cot1 kolonisierte das sich im Torfsubstrat entwickelnde Wurzelsystem von *Photinia* während der 28-tägigen Akklimatisierung im Nebeltreibhaus. Nach Inoculation von 4×10^6 und 3×10^5 CFU/g RFW, blieben die Cot1 Sporenpopulationen zwischen 10^5 und 10^6 CFU/g RFW in den älteren Wurzelabschnitten und zwischen 10^4 und 10^5 CFU/g RFW in den Wurzelspitzenabschnitt. Der Wirkungsgrad in *Daphne* war sehr gering. Die Hemmung von *B. subtilis* Cot1 durch Wurzelexudate in *Daphne* Gewebekulturmedien und die geringe Persistenz von Cot1 im gesammten Wurzelbereich von *Daphne* deutete an, daß der geringe Wirkungsgrad des Antagonisten durch Hemmstoffauscheidung der *Daphne* Wurzeln verursacht wurde.

Fogging glasshouses, which provide continuous high humidity, are now widely used for acclimatization of micropropagated plants. Long periods (3 to 6 weeks) of high humidity are required to prevent desiccation of tissue-cultured plants, which develop poor root systems, reduced cuticular wax layers, and distorted stomata during in vitro growth (15). Both the physiological distortions and the environmental conditions increase the susceptibility of plants to a range of fungal pathogens after transfer to compost media (16). Apart from grey mold (*Botrytis cinerea*), which is endemic, introduction of *Pythium* and *Phytophthora* spp. into high-humidity fogging glasshouses frequently has caused severe damping-off losses in commercial micropropagation companies (23). Mixed infections with both *Pythium* and *Phytophthora* have been associated with some of the most severe damping-off outbreaks (C. Leifert, *unpublished data*). These pathogens also can cause problems in nurseries that propagate conventional seedlings (7,8,28,34). Regular (up to three sprays per week) prophylactic fungicide treatments are the conventional method of protecting plants against fungal attack in fogging glasshouses. However, such frequent fungicide use has resulted in resistance development in pathogen populations (23).

Biocontrol methods could provide an alternative to or reduce the use of fungicides in high-humidity glasshouses. Various microbial antagonists, including strains of the bacterial genera *Bacillus*, *Pseudomonas*, and *Enterobacter* and strains of the fungal genera *Gliocladium*, *Pythium*, and *Trichoderma,* have shown biocontrol activity against damping-off diseases (1,5,6,9,25,32,35,36,40). Some of these biocontrol agents have been developed into commercial products (e.g., a *Bacillus subtilis* strain for cotton and peanuts [Quantum 4000] and a *Gliocladium* strain for glasshouse soil mixes [SoilGard]). However, the available products cover only a small range of crops and have a very small share of the crop-protection market (38).

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The main reason for the very limited commercial impact of biocontrol agents of damping-off is their poor or very variable activity compared to fungicides (23,35). Poor activity has been linked to poor rhizosphere colonization in some studies, but the underlying reasons for poor colonization by antagonists are poorly understood (2,6,13,14,26). Competition by microorganisms colonizing the seedling root in soil was most frequently described as the reason for poor colonization (2,13,14), but exuded plant metabolites, which selectively encourage or prevent root colonization by specific groups of microorganisms (e.g., nutrient, vitamins, and antimicrobial compounds), also may be involved (10). The latter view may be supported by the finding that the activity of certain biocontrol agents is restricted to specific plant species (23,38).

In contrast to fungicides, which often show activity over a wide range of pathogen concentrations in soil, biocontrol agents often lack activity at higher soil pathogen concentrations (37,39). Clear interactions between antagonist and fungal pathogen inocula have been demonstrated in investigations of the biological control of postharvest diseases (19), but similar dose/response trials, to our knowledge, rarely have been reported for damping-off diseases.

In this investigation, we studied (i) the effect of plant species, pathogen inoculum density, and antagonist concentrations on the biocontrol activity of *B. subtilis* Cot1 against damping-off caused by *Pythium* and *Phytophthora* spp.; (ii) the ability of *B. subtilis* Cot1 to persist and colonize the developing root system in peat-based horticultural substrates; and (iii) the effect of root exudates released by *Daphne* microplants in vitro on *B. subtilis* Cot1 to determine whether antibacterial compounds exuded by *Daphne* roots are the reason for poor rhizosphere colonization and biocontrol activity by *B. subtilis* Cot1 on *Daphne*.

MATERIALS AND METHODS

Plant material. *Aster hybrida* L. 'Pearl Star,' *Hemerocallis* 'Stella d'ora,' *Daphne blagayana* Freyer, and *Photinia fraseri* Lindl. were micropropagated and rooted as described previously (4,17,20). Plants were transferred individually to 3 × 3-cm plugs of peat substrates infested with *Pythium* and *Phytophthora* in polystyrene trays (described below). Dutch white cabbage seeds (*Brassica oleracea* var. *capitata* L.) were surface-disinfected (5 min in 10% [vol/vol] commercial bleach [Domestos 10 to 15% (vol/vol) sodium hypochlorite]), germinated, and initially grown in pathogen-free Shamrock multipurpose peat compost (Shamrock, Newbridge, Ireland) in a growth room. When the developing shoots had reached a height of approximately 5 cm, seedlings were transplanted individually to plugs of pathogen-infested substrate in polystyrene trays. Five replicate trays with 25 plants per tray were used per treatment in all experiments. Trays were arranged in randomized complete block designs in the glasshouse or growth room.

Growth substrate and conditions for plants. Biocontrol activity was determined in fertilized Finnish Vapo C2 peat (Croxden Horticulture, Stoke on Trent, UK). Substrates were adjusted to pH 6 (±0.2) with $Ca(OH)_2$ and transferred to 3.5 × 3.5-cm plugs in polystyrene trays and watered to approximately field capacity.

In experiments to determine the effects of levels of pathogen inoculum concentration, antagonist density, and plant species on biocontrol activity, plants were grown for 4 weeks in a heated fogging glasshouse kept at an average daily temperature of 16°C (±4°C) and greater than 93% relative humidity (RH). The percentage of plants killed by damping-off fungi (plants showing completely necrotic shoots and decayed roots) was determined 4 weeks after planting and was used as a measure of biocontrol activity. RH was monitored with a Vaisala 100 humidity probe (Vaisala OY, PL26, Helsinki, Finland).

In experiments to determine the effect of plant species and antagonist inoculum level on antagonist population development in the rhizosphere, plants were planted in pathogen-free peat. Plants were grown for 4 weeks in growth rooms with constant temperatures of 16°C and 16 h of light (220 μE m^{-2} s^{-1} of cool-white fluorescent light). To mimic the high humidity found in the fogging glasshouses, miniature greenhouses were created by attaching metal frames to the polystyrene trays and sealing each tray individually in polyethylene bags. Plants were sprayed four times a day with a mist of sterile distilled water. RH was monitored and remained above 93% throughout the experiments.

Fungal inoculum. A *Pythium ultimum* Trow isolate that was pathogenic to all host plant species used and host-specific strains of *Phytophthora* spp. were isolated from infected bait plants growing in peat in commercial nurseries. The fungal strains used in trials were selected on the basis of their sensitivity to metalaxyl and ease of oospore production. The *Phytophthora* isolate used with cabbage was *Pytophthora megasperma* Drechs., and the strain used for *Aster*, *Daphne*, and *Photinia* was *Phytophthora cactorum* (Lebert & Cohn) J. Schröt. The *Phytophthora* strain used in trials with *Hemerocallis* was not identified to the species level. Oospores were used as inocula for both fungi and were incorporated with the water added to adjust substrate moisture levels prior to placing substrates in polystyrene trays. *Pythium* and *Phytophthora* oospore inocula were produced by previously described methods (12,24).

Biological control and chemical treatments. The antagonist *B. subtilis* Cot1 initially was isolated from contaminated *Cotinus*-tissue cultures (22). When tested in an initial screening program, *B. subtilis* Cot1 was the most active biocontrol agent of 93 isolates tested against oomycete fungi, but it lacked activity against *Botrytis cinerea*, the major foliar disease in fogging glasshouses (F. Berger, Hong Li, D. White, R. Frazer, and C. Leifert, *unpublished data*). *B. subtilis* Cot1 could be selectively isolated and identified with relative ease because it produces a black pigment when grown on Murashige and Skoog's (MS) medium (29) that prevents the growth of a wide range of other bacteria (16,20).

The effect of antagonist inoculum level on biocontrol activity was determined by dipping microplants and seedlings into suspensions containing 10^5, 10^7, 10^8, or 10^9 (±0.2 log units) CFU of *B. subtilis* Cot1, which resulted in 10^2, 3×10^4, 3×10^5, and 4×10^6 (±0.3 log units) CFU/g root fresh weight (RFW; as determined by serial dilution and plating of root washings immediately after dipping in antagonist suspensions). All bacterial inocula were prepared by transferring a loop of cells from a 24-h-old nutrient agar (NA) culture to a 1-liter Erlenmeyer flask containing 200 ml of MS medium (30) with 3% sucrose and incubating on a rotary shaker for 30 h at 20°C. Serial dilutions were made with MS medium with 3% sucrose. Microscopic examination (phase contrast) and plating of inoculum suspensions after heat treatment (80°C for 10 min) showed that more than 95% of CFU in the inoculum were vegetative cells. Control plants were either not treated or were sprayed with a suspension (100 ml/liter [vol/vol]) of the fungicide metalaxyl (Ridomil 240EC; 24% [wt/wt]) at 4-day intervals to prevent Pythium and Phytophthora damping-off. All treatments also were sprayed at 4-day intervals with a suspension (255 g/liter [wt/vol]) of iprodione (Rovral WP; 50% [wt/wt]) to prevent attack by *Botrytis cinerea*. All *Pythium* and *Phytophthora* strains used were resistant to iprodione.

Enumeration of rhizosphere populations of *B. subtilis* Cot1. Roots were removed from the growth substrates after 4 weeks in the growth room. They were then shaken and gently rinsed with sterile distilled water to remove excess soil. Sections (1 cm) were collected from (i) root tissues adjacent to the shoot (section 1), (ii) the approximate center of the root system (section 2), and (iii) the root tip (section 3). Root sections from all 25 plants in a replicate tray were pooled, and a subsample of 2 g of roots from each location was homogenized in a blender for 10 min in 10 ml of 0.25% strength Ringer's solution. The resulting suspension was split into two equal 5-ml samples. One sample was serially diluted and immediately plated on NA (Oxoid) + 60 mg of cyclo-

heximide (NA_c) per liter; the other sample was heated for 10 min at 85°C, serially diluted, and plated on NA_c.

To estimate the amount of *B. subtilis* Cot1 present on roots, we utilized the ability of strain Cot1 to grow and produce a black pigment on KB (11) and MS medium containing high concentrations of sucrose (16,20). MS medium allows selective identification of strain Cot1 because very few bacteria grow on MS medium in the absence of plants, and *B. subtilis* Cot1 was the only *Bacillus* strain that, in our experience, produces a black pigment on MS (16,20). One hundred well-separated colonies from NA_c plates inoculated with heated or nonheated root washings were subcultured with an inoculation needle into wells of immunoassay plates containing 25 µl of liquid MS medium with 30 g of sucrose per liter. Bacterial growth together with the production of a black pigment in the well was used to identify bacterial isolates as *B. subtilis* Cot1. The proportion of CFU originating from vegetative *B. subtilis* cells was estimated by subtracting the number of black pigment-producing CFU found in heated (80°C for 10 min) root homogenates from those found in unheated homogenates.

Testing for antibacterial activity in spent plant tissue-culture medium. To determine whether antimicrobial compounds exuded by *Daphne* roots may be responsible for the poor colonization and biocontrol activity observed in the glasshouse/growth-room trials, we grew aseptically rooted shoot cultures of *Astilbe*, *Hemerocallis*, *Photinia*, and *Daphne* on liquid plant media appropriate for the plant species, as described previously (4,17,20). After 4 weeks of growth, plants were removed from the medium. The spent plant growth medium was either adjusted to pH 6 with NaOH to avoid inhibition of bacterial growth by low pH (some plant species reduce the pH in vitro to below 4.5 and, thereby, prevent growth of *Bacillus* [17,20,21]), filter-sterilized (0.22-µm Millipore filter, Molsheim, France) and used, or rotary evaporated to 50% of its original volume, mixed 1:1 (vol/vol) with MS medium containing 30 g of sucrose, adjusted to pH 6, and filter-sterilized and used. Fresh MS medium was added to test whether *Bacillus* growth was prevented by nutrient deficiency in spent plant medium, because certain plants rapidly deplete tissue-culture media of certain mineral nutrients and carbohydrates (30). Ten milliliters of medium was inoculated with 10^5 CFU of a 24-h-old NA culture of *B. subtilis* Cot1 (>95% vegetative cells) and incubated at 20°C in the dark for 72 h. Bacterial growth in the medium was measured by absorbance at 625 nm with a Grating spectrophotometer (Cecil Instruments, Cambridge).

Statistical analysis. Percent data were arcsine-square root–transformed and microbial density data log-transformed prior to analysis of variance (ANOVA) by Minitab software (Minitab, University of Maryland, College Park) and calculation of Tukey's honestly significant difference test (27). Data from repeated experiments (experiments described in Table 1 were repeated twice for each plant species, and those described in Figures 1 and 2 were repeated five times) were pooled for statistical analysis.

RESULTS

Effect of pathogen and antagonist inoculum on biocontrol activity on *Photinia*. ANOVA showed a very highly significant interaction between oospore density in the growth substrate and antifungal treatment. Treatment with the fungicide metalaxyl resulted in similar and generally very low damping-off losses regardless of fungal inoculum density (Table 2). Disease control by *B. subtilis* Cot1, on the other hand, decreased with increasing pathogen density. Application of Cot1 doses of 4×10^6 and 3×10^5 CFU/g RFW provided control similar to metalaxyl when *Photinia* plants were planted in peat containing a fungal inoculum concentration of $\leq 10^2$ oospores per g of substrate (Table 2). However, at higher fungal inoculum concentrations, plant losses with the two higher *B. subtilis* concentrations were significantly higher than those recorded for fungicide-treated plants but lower compared to untreated control plants (Table 2). When bacterial inocula below 3×10^5 CFU/g RFW were used, plant losses were always higher than in fungicide-treated plants, and at higher pathogen inocula, results were similar to those found in untreated control plants growing in infested peat (Table 2). When untreated plants were planted in noninfested peat, they became infected in the glasshouse and showed losses similar to untreated plants planted in pathogen-infested soil (data not shown).

Biocontrol activity on different host plants. ANOVA generally showed a highly significant difference in damping-off among treatments (biological control, fungicide, and untreated). Damping-off losses on control plants were generally high (>85%) and not significantly different between the three types of pathogen inocula (*Pythium*, *Phytophthora*, and mixed inoculum of the two) in *Aster*, *Daphne*, *Photinia*, and *Brassica* plants. With *Hemerocallis*, damping-off in control plants was significantly lower with *Phytophthora* compared to *Pythium* and mixed inoculum (Table 1). Also with *Hemerocallis*, ANOVA indicated a highly significant interaction between pathogen inoculum and treatment. No significant difference could be found in the activity of the fungicide treatment against the different fungal inocula, and damping-off losses in fungicide-treated plants were generally low (<10%) for all plant species (Table 1).

Treatment with *B. subtilis* Cot1 at a concentration of 4×10^6 CFU/g RFW (10^9 CFU/ml of inoculum suspension) provided protection similar to that obtained by the fungicide for all plant species, except *Daphne* (in which damping-off was higher with all three fungal inocula) and *Brassica* (in which damping-off was higher for the mixed inoculum) (Table 1). Biocontrol activity with *Daphne* was very poor, and with the mixed fungal inoculum, damping-off losses were significantly higher than with *Pythium* alone and not significantly different to damping-off in untreated control plants (Table 1).

Population dynamics of *B. subtilis* Cot1 in the rhizosphere. ANOVA showed significant differences between plant species (*Photinia*, *Brassica*, and *Daphne*) in spore densities 7 and 28 days after

TABLE 1. Percent damping-off in *Aster*, *Daphne*, *Photinia*, and *Hemerocallis* microplants and conventional seedlings of Dutch white cabbage (*Brassica*) after treatment with *Bacillus subtilis* Cot1 or metalaxyl and growth in peat-based compost infested with *Pythium*, *Phytophthora* (10^2 oospores per g of peat), or a mixed inoculum of the two fungi (2×10^2 oospores per g of peat)[x]

	Pythium			*Phytophthora*			Mixed inoculum			Analysis of variance[y] (*P*)		
Plant	Fungic.	Cot1	Untreat.	Fungic.	Cot1	Untreat.	Fungic.	Cot1	Untreat.	Path.	Treat.	Interaction[z]
Aster	6 a	2 a	96 b	2 a	4 a	100 b	7 a	11 a	95 b	0.505	<0.001	0.473
Daphne	4 a	62 b	86 cd	5 a	74 bc	94 d	6 a	89 cd	95 d	0.042	<0.001	0.399
Photinia	4 a	7 a	99 b	6 a	3 a	96 b	3 a	7 a	100 b	0.609	<0.001	0.736
Hemerocallis	7 a	1 a	99 b	9 a	3 a	72 c	8 a	4 a	95 b	0.123	<0.001	0.004
Brassica	1 a	9 a	91 c	7 a	4 a	100 c	5 a	17 b	100 c	0.025	<0.001	0.118

[x] Disease control activity of *B. subtilis* Cot1 and metalaxyl was determined in separate experiments for each plant species. For treatments of the same plant species, different letters indicate significant differences ($P < 0.05$) according to Tukey's honestly significant difference test. No comparison was made between different plant species. Fungic. = fungicide; Untreat. = untreated; Path. = pathogen; Treat. = treatment.

[y] Analysis of variance was carried out on arcsine-transformed data.

[z] Pathogen-treatment.

inoculation and between different sections of the root system but no significant difference between assessment dates (7 and 28 days after inoculation) (Figs. 1 and 2). The highest Cot1 spore densities (10^5 to 2×10^6 CFU/g RFW) were found on *Photinia* microplants. Spore densities on conventional *Brassica* seedlings were similar to those found on *Photinia* for the middle section of the root but significantly (1 log unit) lower in the upper and root-tip sections. Compared to both *Photinia* and *Brassica*, spore densities on *Daphne* roots were significantly lower ($<3 \times 10^3$) throughout the root system (Fig. 1).

After inoculation of *Photinia* with three inoculum levels of Cot1 (3×10^4, 3×10^5, and 4×10^5 CFU/g RFW), significant differences in spore density were found between initial inoculum densities and root section but not between the two assessment dates. Spore densities of Cot1 generally decreased with decreasing initial Cot1 inoculum and toward the tip of the root system.

Due to variability in experiments in which enumeration was based on serial dilution and plate counts and due to the requirement of subculturing colonies from isolation plates in liquid MS medium, the total number (spores plus vegetative cells) of *B. subtilis* Cot1 could be determined only when the total number of *B. subtilis* CFU was not more than 1.5 log units lower than the total number of CFU. Therefore, the total number of *B. subtilis* CFU was determined only for *Photinia*, in which we calculated (total viable CFU of Cot1 – Cot1 spores) that 40% (±30%) of the CFU of *B. subtilis* Cot1 were present as spores (60% vegetative cells).

Inhibition of *B. subtilis* Cot1 by *Daphne* root exudates. Spent *Daphne* tissue-culture medium inhibited the growth of *B. subtilis* Cot1 (there was no increase in absorbance with or without addition of MS nutrients). The spent plant tissue-culture medium of *Aster*, *Hemerocallis*, and *Photinia* supported growth of *B. subtilis* Cot1 (absorbencies were generally >2.0 due to the production of black pigment associated with growth of *B. subtilis*) with or without additional MS nutrients.

DISCUSSION

B. subtilis Cot1 provided disease control similar to commercial fungicides with moderate fungal inocula (≤10^2 oospores per g of peat) but failed to match the metalaxyl treatment at higher fungal inoculum levels. Similar results have been described for several other antagonist-pathogen-plant interactions, including *Coniothyrium minitans/Sclerotinia sclerotiorum* on lettuce in glasshouses (39) and *Pseudomonas fluorescens/Botrytis cinerea* on cold-stored cabbage (18,19). *Pythium* density in a range of compost products based on Finnish and Irish peat were always below 5×10^1 propagules per g of peat (C. Leifert, *unpublished data*), and inocula above 10^2 propagules per g of peat, to our knowledge, have not been published for commercial peat substrates. In the absence of additional inoculum sources within the glasshouse (irrigation water and infected plants), the antagonist, therefore, would be expected to provide good protection in commercial practice.

High inocula levels of *B. subtilis* Cot1 (4×10^6 CFU/g RFW) provided good control on a wide range of plant species (*Aster*, *Brassica*, *Hemerocallis*, and *Photinia*) but very poor control on *Daphne*. A comparison of antagonist populations on *Photinia*, *Brassica*, and *Daphne* roots 7 and 21 days after application of antagonists showed considerably lower Cot1 population densities on *Daphne* roots. This supports earlier reports that described a

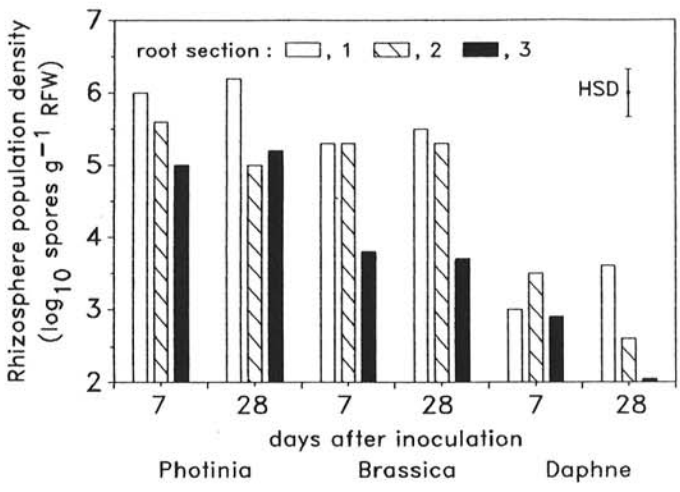

Fig. 1. Populations of *Bacillus subtilis* Cot1 spores in the rhizosphere of micropropagated *Daphne* or *Photinia* plants or conventionally raised Dutch white cabbage (*Brassica*) seedlings 7 and 28 days after inoculation with 4×10^6 CFU/g root fresh weight (RFW) of strain Cot1. Plants were assessed 7 and 28 days after inoculation with strain Cot1, and means were from five replicates. Spore counts were log-transformed prior to analysis of variance and calculation of Tukey's honestly significant difference test (HSD). There were significant differences between plant species ($P < 0.001$) and root sections ($P < 0.001$) but not between the assessment dates ($P = 0.073$). Significant interactions were found between plant species and root section ($P < 0.001$), plant species and assessment date ($P = 0.034$), assessment date and root section ($P < 0.001$), and plant species, antifungal treatment, and root section ($P = 0.003$).

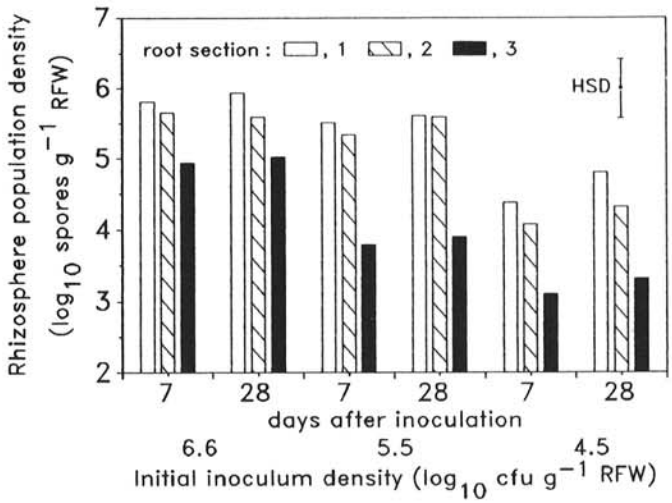

Fig. 2. Effect of antagonist inoculum density on the population of *Bacillus subtilis* Cot1 in the rhizosphere of micropropagated plants grown in a pathogen-free peat substrate. RFW = root fresh weight. Means were from five replicates. Spore counts were log-transformed prior to analysis of variance and calculation of Tukey's honestly significant difference test (HSD). There were significant differences between inoculum density ($P < 0.001$) and root section ($P < 0.001$) but not between assessment dates. Significant interactions were found between inoculum density and root section ($P = 0.048$) but not between inoculum density and time ($P = 0.625$), root section and time ($P = 0.951$), or inoculum density, root section, and time ($P = 0.976$).

TABLE 2. The effect of inoculum densities of *Bacillus subtilis* Cot1 and *Pythium ultimum* on damping-off of *Photinia*

Antifungal treatment	Dose	*Pythium* inoculum (oospores/g of peat)[z]			
		10^1	10^2	10^3	10^4
Metalaxyl		6 aA	2 aA	6 aA	3 aA
B. subtilis	10^9	6 aA	7 aA	14 bB	61 cB
	10^8	2 aA	6 aA	54 bC	69 cB
	10^7	30 aB	74 bB	97 cD	95 cC
	10^5	95 aC	94 aC	96 aD	98 aC
Untreated control		94 aC	99 aC	96 aD	98 aC

[z] Different letters indicate significant differences ($P < 0.01$) according to Tukey's honestly significant difference test after arcsine transformation. Lowercase letters indicate differences between fungal inocula in the same antifungal treatment. Uppercase letters indicate differences between antifungal treatments at the same fungal inoculum. Analysis of variance of arcsine-transformed data showed significant differences between fungal inocula ($P < 0.001$), antifungal treatments ($P < 0.001$), and interaction between fungal and bacterial inocula ($P < 0.001$).

strong relationship between rhizosphere colonization by beneficial rhizobacteria and plant health (13,16,17). However, because there was no rhizosphere flora on *Daphne* and *Photinia* prior to antagonist application (aseptic tissue-cultured plants were used) and competition pressure by bacteria colonizing from the peat substrate can be assumed to be the same for both plant species, it is likely that poor colonization on *Daphne* was due to differences in plant rhizosphere conditions rather than competition by the microflora in the peat substrate. This view is further supported by the finding that *B. subtilis* Cot1 failed to grow on tissue-culture media on which *Daphne* had been grown; this indicates that *Daphne* produces an antimicrobial compound in the rhizosphere that prevents colonization and biocontrol activity of *Bacillus*. However, root exudate composition in in vitro cultures is quite different (10, 16) from that of field-grown plants, and production of antimicrobial compound(s) in peat will have to be demonstrated before such a mechanism can be established.

It is interesting that *Hemerocallis* plants prevented the growth of *B. subtilis* Cot1 in plant tissue culture by reducing the agar medium pH to below 4 (16,20) but did not suppress the growth of the bacterium in the rhizosphere when *Hemerocallis* was grown in peat substrates. The acidity produced by *Hemerocallis* plants is thought to be a result of rapid ammonium uptake, which is associated with medium acidification in many plants (17). Acidification was not observed in Vapo C2 peat, probably because the peat contained no NH_4^+ fertilizer and had a higher buffering capacity than tissue-culture media.

Good control (not significantly different from the commercial fungicide metalaxyl) was obtained only under environmental conditions in which the biocontrol agent was able to persist in densities of between 10^5 and 10^6 spores per g of root tissue. *B. subtilis* Cot1 produces a sticky extracellular slime (the organism is extremely difficult to remove from agar plates) after more than 48 h of growth, which may aid in adsorption on roots and may result in the development of biofilms of the organism on the root surface. This also may explain the higher proportion of *Bacillus* cells that remained on the root with more highly concentrated inoculum suspensions. It should be pointed out that the tissue-cultured plants used in the experiments had no detectable microbial populations at planting. Colonization by strain Cot1, therefore, was initially free of competition from resident rhizosphere microflora. However, large rhizosphere populations of Cot1 and good biocontrol activity also were obtained with conventional cabbage seedlings that developed microbial rhizosphere flora prior to inoculation with strain Cot1 (C. Leifert, *unpublished data*). This indicates that Cot1 also can colonize the rhizosphere when competing with already established populations of rhizobacteria. However, the peat compost used had much lower numbers of microorganisms than most agricultural soils (S. Chidburee and C. Leifert, *unpublished data*), and future work needs to test the competitiveness of Cot1 against microbial populations found in soil substrates.

The primary mode of action of *B. subtilis* Cot1 is thought to be production of antibiotics, most of which are formed only at the onset of sporulation (6,23,33; C. Leifert, *unpublished data*). The ability to form spores and the number of sporulating cells in the rhizosphere, therefore, is likely to be a major factor affecting biocontrol activity. A ratio of 3:2 vegetative cells/spores of *B. subtilis* Cot1 was found on *Photinia* at both 7 and 28 days after inoculation. This may indicate that the antagonist grows in the rhizosphere and that both sporulation and germination take place. One limitation of our investigation was the inability to monitor the number of vegetative, metabolically active *B. subtilis* Cot1 cells on *Daphne*, on which biocontrol activity remained lower than in fungicide controls. Therefore, we do not know whether growth, sporulation, or both were inhibited. Such knowledge could be important when devising methods for improving biocontrol activity. For example, if growth was inhibited but not sporulation, repeated applications of the antagonist (similar to a fungicide treatment) might allow continuous protection. However, if sporulation and, thereby, antibiotic production were inhibited, promotion of sporulation (e.g., by addition of manganese to the inoculum suspension) might increase biocontrol activity (3,23).

It is now possible to introduce *lux* marker genes into rhizosphere-colonizing *B. subtilis* Cot1 (31). A *lux*-marked *B. subtilis* Cot1 strain would allow the study of vegetative, metabolically active populations of Cot1 in situ.

LITERATURE CITED

1. Adams, P. B. 1990. The potential of mycoparasites for biological control of plant diseases. Annu. Rev. Phytopathol. 28:59-72.
2. Bull, C. T., Weller, D. M., and Thomashow, L. S. 1991. Relationship between root colonization and suppression of *Gaeumannomyces graminis* var. *tritici* by *Pseudomonas fluorescens* strain 2-79. Phytopathology 81: 954-959.
3. Claus, D., and Berkeley, R. C. W. 1986. Genus *Bacillus*. Pages 1105-1139 in: Bergey's Manual of Systematic Bacteriology, vol. 2. P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt, eds. Williams and Wilkins, Baltimore.
4. Cooke, D. L., Waites, W. M., and Leifert, C. 1992. Effects of *Agrobacterium tumefaciens*, *Erwinia carotovora*, *Pseudomonas syringae* and *Xanthomonas campestris* on plant tissue cultures of *Aster*, *Cheiranthus*, *Delphinium*, *Iris* and *Rosa*; Disease development *in vivo* as a result of latent infection *in vitro*. J. Plant Dis. Prot. 99:469-481.
5. Hadar, Y., Harman, G. E., Taylor, A. G., and Norton, J. M. 1983. Effects of pregermination of pea and cucumber seeds and of seed treatment with *Enterobacter cloacae* on rots caused by *Pythium* spp. Phytopathology 73: 1322:1325.
6. Handelsman, J., Raffel, S., Mester, E. H., Wunderlich, L., and Grau, C. R. 1990. Biological control of damping-off of alfalfa seedlings with *Bacillus cereus* UW85. Appl. Environ. Microbiol. 56:713-718.
7. Hausbeck, M. K., Stephens, C. T., and Heins, R. D. 1989. Damping-off, root rot, and lower root rot of seed-propagated geraniums caused by *Pythium ultimum*. Plant Dis. 73:625-627.
8. Hendrix, F. F., Jr., and Campbell, W. A. 1983. *Pythium* as plant pathogen. Annu. Rev. Phytopathol. 11:77-98.
9. Howell, C. R. 1991. Biological control of Pythium damping-off of cotton with seed-coating preparations of *Gliocladium virens*. Phytopathology 81: 738-741.
10. Killham, K. 1994. Soil Ecology. Cambridge University Press, Cambridge.
11. King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. 44: 301-307.
12. Kittle, D. R., and Gray, L. E. 1979. Storage and use of *Phytophthora megasperma* var. *sojae* oospores as inoculum. Phytopathology 69:821-823.
13. Kloepper, J. W., and Schroth, M. N. 1981. Relationship of in vitro antibiosis of plant growth-promoting rhizobacteria to plant growth and the displacement of root microflora. Phytopathology 71:1020-1024.
14. Kloepper, J. W., Schroth, M. N., and Miller, T. D. 1980. Effects of rhizosphere colonization by plant growth-promoting rhizobacteria on potato plant development and yield. Phytopathology 70:1078-1082.
15. Leifert, C., Clark, E., and Rothery, C. 1993. Micropropagation: The propagation of plants by tissue culture. Biol. Sci. Rev. 5:31-35.
16. Leifert, C., Morris, C., and Waites, W. M. 1994. Ecology of microbial saprophytes and pathogens in field grown and tissue cultured plants. CRC Crit. Rev. Plant Sci. 13:139-183.
17. Leifert, C., Pryce, S., Lumsden, P. J., and Waites, W. M. 1992. Effect of medium acidity on growth and rooting of different plant species growing *in vitro*. Plant Cell Tis. Org. Cult. 30:171-179.
18. Leifert, C., Sigee, D. C., Epton, H. A. S., Stanley, R., and Knight, C. 1992. Isolation of bacteria antagonistic to post-harvest fungal diseases of cold-stored *Brassica* spp. Phytoparasitica (Suppl.) 20:143-149.
19. Leifert, C., Sigee, D. C., Stanley, R., Knight, C., and Epton, H. A. S. 1993. Biocontrol of *Botrytis cinerea* and *Alternaria brassicicola* on Dutch white cabbage by bacterial antagonists at cold store temperatures. Plant Pathol. 42:270-279.
20. Leifert, C., and Waites, W. M. 1992. Bacterial growth in plant tissue culture media. J. Appl. Bacteriol. 72:460-466.
21. Leifert, C., Waites, B., Keetley, J. W., Wright, S. M., Nicholas, J. R., and Waites, W. M. 1993. Effect of medium acidification on filamentous fungi, yeasts and bacterial contaminants in *Delphinium* tissue cultures. Plant Cell Tis. Org. Cult. 31:1-7.
22. Leifert, C., Waites, W. M., and Nicholas, J. R. 1989. Bacterial contaminants of micropropagated plant cultures. J. Appl. Bacteriol. 67:353-361.
23. Leifert, C., Workman, S., Li, H., Chidburee, S., Hampson, Sigee, D. C., Epton, H. A. S., and Harbour, A. 1995. Antibiotic production and biocontrol

activity by *Bacillus subtilis* CL27 and *Bacillus pumilus* CL45. J. Appl. Bacterial. 78:97-108.
24. Lifshitz, R., and Hancock, J. G. 1984. Environmental influences on the passive survival of *Pythium ultimum* in soil. Phytopathology 74:128-132.
25. Lifshitz, R., Windham, M. T., and Baker, R. 1986. Mechanism of biological control of preemergence damping-off of pea by seed treatment with *Trichoderma* spp. Phytopathology 76:720-725.
26. Liu, Z. L., and Sinclair, J. B. 1992. Population dynamics of *Bacillus megaterium* strain B153-2-2 in the rhizosphere of soybean. Phytopathology 82:1297-1301.
27. Milliken, G. A., and Johnson, D. E. 1984. Analysis of Messy Data. Vol. 1, Designed Experiments. Wadsworth, Balmont, CA.
28. Mitchell, D. J., and Kannwischer-Mitchell, M. E. 1991. *Phytophthora*. Pages 31-39 in: Methods for Research on Soilborne Phytopathogenic Fungi. L. L. Singleton, J. D. Mihail, and C. M. Rush, eds. The American Phytopathological Society, St. Paul, MN.
29. Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.
30. Pryce, S., Lumsden, P. J., Berger, F., and Leifert, C. 1993. Effect of plant density and macronutrient nutrition on *Delphinium* shoot cultures. J. Hortic. Sci. 68:807-813.
31. Rattray, E. A. S., Prosser, J. I., Glover, L. A., and Killham, K. 1992. Construction of bioluminescent inocula to assess the impact of genetically modified biological agents in the rhizosphere. J. Sci. Food Agric. 60: 391-396.
32. Sreenivasaprasai, S., and Manibhushanrao, K. 1990. Biocontrol potential of fungal antagonists *Gliocladium virens* and *Trichoderma longibrachiatum*. J. Plant Dis. Prot. 97:570-579.
33. Sterlini, J. M., and Mandelstam, J. 1969. Commitment to sporulation in *Bacillus subtilis* and its relationship to development of actinomycin. Biochem. J. 113:29-37.
34. Stevens, C. T., Powell, C. C., and Schmitthenner, A. F. 1981. A method for evaluating postemergence damping-off pathogens of bedding plants. Phytopathology 71:1225-1228.
35. Taylor, A. G., and Harman, G. E. 1990. Concepts and technologies of selected seed treatments. Annu. Rev. Phytopathol. 28:321-339.
36. Weller, M. 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Annu. Rev. Phytopathol. 26:379-407.
37. Whipps, J. M. 1992. Aspects of selection and use of fungal antagonists for biological control of *Sclerotinia sclerotiorum* in glasshouse celery and lettuce. IOBC/WPRS Bull. 16:60-63
38. Whipps, J. M. 1994. Advances in biological control in protected crops. Brighton Crop Prot. Conf. Pests Dis. 9B:1259-1264.
39. Whipps, J. M., Budge, S. P., and McQuilken, M. P. 1992. Use of *Coniothryium minutans* and *Pythium oligandrum* as disease biocontrol agents. Phytoparasitica (Suppl.) 20:107-112.
40. Wolfhechel, H., and Jensen, D. F. 1992. Use of *Trichoderma harzianum* and *Gliocladium virens* for the biological control of post-emergence damping-off and root rot of cucumber caused by *Pythium ultimum*. J. Phytopathol. 136:221-230.