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Amendment with Peony Root Bark Improves the Biocontrol Efficacy of *Trichoderma harzianum* against *Rhizoctonia solani*

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We tested *Trichoderma harzianum* as a biocontrol agent for *Rhizoctonia solani* AG2-1, using six natural antifungal materials to improve its efficacy. Among the six materials tested, peony (*Paeonia suffruticosa*) root bark (PRB) showed the strongest antifungal activity against *R. solani* AG2-1, and was not antagonistic to *T. harzianum*. Scanning electron microscopy showed that treatment with PRB extract resulted in shortened and deformed *R. solani* AG2-1 hyphal cells. The control of radish damping-off caused by *R. solani* AG2-1 was greatly increased by combined treatments of *T. harzianum* and PRB, as compared with either of the two treatments alone, with the control effect increased from 42.3–51.5% to 71.4–87.6%. The antifungal compound in PRB, which was isolated in chloroform and identified as paeonol by mass spectrometry, ^{1}H NMR, and ^{13}C NMR analyses, inhibited the growth of *R. solani* AG2-1 but not that of *T. harzianum*. Thus, PRB powder or extract may be used as a safe additive to *T. harzianum* to improve the control of the soil borne diseases caused by *R. solani* AG2-1.

Keywords: Biocontrol, damping-off, peony, *Rhizoctonia solani*, *Trichoderma harzianum*

Rhizoctonia solani Kühn is an important cosmopolitan necrotrophic soilborne fungus. Diseases caused by *R. solani* result in yield losses in more than 200 crops globally [43]. In Korea, limited arable land forces farmers to employ high-input-intensive cultural practices and continuous monocropping, which results in the accumulation of soilborne pathogens. Perennial and vegetable crop losses caused by *R. solani*, which is responsible for damping-off and seedling blight, are high.

The management of *Rhizoctonia* damping-off relies mainly on the use of chemical pesticides, leading to increased cultivation costs and adverse impacts on human and animal health. Moreover, pathogens may develop resistance against chemical pesticides, increasing the challenge of plant protection [42]. Biocontrol appears to offer an environmentally safe and economically feasible option for plant protection and has great potential for promoting sustainable agriculture. Biocontrol of phytopathogens involves the use of biological processes to reduce pathogen populations to levels below disease thresholds, thus reducing crop losses while interfering minimally with ecosystems.

Although several microorganisms have been identified to suppress plant pathogens, only a few have been commercialized [5, 10]. *Trichoderma* strains have been used on a variety of crops under greenhouse [25] and field [9, 36] conditions to manage *R. solani*. The efficacy of individual biocontrol agents [3, 13, 17, 20, 24, 38], as well as mixtures of antagonists [6, 8, 14, 23, 30, 32, 35, 37, 39], has been demonstrated in several crops.

The efficacy of soil amendments, including plant extracts, essential oils, and organic materials, against plant pathogens has been investigated [4, 33, 34]. Combinations of antagonist and synthetic chemicals or natural materials often provide better plant protection than individual treatments [2, 7, 16]. However, even mixtures of chemicals may prove ineffective, owing to resistant fungal populations [15]. Thus, the development of nonchemical mixtures is an important strategy for enhancing disease control efficacy. We screened antifungal plant materials against *R. solani* AG2-1 *in vitro* and evaluated the efficacy of individual and combined treatments of peony (*Paeonia suffruticosa*) root bark and *Trichoderma harzianum* on controlling radish damping-off caused by *R. solani* AG2-1 under greenhouse conditions.

Materials and Methods

Biocontrol Organism, Pathogen, and Plant Cultivar

The biocontrol organism *Trichoderma harzianum*, which we have used previously [14] and maintain in the laboratory, was used in all of the trials. *Rhizoctonia solani* AG2-1 was obtained from KT&G Central

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Institute, Suwon, Korea. The test plant was radish (*Raphanus sativus* L.) cv. Daeseung (seeds from Jeil Seed and Agricultural Products Co., Jeung Pyeong-gun, Korea).

In Vitro Experiments

Six dried plant materials purchased from an Oriental medicine store were tested for antifungal activities against *R. solani* AG2-1 and *T. harzianum*. The plant materials were *Paeonia suffruticosa* root bark (PRB), *Phellodendron amurense* stem bark (PSB), *Coptis chinensis* root (CCR), *Cnidium officinale* rhizome (COR), *Anethum graveolens* fruit (AGF), and *Cinnamomum cassia* stem bark (CSB). For each, 100 g of dried materials was ground using an electric grinder (Alozen; Alona Electronic Co. Ltd., Daejon, Korea), extracted in 1 l of ethanol overnight, filtered through filter paper (Grade 3 Qualitative, 15-cm diameter; Whatman, Kent, U.K.), and fully dried using a rotary vacuum evaporator (Büchi Rotavapor R-200; Flawil, Switzerland) under reduced pressure at 37°C. The residue was dissolved in 100 ml of ethanol. Paper discs (8-mm diameter) were soaked in each of the ethanol extracts, dried overnight, and placed on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, U.S.A.) plates 3 cm away from the fungi. Paper discs soaked in absolute ethanol served as the control. Four days later, the mycelial growth of *R. solani* AG2-1 and *T. harzianum* was examined. Each experiment was performed with five replications.

Scanning Electron Microscopy

To observe the antifungal activity of PRB against *R. solani* AG2-1, *R. solani* AG2-1 was incubated on PDA with a paper disc soaked in the PRB ethanol extract. A paper disc soaked in absolute ethanol was used as the control. Agar discs (1-mm thick) were cut from the interaction zone of the fungal colony and fixed at 4°C with modified Karnovsky's fixative, consisting of 5% glutaraldehyde and 5% paraformaldehyde in 0.2 M phosphate buffer (pH 7.2). After 4 h, the fixed specimens were washed with 0.1 M phosphate buffer three times for 15 min per wash. The specimens were then fixed in 2% OsO_4 in 0.2 M phosphate buffer (pH 7.2) at 4°C for 4 h. The specimens were washed again with 0.1 M phosphate buffer solution three times for 15 min per wash, and then dehydrated in an ethanol series of 50%, 70%, 80%, 90%, and 100% for 15 min at each concentration. The final exposure to 100% ethanol was repeated three times. The specimens were critical-point dried, sputter-coated with gold using a Sputter Coater (JFC-1110E, JEOL, Tokyo, Japan), and observed under a scanning electron microscope (SEM; JSM-5410LV, JEOL, Tokyo, Japan) at 20 kV.

Extraction and Identification of Antifungal Materials

As described above, 1 kg of ground PRB was extracted in 10 l of methanol, filtered (Grade 3 Qualitative, 15-cm diameter; Whatman), and evaporated to dryness. The PRB methanol extracts were separated in separation funnels using 300 ml of hexane and 300 ml of H_2O. The water layer was separated again with an equal volume of chloroform, and the resulting water layer was further separated as described above, using ethyl acetate and then *n*-butanol.

Each extracted layer was evaporated to dryness. A one-tenth volume of absolute ethanol relative to the weight of the material used was then added to each extract. Paper discs (8-mm diameter) were soaked with each ethanol solution, dried overnight, and placed in the center of PDA plates seeded with spores of the plant pathogenic fungi *Fusarium*, *Alternaria*, *Colletotrichum*, and *Penicillium* spp. The paper discs were also placed on PDA plates 3 cm from the *R. solani* AG2-1 and *T. harzianum* colonies.

Purification of Antifungal Materials

Well-ground PRB was extracted with methanol in a shaking incubator overnight and then filtered through filter paper. The chloroform extract, which had antifungal activity, was dissolved in a minimal volume of methanol and applied to a silica-gel column (64×500 mm, 63/200 mesh; Silica Gel 60; Merck, Darmstadt, Germany). The column was eluted with the following stepwise solvent rates of hexane:ethyl acetate: 100:0→99:1→9:1→8:2→7:3→1:1 (v/v), which was modified from the methods described in other studies [21, 22]. Each elution from the silica-gel column was collected and evaporated, after which absolute ethanol was added to adjust the solution concentration to 10%. Antifungal activity was evaluated using the method described above.

Isolation of Antifungal Material by Thin-Layer Chromatography

Antifungal materials were separated by thin-layer chromatography (TLC). The active hexane:ethyl acetate (9:1) fraction was concentrated and developed on a silica-gel TLC plate (Silica Gel 60 F_{254}; Merck). Each separation was tested for antifungal activity against *R. solani* AG2-1 and *T. harzianum*.

Mass Spectrometry, ^{1}H-NMR, and ^{13}C-NMR

To identify the purified active component of the antifungal material, mass measurements were performed on a JMS-AX50510A mass spectrometer (Joel, Co. Ltd, Japan), and NMR spectra were recorded on a Jeol JNM-LA 400 spectrometer (Jeol, Co. Ltd, Japan) at 399.65 MHz and 100.40 MHz for ^{1}H-NMR and ^{13}C-NMR, respectively.

Effects of Soil Amendments of Medicinal Plant Materials on *Rhizoctonia* Radish Damping-Off

Seeds of the radish cultivar Daeseung were sown in plastic containers (11×9×4.5 cm) containing a soil mixture (organic matter:sand=1:1) that had been autoclaved twice at 121°C for 1 h on two successive days. Each container held 20 plants. For pathogen inoculation, *R. solani* AG 2-1 was cultured on PDA plates (9-cm diameter) at 25°C for ten days, and then mixed thoroughly with soil three days before sowing. Nine plastic containers were inoculated with one culture plate, a condition that had resulted in damping-off incidences of 70% in a preliminary study. The inoculated soil was then thoroughly mixed with ground dried medicinal plant materials, which were incorporated into the soil at 1% w/v. Each treatment was replicated five times. The plants were maintained at room temperature in a greenhouse and watered daily to field capacity. The occurrence of pre- and post-emergence damping-off was examined five days after planting.

Effect of Individual and Combined Treatments of PRB and *T. harzianum* on *Rhizoctonia* Radish Damping-Off

Rhizoctonia solani AG2-1 inoculum was prepared as described above and used as the pathogen. To prepare the antagonist *T. harzianum* inocula, a sawdust medium consisting of pine wood sawdust, rice bran, and water at a 4:1:3 ratio (v/v) that was autoclaved for 1 h at 121°C was used. The medium was inoculated with *T. harzianum* mycelial plugs grown on PDA and incubated at 25°C for ten days. Pathogen inoculation and PRB treatment were performed following the above methods. *T. harzianum* and PRB were added to the soil alone and in combination

Table 1. Effect of various medicinal plant materials on mycelial growth of *Trichoderma harzianum* and *Rhizoctonia solani* AG2-1.

Species	Plant parts used	*T. harzianum*		*R. solani* AG2-1	
		Treated	Control	Treated	Control
Anethum graveolens	Fruit	4.5±0.1	4.5±0.0	2.3±0.1	2.6±0.2
Cinnamomum cassia	Stem bark	4.2±0.2	4.4±0.0	1.1±0.4	2.2±0.2
Cnidium officinale	Rhizome	4.2±0.1	4.4±0.0	2.1±0.4	2.5±0.1
Coptis chinensis	Rhizome	4.5±0.0	4.5±0.0	2.5±0.6	2.5±0.4
Paeonia suffruticosa	Root bark	4.4±0.2	4.5±0.0	0.8±0.1	2.5±0.2
Phellodendron amurense	Stem bark	4.5±0.0	4.5±0.1	2.5±0.2	2.5±0.2

Data are means±standard deviations of five replications.

at the dose concentrations shown in Table 4, and mixed thoroughly to evaluate their control of *Rhizoctonia* damping-off. Each treatment was performed with five replications.

Effect of PRB on the Culture of *T. harzianum*

PRB powder was incorporated into the autoclaved sawdust medium at the rates of 0.1% and 1.0% (w/v). The prepared media were inoculated with *T. harzianum* mycelial plugs grown on PDA and incubated at 25°C in an incubator. To examine population growth, 5 g of the culture media incubated with or without PRB was harvested after 5, 7, 9, 11, 13, 15, or 20 days of incubation, dissolved in 10 ml of distilled water, serially diluted, and spread on acidic PDA in 0.1-ml aliquots. Each treatment was performed with five replications. Colony-forming units (CFU) of each treatment were examined two days after inoculation.

Statistical Analysis

Data from the repeated experiments were analyzed by one-way analysis of variance (ANOVA) using StatGraphics Plus ver. 2.1 (Statistical Graphics Corp., Baltimore, MD, U.S.A.). Mean values were compared using the least significant difference (LSD) test at P=0.05.

RESULTS

Screening of Antifungal Materials Against *R. solani* AG2-1

Mycelial growth of *R. solani* AG2-1 was inhibited by all of the medicinal plant materials tested except PSB and CCR; however, compared with the control, *T. harzianum* was not inhibited (Table 1). Of the four medicinal plant materials that displayed antifungal activity against *R. solani* AG2-1, PRB and CSB were highly effective, inhibiting *R. solani* AG2-1 growth by 68% and 50%, respectively, as compared with the control (Table 1).

A whitish fungal colony of *R. solani* AG2-1 was formed on PDA in the untreated control (Fig. 1A). SEM of the colony revealed slender and straight hyphal cells (Fig. 1B). However, the fungal colony turned brownish with narrowly spaced mycelial rings toward the PRB treatment (Fig. 1C). In this area, the hyphal cells were shortened in length and swollen, as shown by SEM (Fig. 1D), indicating growth retardation.

Antifungal Activity of Column Chromatography Eluate

The active ingredients in PRB, which had the strongest antifungal activity, were investigated. Only the chloroform layer showed antifungal activity against the pathogenic fungi tested, *Fusarium*, *Alternaria*, *Colletotrichum*, and *Penicillium* spp. (data not presented).

The chloroform extract was subjected to silica-gel column chromatography for further purification. Only the 9:1 hexane: ethyl acetate solvent ratio eluate showed a strong inhibitory activity against *R. solani* AG2-1 (Fig. 2).

Isolation of an Antifungal Compound

The compound responsible for the antifungal activity of PRB was identified. The 9:1 hexane:ethyl acetate eluate was collected for TLC. The TLC *Rf* 0.41 line was almost black under 365 and 254 nm UV light. At 365 nm, another fluorescent white layer was present just above the first black layer. The first black layer at 365 and 254 nm contained antifungal products that inhibited the growth of *R. solani* AG2-1 (Fig. 3A), but not *T. harzianum* (Fig. 3B). The fluorescent layer did not inhibit the growth of either *R. solani* AG2-1 or *T. harzianum*.

Fig. 4 shows the mass spectrum of the purified antifungal material (molecular weight of 166) and its total and severed

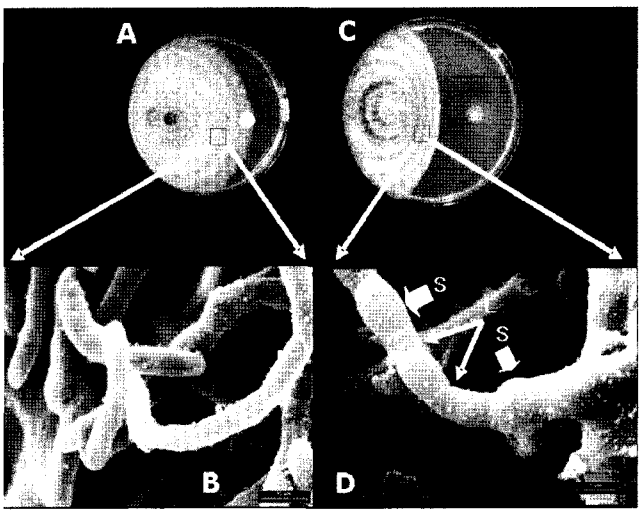

Fig. 1. Inhibition of *Rhizoctonia solani* AG2-1 by *P. suffructicosa* root bark ethanol extract: paper disc method (**A**, **C**); electron microscopy (**B**, **D**).
Hyphal cells shortened in length (L) and swelled (S). **A**, **B**: Control; **C**, **D**: treated with *P. suffructicosa*.

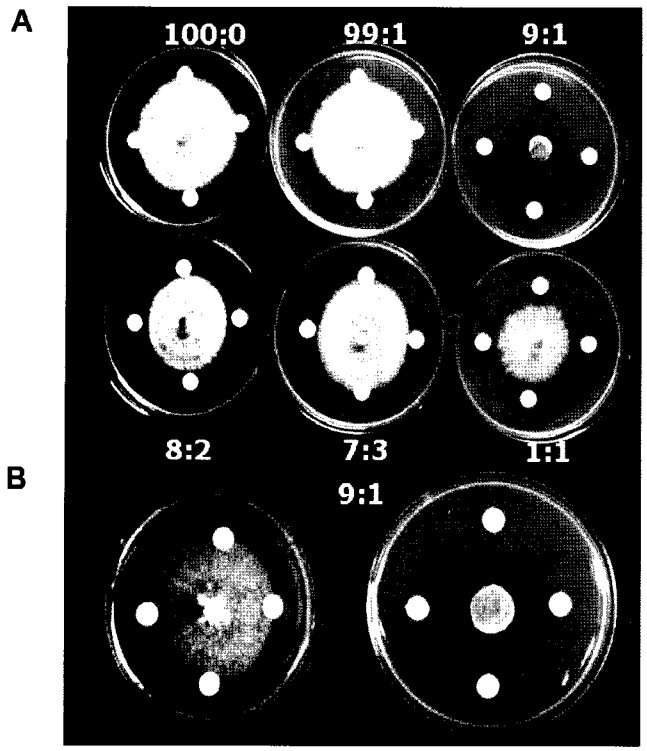

Fig. 2. Antifungal activity of column chromatography eluents against *Rhizoctonia solani* AG2-1.
A. Antifungal activity of different solvent rates of hexane: ethylacetate against *R. solani* AG2-1. **B.** Comparison of antifungal activity of the eluates from 9:1 solvent system against *T. harzianum* (left) and *R. solani* AG2-1 (right).

structure, which is identical to paeonol. ^{1}H-NMR and ^{13}C-NMR spectra were recorded with CD_3OD as the solvent, and the chemical shift ∂ (ppm) was measured and summarized in Table 2. ^{1}H NMR of the antifungal material showed two methyl

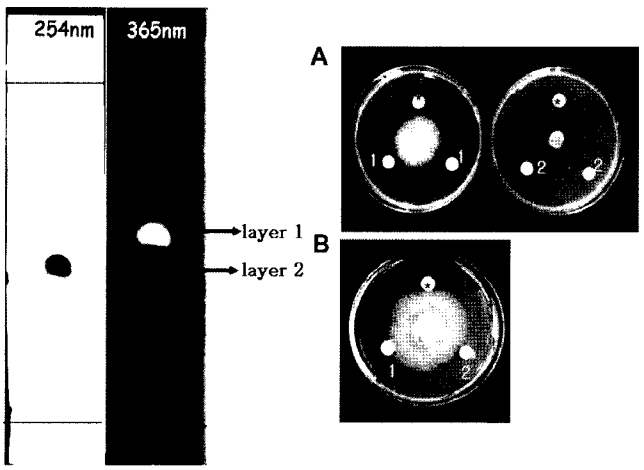

Fig. 3. Isolation of antifungal material by thin-layer chromatography (TLC).
The chloroform extract was developed on silica-gel TLC plates using a hexane and ethylacetate (9:1) solvent system. TLC plates were observed under UV light at 254 nm and 365 nm. Antifungal activity of each layer against *Rhizoctonia solani* AG2-1 (**A**) and *Trichoderma harzianum* (**B**). *, Control; 1, layer 1; 2, layer 2.

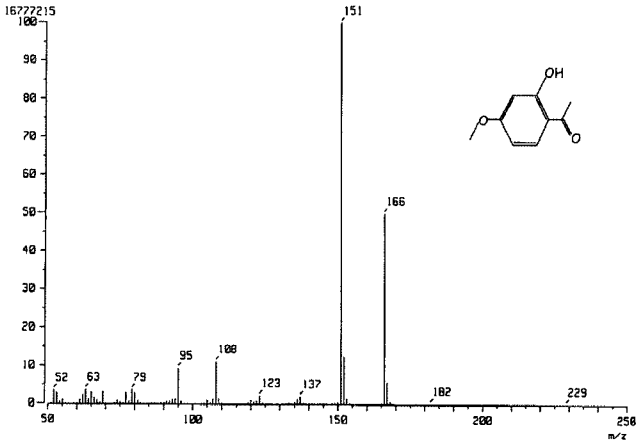

Fig. 4. Mass spectrum of paeonol from *Paeonia suffructicosa* root bark.

signals at 2.54 and 3.83 ppm and three aromatic methine signals at 6.40, 6.48, and 7.77 ppm. ^{13}C-NMR spectra revealed signals assigned to the following; one carbonyl carbon (204.50 ppm), two oxygenated sp^2 quaternary carbons (166.18, 167.72 ppm), three aromatic methane carbons (101.72, 108.32, 133.98 ppm), one sp^2 quaternary carbon (204.50 ppm), and two sp^3 carbons (26.32, 56.09 ppm). These NMR data matched well with paeonol, having the molecular formula of $C_9H_{10}O_3$ with the benzene ring. Thus, the antifungal compound isolated from PRB was confirmed to be paeonol by mass spectrometry, ^{1}H-NMR, and ^{13}C-NMR analyses.

Effect of Medicinal Plant Materials on *Rhizoctonia* Damping-off of Radish

Soil amended with medicinal plant materials at 1% (w/v) reduced the incidence of damping-off caused by *R. solani* AG2-1 (Table 3). Among the medicinal plant materials tested, PRB and CSB displayed high efficacy, with control values of 95.1% and 82.9%, respectively. The control values of COR and CCR were both ~64%, and those of AGF and PSB were 28.0% and 30.9%, respectively (Table 3). The radish seed germination rate of >90% was not affected by

Table 2. ^{1}H and ^{13}C NMR spectral data for paeonol in CD_3OD.

No.	Chemical shifts (ppm)	
	^{1}H	^{13}C
1		115.02
2		166.18
3	6.40 (s)	101.72
4		167.72
5	6.48 (d, J=8.2 Hz)	108.32
6	7.77 (d, J=8.2 Hz)	133.98
7		204.50
8	2.54 (s)	26.32
9	3.83 (s)	56.09

Table 3. Effect of medicinal plant materials on controlling radish damping-off caused by *Rhizoctonia solani* AG2-1.

Treatment*	Disease incidence (%)	Control value (%)
Paeonia suffruticosa	2.3±2.5	95.1[a]
Cinnamomum cassia	8.1±3.6	82.9[ab]
Coptis chinensis	16.7±1.8	64.6[abc]
Cnidium officinale	16.9±3.1	64.2[abc]
Phellodendron amurense	32.7±9.6	30.9[d]
Anethum graveolens	34.0±7.4	28.0[d]
Untreated (control)	47.3±2.8	0.0[e]

*1% soil amendment (w/v).
Data are means ± standard deviations of two trials with five replications per treatment.
[abcde]Values followed by the same letter are not significantly different from each other at P=0.05 according to the least significant difference (LSD) test.

any of these treatments and was similar to that of the control (data not presented).

Effect of Individual and Combined Treatments of PRB and *T. harzianum* on *Rhizoctonia* Damping-off of Radish

Disease suppression by individual and combined treatments of PRB and *T. harzianum* was investigated using potted soil under greenhouse conditions. Application of PRB and *T. harzianum* individually or in combination substantially reduced the severity of *Rhizoctonia* damping-off of radish at all doses, but was more effective when the two were combined than with either of the treatments alone (Table 4). Separate PRB and *T. harzianum* treatments at concentrations of 0.05–0.1% and 0.2–0.6% (w/v) resulted in 43–55% and 42–52% disease control, respectively. However, combined treatment with PRB and *T. harzianum* enhanced disease control by 23–38% as compared with the individual treatments.

Table 4. Effect of individual and combined treatments of *Paeonia suffruticosa* root bark (PRB) and *Trichoderma harzianum* on the control of radish damping-off caused by *Rhizoctonia solani* AG2-1.

Treatment concentration (%)		Disease incidence (%)	Control value (%)
PRB	*T. harzianum*		
0	0.0	90.9±15.8	0.0[d]
	0.2	53.4±4.8	42.3[c]
	0.4	46.3±5.4	49.1[c]
	0.6	44.1±8.7	51.5[c]
0.05	0.0	51.7±3.7	43.1[c]
	0.2	22.6±8.9	75.1[ab]
	0.4	21.1±2.4	77.9[ab]
	0.6	16.3±2.4	78.5[ab]
0.08	0.0	42.5±1.7	53.2[c]
	0.2	23.3±12.6	74.3[b]
	0.4	13.2±4.5	85.5[ab]
	0.6	11.2±6.2	87.6[a]
0.1	0.0	41.2±10.6	54.7[c]
	0.2	26.9±7.6	71.4[ab]
	0.4	12.3±3.1	86.4[ab]
	0.6	14.9±10.7	83.6[ab]
$LSD_{0.05}$			12.9

Data are means±standard deviations of five replications.
[abcd]Values followed by the same letter are not significantly different from each other at P=0.05 according to the least significant difference (LSD) test.

Effect of PRB Amendment on the Culture of *T. harzianum*

The *T. harzianum* population in sawdust medium without PRB differed significantly at P=0.05 from that with 1.0% PRB, but not from that with 0.1% PRB at five days after inoculation, with *T. harzianum* populations of 5.47, 5.53, and 4.83 Log CFU/g medium in the control (0.0%) and in medium containing 0.1% and 1.0% PRB, respectively (Fig. 5). The *T. harzianum* population in medium containing 1.0% PRB began to decline after seven days of inoculation.

DISCUSSION

The six medicinal plant materials tested showed variable antifungal activities against *R. solani* AG2-1 but had no adverse effect on *T. harzianum*. PRB and CSB were highly antagonistic to *R. solani* AG2-1, with PRB showing somewhat higher control activity than CSB. SEM revealed that the PRB ethanol extract caused morphological abnormalities (shortening and swelling) of *R. solani* AG2-1 hyphal cells. The purified antifungal compound separated from the PRB chloroform extract inhibited the growth of several plant pathogenic fungi, including *R. solani* AG2-1, but not *T. harzianum*. The mode of antifungal action against *R. solani* AG2-1 may involve the release of volatile antimicrobial substances, such as paeonol, which inhibits fungal mycelial growth. The fact that volatile constituents of *Paeonia clusii* were active against several microorganisms, including pathogenic fungi, and exhibited the highest antifungal activity among the *Paeonia* taxa tested, may be attributed to the existence of paeonol, because *P. clusii* was found to contain a high percentage (32.57%) of paeonol [31]. Very recently, paeonol and benzoic acid were identified in *Paeonia suffruticosa* root bark that showed acaricidal activities against the copra mite *Tyrophagus putrescentiae*; these activities were much higher in closed containers than in open ones, indicating that the effects of these compounds were largely due to action in the vapor phase [41]. *Paeonia albiflora* and *P. moutan* root bark display antifungal activity against *Alternaria alternata* [18]. Medicinal plant soil amendments, including PRB and CSB, reduced tomato root-knot nematode [19]. Considering the similarities of the biological activities of PRB and paeonol, the main active component of PRB is likely to be paeonol.

Treatment with a combination of PRB and *T. harzianum* resulted in greater control of *Rhizoctonia* damping-off

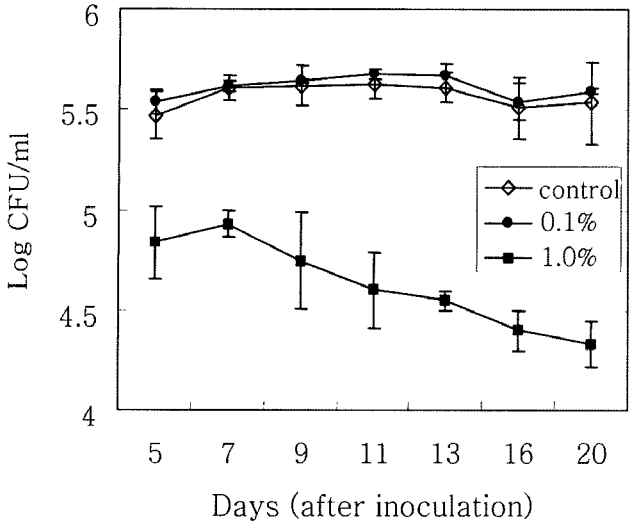

Fig. 5. Effect of *Paeonia suffructicosa* amendment doses on *Trichoderma harzianum* growth in sawdust medium.

(P=0.05) than the use of either treatment alone. Various modes of action have been postulated and demonstrated for the antagonistic effect of *Trichoderma* strains in controlling soilborne diseases, including the production of antibiotic substances [12, 40], the presence of cell wall-degrading chitinases [11], nutrient competition [1], and the induction of systemic resistance [44]. Applied at low concentrations, PRB displayed antifungal activity against *R. solani* AG2-1, but not against *T. harzianum*. This property may have enhanced the competitiveness of the biocontrol agent, thus increasing the efficacy of PRB as a soil amendment. The differential antibiosis of PRB to the pathogen and the antagonistic fungus may also have led to enhanced disease suppression. At a dose concentration of 1.0%, PRB inhibited *T. harzianum* growth remarkably, but at 0.1% PRB the *T. harzianum* multiplication rate was not affected and was similar to that of the fungus alone. Thus, selection of an optimal PRB dose used in conjunction with *T. harzianum* is of primary importance. Together, the antagonist *T. harzianum* and PRB produce a greater pathogenic effect with no phytotoxicity. Peony has analgesic, sedative, anti-inflammatory, and antimicrobial properties and has been used as a human and animal disease remedy [26–29], indicating that it is a very safe plant material. Thus, combining PRB or its extract with *T. harzianum* as a biofungicide is an attractive alternative to chemicals, especially in intensive organic farming systems.

Acknowledgments

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