Evaluation of the Biocontrol Potential of Some Medicinal Plant Materials Alone and in Combination with *Trichoderma harzianum* Against *Rhizoctonia solani* AG 2-1

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(Received on December 8, 2010; Accepted on February 4, 2011)

Fifty five species of medicinal plant materials were tested for their antifungal activity *in vitro* against *Rhizoctonia solani* AG 2-1 and *Trichoderma harzianum* to select plant species that can be used to improve the biocontrol efficacy of *T. harzianum*. Six species were effective against *R. solani* AG 2-1 but were also antagonistic to *T. harzianum*, except for *Cinnamomum loureirii* stem bark (CSB). CSB inhibited mycelial growth of *R. solani* AG 2-1 by 73.7% but showed an inhibitory effect on mycelial growth of *T. harzianum* by only 2.2%. Scanning electron microscophs showed that the CSB treatment resulted in deformed *R. solani* AG 2-1 hyphal cells, and transmission electron microscophs revealed degenerated cell structures such as degenerated cytoplasm and disentangled cell wall and the accumulation of electron-dense inclusions (asterisks) in the CSB treatment. The biocontrol efficacy of radish damping-off increased greatly following the combined treatments of *T. harzianum* and CSB and the combined treatment increased efficacy from 6.4-23.1% to 37.1-87.3% compared with either treatment alone. CSB did not affect *T. harzianum* population growth, as it was almost the same in rice-bran peat medium (culture) amended with 0.1% and 1.0% CSB powder as in non-amended medium. The formulation of *T. harzianum* in rice-bran peat medium amended with CSB powder reduced the severity of radish damping-off by 80.6%, suggesting that *T. harzianum* and CSB can be formulated as a biocontrol product for the control of *R. solani* AG 2-1.

Keywords : Biocontrol, *Cinnamomum loureirii* stem bark, *Rhizoctonia solani*, *Trichoderma harzianum*

Rhizoctonia solani Kühn is a fungal pathogen causing damping-off in several crops under both greenhouse and open field conditions (Berta et al., 2005). Managing

Rhizoctonia damping-off mainly relies on the use of chemicals, but the overuse of chemical fungicides leads to adverse impacts on the environment, humans, and animal health and induces a pathogenic resistance to chemicals. These issues and a means to develop eco-friendly and safer control methods have encouraged research efforts aimed at finding new and effective methods for controlling plant diseases. Emerging strategies for plant disease management involve biological and integrated control by applying antagonistic microorganisms alone or in combination with and/or alternating with fungicides or natural materials (Lima and De Cicco, 2006; Paulitz and Belanger, 2001). Biological control appears to offer an environmentally safe and economically feasible strategy for plant protection and has great potential to promote sustainable agriculture. However, although numerous microorganisms have been considered potential agents for suppressing plant pathogens, only a few have been commercialized (Brannen and Kenney, 1997; Gerhardson, 2002).

Trichoderma harzianum is a filamentous soil fungus that functions as a biocontrol agent for a wide range of economically important plant pathogens (Chet, 1987; Papavizas, 1985). *Trichoderma* spp. are worldwide in occurrence and easily isolated from soil, decaying wood, and other forms of plant organic matter (Howell, 2003). The potential of *Trichoderma* spp. as biocontrol agents of plant diseases was first recognized in the early 1930s (Weindling, 1932), and subsequently, control of many diseases has been added to the list (Harman, 2000). *Trichoderma* strains have been used against *Rhizoctonia solani* on several crops (Elad et al., 1981; Lee et al., 2008; Lewis et al., 1998; Ruppel et al., 1983). *Trichoderma* produces a large range of secondary metabolites with inhibitory traits, including direct mycoparasitism as well as lysis and degradation of the cell wall by enzymes (Howell, 2003). The mycoparasitic activity of this organism is attributable to a combination of successful nutrient competition (Brannen and Kenney, 1997), production of cell wall-degrading enzymes (Pierson and Weller, 1994), and antibiosis (Hjelord and Tronsmo,

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1998; Nandakumar et al., 2001). Several strains of *Trichoderma* have been tested as an alternative to chemical fungicides (Howell, 1998). However, achieving full disease control in soil is difficult mainly because of the complexity of the plant-soil system, whereby the microbial inoculum is affected by biotic and abiotic factors and shows relatively lower efficacies compared to those of applying chemicals under various environmental conditions. Therefore, the integrated use of microorganisms and chemical pesticides and/or natural materials may be an effective method applicable in the field. Using mixtures or combinations of biological agents may improve biological control, particularly when they exhibit different or complementary modes of action. Combined applications of a biocontrol agent and synthetic chemicals or plant materials often provide better plant protection than individual treatments (Bardin et al., 2003; Duffy, 2000). The combined application of *Paeonia suffruticosa* cortex (medicinal plant) and *T. harzianum* is more effective than either treatment alone for controlling *Rhizoctonia* damping-off (Lee, 2005; Lee et al., 2008). A combined application of metalaxyl with either *T. harzianum* or *Pseudomonas cepacia* resulted in lower disease incidences of red pepper phytophthora blight than by any single treatment (Kim et al., 1991). Two different biocontrol agents used in a mixture of *Pichia guilliermondii* and *Bacillus mycoides* are higher in control efficacy than that achieved by separate inoculation, and the variability of suppression was reduced (Guetsky et al., 2001, 2002).

The antimicrobial activity of plant materials and their extracts have been recognized for many years. In particular, the antimicrobial activities of plant materials and essential oils have formed the basis of preservation, pharmaceutical, alternative medicine, and natural therapies (Lis-Balchin and Deans, 1997; Reynolds, 1996). Natural plant materials are possible sources of environmentally friendly agrochemicals that are effective against pathogens (Zaika, 1988). In this study, we conducted an *in vitro* screening of antifungal medicinal plant materials antagonistic to *R. solani* AG 2-1 but not to *T. harzianum* to select potential plant materials useful for controlling the pathogen with the biocontrol agent in combination. The efficacies of individual and combined treatments of antifungal plant materials and the biocontrol agent, *T. harzianum*, were evaluated for the control of radish damping-off of *R. solani* AG 2-1 under greenhouse conditions.

Materials and Methods

Source of fungal inoculums and preparation of fungal culture. *R. solani* AG 2-1 was obtained from KT&G Central Research Institute, Suwon, Korea. The biocontrol organism, *T. harzianum*, was isolated from soil and main-

tained at the Department of Agricultural Biotechnology, Seoul National University, Seoul, Korea, and used for artificial inoculations. A pure mycelia culture generated through a single spore of this isolate, maintained in potato-dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA) at 28 °C, served as an initial inoculum source. For the mass culture, both fungi were grown in potato-dextrose broth (PDB; Alpha Biosciences, Baltimore, MD, USA) at 25 °C for 2 weeks.

Screening of medicinal plant extracts for their anti-fungal activity. Fifty-five dried medicinal plant materials were collected from Kyung-Dong Mart, Seoul, Korea, to test their antifungal activity against *R. solani* AG 2-1 and *T. harzianum*. They were one species each in 21 families including Alismataceae, Cornaceae, Cyperaceae, Discoraceae, Eucommiaceae, Lardizabalaceae, Lobeliaceae, Magnoliaceae, Oleaceae, Orobanchaceae, Pinaceae, Polygalaceae, Rhamnaceae, Rosaceae, Rubiaceae, Sapindaceae, Schisandraceae, Scrophulariaceae, Solanaceae, Umbelliferae, and Zingiberaceae; four species including *Angelica gigas* root in Apiaceae, four including *Atractylodes macrocephala* root in Asteraceae; three including *Adenophora verticillata* root in Campanulaceae; and the other 23 species in Fabaceae (4), Labiatae (2), Lauraceae (3), Myrtaceae (3), Paeoniaceae (2), Polyporaceae (6), and Rutaceae (3). The collected medicinal plant materials were washed in sterilized distilled water and air-dried, then 10 g of each plant material was ground separately in an electric grinder (Alozen; Alona Electronic Co. Ltd., Daejeon, Korea) and extracted in 20 ml of ethanol overnight. The resultant solution was passed through filter paper (grade 3 qualitative, 150 mm diameter; Whatman, Kent, UK), which were used to test the antifungal efficacy on *R. solani* AG 2-1 and *T. harzianum*. For this, 8-mm-diameter paper disks (Toyo Roshi Kaisha, Ltd., Tokyo, Japan) were soaked with 50 µl of each ethanol extract, dried under ultraviolet light, and placed on PDA plates aseptically under laminar flow 3 cm away from the fungi. Paper disks soaking in absolute ethanol served as the control. Four days later, *R. solani* AG 2-1 and *T. harzianum* mycelial growth was examined and antagonistic potentials of plant extracts were calculated as given below:

$$I = (C - T)/C \times 100,$$

where I is the percent inhibition in mycelial growth, C is the growth of mycelium in the control (mm), and T is the growth of the mycelium in the treatment (mm). Each experiment was performed with five replicates.

Efficacy of the selected medicinal plant extracts on mycelial growth. Fifty-five medicinal plant materials were tested for their antifungal potential; six showed moderate to

strong antifungal activity against *R. solani* AG 2-1 but were not so antagonistic to *T. harzianum* in a preliminary study. These selected medicinal plant materials were *Cinnamomum loureirii* stem bark (CSB), *Eugenia caryophyllata* flower bud (Bud Clove, EFB), *Zanthoxylum piperitum* fruit (Fructus Zanthoxyli, ZF), *Curcuma aromatica* rhizome (Rhizobium Curcumae aromatase, CR) and *Polygala tenuifolia* rhizome (Radix Polygalae, PR). For each, 10 g of dried material was extracted in 100 ml ethanol as described in the above section and fully dried using a rotary vacuum evaporator (Rotavapor R-200; Büchi, Flawil, Switzerland) under reduced pressure at 37 °C. The extracted residue was dissolved again in 10 ml of ethanol, which was considered a standard solution. For further dilution, 1 ml of standard ethanol extracts was added separately to 100 ml melted PDA aseptically, under laminar flow, and mixed thoroughly to obtain a 1% concentration of the amendments. The amended medium was poured onto sterilized Petri dishes under laminar flow and allowed to solidify. Each plate was inoculated with 2-mm disks of a mycelial bit taken from the periphery of a 7-day-old colony of *R. solani* AG 2-1 and *T. harzianum*, then placed 3 cm away from one another and incubated at 25 °C. Mycelial growth of *R. solani* AG 2-1 and *T. harzianum* was measured after a 4-day incubation. Medium without medicinal plant extracts served as a control. The synergistic effect of *T. harzianum* and the medicinal plant materials on the growth of *R. solani* AG 2-1 was measured by comparing the mycelial growth of the pathogen and the antagonistic fungus on the media incorporated with and without medicinal plant extracts. Each experiment was performed with five replicates.

Effect of a combined treatment of *T. harzianum* and medicinal plant materials on *R. solani* AG 2-1 mycelial growth. The synergistic effect of *T. harzianum* and the two plant materials, FEB and CSB, on *R. solani* AG2-1 mycelial growth was investigated by coculturing the pathogen with *T. harzianum* on PDA amended with various concentrations of plant material ethanol extracts (Table 2). To obtain the desired concentrations of amendment, the required amounts of plant extracts were added to the PDA as described in the previous section.

Antagonistic actions of *T. harzianum* on *R. solani* AG 2-1. The two fungi were cocultured on glucose agar (water agar containing 0.5% glucose) to observe the antagonistic action of *T. harzianum* on *R. solani* AG 2-1. After a 24-h incubation, when the *T. harzianum* mycelia grew over (interacted with) the *R. solani* AG 2-1 mycelia, samples containing the interacting mycelial zones were observed by scanning electron microscopy (SEM). For SEM, agar disks

(1 mm thick) were cut from interacting zones of *T. harzianum* and *R. solani* AG 2-1 on glucose agar and prepared for SEM observation as described below. Agar disks were fixed at 4 °C in modified Karnovsky's fixative consisting of 5% paraformaldehyde and 5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 4 h. The fixed samples were washed with 0.2 M phosphate buffer three times for 15 min per wash. The samples were post-fixed again in 2% osmium tetroxide (OsO₄) in 0.2 M phosphate buffer (pH 7.2) at 4 °C for 4 h. The samples were washed again with 0.1 M phosphate buffer solution three times for 15 min per wash and then dehydrated in an ethanol series (30%, 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 SEM (JSM-5410LV; JEOL) at 20 kV.

Antifungal activity of the CSB extract against *R. solani* AG 2-1. To observe the antifungal activity of the CSB extract against *R. solani* AG 2-1, *R. solani* AG 2-1 fungal disks were incubated on PDA plates with a paper disk (8 mm diameter) soaked in CSB ethanol extract. A paper disk soaked in absolute ethanol was used as the control. An inhibition zone formed around the CSB-treated paper disk; agar disks (1 mm thick) were cut from the interaction zone of the fungal colony and prepared for SEM observations as described in above section. The samples were also observed under transmission electron microscopy (TEM). For this, agar disks were fixed with modified Karnovsky's fixative consisting of 2% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde in 0.05 M sodium cacodylate buffer (pH 7.2) at 4 °C overnight and washed with the same buffer three times for 10 min each. The specimens were post-fixed with 1% (w/v) OsO₄ in the same buffer at 4 °C for 2 h and washed with distilled water two times briefly. The post-fixed specimens were en bloc stained in 0.5% (w/v) uranyl acetate at 4 °C overnight. They were dehydrated in a graded ethanol series for 10 min each as described above, and the final exposure to 100% ethanol was repeated three times. The samples were further treated with propylene oxide as a transition fluid twice for 15 min each and embedded in Spurr's epoxy resin. The embedded samples were sectioned (80-90 nm thick) with a glass knife on an ultramicrotome (MT-X; RMC, Inc., Tucson, AZ, USA). Sections were mounted on copper grids and stained with 2% uranyl acetate and lead citrate for 10 min each and observed under TEM (JEM-1010; JEOL) at 80 kV. Electron micrographs were recorded and digitized using an image processing system (FDL 5000; Fuji Photo Film Ltd.).

Antifungal activity of combined treatments of *T. harzianum* and CSB ethanol extract on *R. solani* AG 2-1. The pathogen and *T. harzianum* were cocultured on PDA containing 0.1% CSB ethanol extract to observe the synergistic effect of *T. harzianum* and CSB ethanol extract on *R. solani* AG 2-1. The interacting zones between *T. harzianum* and *R. solani* AG 2-1 on PDA were cut and prepared for SEM observation as described in earlier sections.

Effect of medicinal plant materials on *Rhizoctonia* radish damping-off. Radish seeds (*Raphanus sativus* L.) cv. Guem-jung, obtained from Kyungshin seeds Co. Ltd., Seoul, Korea, were sown in 10-cm diameter plastic pots containing a soil mixture (organic matter:sand:soil = 1:1:1) that had been autoclaved twice at 121 °C for 1 h on 2 consecutive days. For pathogen inoculation, *R. solani* AG 2-1 was cultured in oatmeal-sand medium (consisting of sand, oatmeal powder, and moisture in the ratio of 20:1:4, v/v) at 25 °C for 2 weeks, and then incorporated into sterilized soil mixture at a 0.03 ratio (w/w) 2 days before seed sowing. The medicinal plant materials, EFB and CSB, which were ground to a powder, were added to the soil mixture at the rate of 0.1%, 0.2%, 0.4%, 0.6%, 0.8%, and 1.2% (w/w) 2 days before seed sowing. Prior to sowing, radish seeds were surface-sterilized in 1% NaOCl (Ducksan, Seoul, Korea) for 1 min and rinsed three times in sterilized distilled water. Ten seeds were sown into each container with five replicates for each treatment. Untreated pots served as controls. The plants were maintained in a greenhouse at room temperature and watered daily to field capacity. The occurrence of pre- and post-emergence damping-off was examined at 7 days after planting.

Effect of various components of *T. harzianum* on *Rhizoctonia* radish damping-off. *R. solani* AG 2-1 inoculum was prepared as described above and used as the pathogen to evaluate the control effect of various *T. harzianum* components on radish damping-off. The biocontrol agent, *T. harzianum*, was cultured in PDB at 25 °C for 2 weeks. The mycelial masses were saved by carefully draining the culture filtrates from the fungal cultures. The separated mycelial masses and drained *T. harzianum* culture filtrates were applied to the soil 2 days before seed sowing as follows: a 1.0% (w/w) mycelial mass with 10 ml of culture medium, 1.0% (w/w) mycelial mass washed in sterilized distilled water, and 10 ml of *T. harzianum* culture filtrate, and all of these treatments were followed by heat treatment at 100 °C for 1 h. Ten seeds were sown into the pots with five replicates for each treatment. The plants were maintained in a greenhouse at room temperature and watered daily to field capacity. The occurrence of pre- and post-emergence damping-offs was examined at 7 days after planting.

Table 1. Inhibitory effect of selected medicinal plant materials on mycelial growth of *Rhizoctonia solani* AG 2-1 and *Trichoderma harzianum*

Medicinal plant materials ^a	<i>R. solani</i> AG 2-1		<i>T. harzianum</i>	
	Mycelial growth (cm)	Mycelial inhibition (%)	Mycelial growth (cm)	Mycelial inhibition (%)
EFB	1.0 ± 0.1	73.7	3.2 ± 0.2	28.9
CSB	1.1 ± 0.1	71.1	4.4 ± 0.1	2.2
ZF	1.4 ± 0.2	63.2	3.3 ± 0.5	26.7
CR	2.3 ± 0.1	39.5	3.2 ± 0.5	28.9
PR	2.5 ± 0.7	34.2	3.9 ± 0.3	13.3
MSB	2.8 ± 0.1	26.3	2.2 ± 0.1	51.5
Control ^b	3.8 ± 0.2	–	4.5 ± 0.1	–

^a EFB (*Eugenia caryophyllata* flower bud), CSB (*Cinnamomum loureirii* stem bark), ZF (*Zanthoxylum piperitum* fruit), CR (*Curcuma aromatica* rhizome), PR (*Polygala tenuifolia* rhizome), MSB (*Machilus thunbergii* stem bark)

^b Control (soaked with absolute ethanol and dried)

Table 2. Synergistic effect of medicinal plant materials and *Trichoderma harzianum* on mycelial growth of *Rhizoctonia solani* AG 2-1

Treatment conc. (v/v %) ^a	Mycelial growth of <i>R. solani</i> AG 2-1 (cm)			
	EFB ^b		CSB	
	Without TH ^c	With TH	Without TH	With TH
1.00	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
0.10	0.6 ± 0.1	0.5 ± 0.1	2.1 ± 0.2	1.1 ± 0.2
0.01	2.2 ± 0.1	2.0 ± 0.2	2.3 ± 0.2	1.1 ± 0.1
0.00	3.5 ± 0.1	2.9 ± 0.5	3.6 ± 0.1	2.9 ± 0.6

^a Ethanol extracts of medicinal plant materials added to potato dextrose agar (PDA) at various concentrations.

^b EFB (*Eugenia caryophyllata*), CSB (*Cinnamomum loureirii*).

^c Mycelial growth of *R. solani* AG 2-1 cultured with TH (*T. harzianum*) or without TH on PDA containing ethanol extracts of plant materials

Effect of individual and combined treatments of CSB powder and *T. harzianum* on *Rhizoctonia* radish damping-off. *In vitro* experiments have shown that CSB extracts do not inhibit *T. harzianum* mycelial growth (Table 1) but that their combined application enhances the inhibition of *R. solani* AG 2-1 mycelia growth compared to an individual application (Table 2). Therefore, an attempt was made to evaluate the biocontrol potential of CSB powder and *T. harzianum* as individual agents or in combination against *Rhizoctonia* radish damping-off in potted soil. *R. solani* AG 2-1 inoculum was prepared and incorporated in potted soil as described above. Mycelia of *T. harzianum* cultured on PDB at 25 °C for 15 days were harvested and partially dried for 1 h at room temperature to prepare biocontrol agent inocula. *T. harzianum* and the CSB powder

were added to the potted soil alone or in combination at various dose concentrations (Table 5). Radish seeds were surface-sterilized in 1% NaOCl for 1 min and rinsed three times in sterilized distilled water. Ten seeds were sown into each container with five replicates for each treatment. Untreated pots served as controls. The plants were maintained in a greenhouse at room temperature and watered daily to field capacity. The occurrence of pre- and post-emergence damping-off was examined at 7 days after planting.

Effect of CSB amendments on growth of *T. harzianum* in rice-bran peat medium. CSB powder was added to the autoclaved rice-bran peat medium (consisting of peat, rice bran, and moisture in a ratio of 8:2:4, v/v/v) at the rate of 1.0% and 0.1% (w/v) and mixed thoroughly. Prepared media were inoculated with 5 ml of 1.0×10^3 /ml spore suspension of *T. harzianum* and incubated at 25 °C in an incubator. The population of *T. harzianum* was observed on 5, 7, 14, 18, and 24 days post inoculation with CSB powder. For this, 1 g of *T. harzianum* inoculated medium was taken and dissolved in 9 ml of distilled water, serially diluted, and spread on *Trichoderma* selective medium (TSM medium containing 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.9 g K_2HPO_4 , 1.0 g NH_4NO_3 , 0.15 g KCl, 0.15 g rose bengal, 3 g glucose, and 20 g agar per liter of distilled water, after autoclave sterilization, and 0.25 g chloramphenicol, 90 mg streptomycin, 0.2 g quitozine were added) (Askew, 1993). The colony-forming units (CFU) for each treatment on the selective medium were examined 2 days after incubation.

Effect of formulated *T. harzianum* in rice-bran peat medium amended with CSB on radish damping-off. To evaluate the efficacy of the formulated *T. harzianum* in rice bran on *Rhizoctonia* damping-off of radish, *T. harzianum* was cultured for 14 days at room temperature in rice-bran peat medium containing 1.0%, 0.1%, and 0.0% (w/w/w) CSB powder. The *R. solani* AG 2-1 inoculum was prepared as described above and the formulated *T. harzianum* was added to potted soil at a rate of 0.1% (w/w) 2 days before seed sowing. Ten seeds were sown into each pot with five replicates for each treatment. The plants were maintained in a greenhouse at room temperature and watered daily to field capacity. The occurrence of pre- and post-emergence damping-off was examined at 7 days after planting.

Statistical analysis. Data from repeated experiments were analyzed by two-way analysis of variance performed on the data followed by Duncan's multiple range test to compare between treatments at $P \leq 0.05$. All statistical analyses were done using Analyse-it software for Excel (Microsoft, Redmond, WA, USA). No significant differences were observed

between the repeated experiments, and thus data from duplicate tests were pooled for analysis.

Results

Screening of antifungal materials against *R. solani* AG 2-1.

Fifty five medicinal plant materials were tested for their antifungal activity against *R. solani* AG 2-1 and *T. harzianum*. No significant reduction in mycelial growth of *R. solani* was noted by most medicinal materials tested (data not shown), except for six medicinal plant materials including CSB, EFB, PR, CR, ZF, and *Machilus thunbergii* stem bark (Cortex Manoliae, MSB; Table 1). These plant materials had significantly inhibited mycelia growth of the biocontrol agent, *T. harzianum* ($P \leq 0.05$), except CSB, which inhibited mycelial growth of *T. harzianum* by only 2.2%. Among these six medicinal plant materials, EFB and CSB were the most effective against *R. solani* AG 2-1 mycelial growth, revealing an inhibitory efficacy of 73.7% and 71.1%, respectively, compared to untreated controls.

Effect of combined treatment of medicinal plant materials and *T. harzianum* on mycelial growth of *R. solani* AG 2-1.

The synergistic effect of *T. harzianum* and the two most effective medicinal plant materials, FEB and CSB, against *R. solani* AG 2-1 was investigated by coculturing the pathogen, *R. solani* AG 2-1, with *T. harzianum* on PDA amended separately with FEB and CSB. The inhibition of *R. solani* AG 2-1 mycelial growth was significantly enhanced on the PDA amended with CSB compared to non-amended media at all concentrations tested, except 1.0%, at which no fungal growth was observed (Table 2). In contrast, the combined treatment of *T. harzianum* and FEB did not enhance the inhibition of mycelial growth compared to either treatment alone ($P \leq 0.05$).

Interactions between *T. harzianum* and *R. solani* AG 2-1.

SEM revealed that the *T. harzianum* hyphae grew along and over *R. solani* AG 2-1, frequently branching and coiling around the host hyphae (Fig. 1A, B). The main hyphae of *T. harzianum* coiled around the host (*R. solani* AG 2-1) and produced short branches that tightly surrounded the host hyphae. The detached *T. harzianum* hyphae revealed a hole in the host cell wall and the *T. harzianum* hyphae were growing inside the host hyphae (Fig. 1B).

Inhibitory effect of CSB ethanol extracts on *R. solani* AG 2-1.

The SEM observation of the *R. solani* AG 2-1 untreated control showed smooth, straight, and normally growing hyphae (Fig. 2A), whereas the pathogen hyphae treated with CSB ethanol extracts showed a knotty-look with distorted hyphae and local swelling on hyphal tips and

Fig. 1. Scanning electron micrographs showing interactions between *Trichoderma harzianum* (T) and *Rhizoctonia solani* AG 2-1 (R). *T. harzianum* coiling around *R. solani* AG 2-1 hyphae (A) and detached hyphae of *T. harzianum* revealing a hole in an *R. solani* AG 2-1 cell (arrow). Bars = 10 μ m.

Fig. 2. Scanning electron micrographs showing inhibited growth of *Rhizoctonia solani* AG 2-1 hyphae caused by *Cinnamomum loureirii* stem bark (CSB) ethanol extracts. A: Control, smooth, straight and normally-growing hyphae; B: Treated with CSB ethanol extracts, showing knotty and distorted hyphae with local swelling (arrow) on hyphal tips and interhyphal cells. Bars = 20 μ m.

inter-hyphal cells (Fig. 2B). The TEM structural features of *R. solani* AG 2-1 without CSB treatment were normal-looking with a dolipore septum (with glucan deposits), vacuoles, vesicles, and intact organelles such as mitochondria and paramural bodies, whereas the cytoplasm of the pathogen-infested hyphal cells was degenerated with an occasional accumulation of electron-dense inclusion bodies (Fig. 3). Cell wall degradation was noted in the *R. solani* AG 2-1 hyphal cells treated with CSB, which was indicated by the disentangled outer layer of hyphal cells (Fig. 3C). The morphological features of the fungal hyphae treated with CSB indicated that it inhibited hyphal growth.

Effect of a combined treatment of CSB and *T. harzianum* on *R. solani* AG 2-1 mycelial growth. SEM on *R. solani* AG 2-1 treated with CSB ethanol extract and *T. harzianum* in combination revealed severely distorted and destroyed hyphae, which were coiled by *T. harzianum* (Fig. 4A). The severely distorted pathogen hyphal cells were penetrated by *T. harzianum* hyphae, indicating hyperparasitism (Fig. 4B).

Fig. 3. Transmission electron micrographs of *Rhizoctonia solani* AG 2-1 hyphae, showing intact cell structures such as the cell wall (W), dolipore septum (DS) with deposit of glucan (G), vacuoles (V), vesicles (Ve), and paramural bodies (P) in the control (A, B), and degenerated cell structures such as degenerated cytoplasm (DC) and disentangled cell wall (arrow, W) and the accumulation of electron-dense inclusions (asterisks) in the *Cinnamomum loureirii* stem bark (CSB) treatment (C, D). Bars = 0.5 μ m.

Fig. 4. Scanning electron micrographs showing interactions between *Trichoderma harzianum* (T) and *Rhizoctonia solani* AG 2-1 (R) on medium amended with *Cinnamomum loureirii* stem bark (CSB), showing *T. harzianum* coiling around severely distorted *R. solani* AG 2-1 hyphae (A) and penetrating the *T. harzianum* hypha in a *R. solani* AG 2-1 cell (arrow) (B). Bars = 20 μ m.

Effect of EFB and CSB amendments on *Rhizoctonia* radish damping-off. Soil amendments with EFB and CSB at various concentrations significantly reduced the incidence of *Rhizoctonia* damping-off of radish (Table 3). The control effect increased with increasing application concentrations. The highest control effect (90.9%) of EFB powder was recorded when applied at a rate of 0.8% and 1.2% (w/w). However, the control effect decreased to 54.5% when applied at the lowest concentration of 0.1%. Applying CSB

Table 3. Effect of various doses of medicinal plant materials (EFB and CSB) on *Rhizoctonia* damping-off of radish

Treatment conc. (% w/w) ^a	EFB treatment ^b		CSB treatment	
	Disease incidence (%)	Control effect (%)	Disease incidence (%)	Control effect (%)
0.0	76.7 dc	0.0	70.5 f	0.0
0.1	34.9 c	54.5	54.5 de	22.7
0.2	23.3 bc	69.6	50.0 cde	29.1
0.4	14.0 ab	81.8	45.5 bcd	35.5
0.6	9.3 ab	87.9	38.6 bc	45.3
0.8	7.0 a	90.9	36.4 b	48.4
1.2	7.0 a	90.9	36.0 b	48.9
Control	0.0 a	–	0.0 a	–

^a Concentrations of medicinal plant materials amended in soil.

^b EFB (*Eugenia caryophyllata* powder), CSB (*Cinnamomum loureirii* powder).

^c Means within columns followed by the same letters are not significantly different by Duncan's multiple range test at $P = 0.05$.

Table 4. Effect of various components of *Trichoderma harzianum* on *Rhizoctonia* damping-off of radish

Treatments ^a		Disease incidence (%)	Control effect (%)
Without heat treatment	TH culture	38.8 c ^b	55.1
	TH culture filtrate	44.1 c	49.0
	TH washed mycelium	17.8 b	79.4
With heat treatment	TH culture	72.7 de	15.9
	TH culture filtrate	65.9 d	23.8
	TH washed mycelium	70.5 d	18.5
	RS	86.4 e	0.0
Control		0.0 a	–

^a RS (inoculated with *R. solani* AG 2-1 at 3% (w/w)), control (uninoculated)

^b Means followed by the same letters within the column are not significantly different at $P = 0.05$ by Duncan's multiple range test. TH, *Trichoderma harzianum*

was less effective than applying EFB, as the highest control value of CSB was 48.9% at an application concentration of 1.2%. The control efficiency of CSB also increased significantly with the increase of dose concentration up to certain level (0.8%) (Table 3). However, soil amendments with CSB at a rate of 1.2% showed adverse effects on radish growth, including much reduced plant height and weight compared to uninoculated controls (data not shown).

Effect of various components of *T. harzianum* on radish damping-off. Radish damping-off was suppressed by all of the *T. harzianum* components used (Table 4). However, the highest disease suppression was achieved in pots treated with the washed mycelia of *T. harzianum* with its control

Table 5. Effect of individual and combined treatments of *Trichoderma harzianum* and *Cinnamomum loureirii* on *Rhizoctonia* damping-off of radish

Treatments ^a		Disease incidence (%)	Control effect (%)
CSB (%)	TH (%)		
0.0	0.0	95.9 e ^b	0.0
	0.3	89.8 e	6.4
	0.6	74.3 c	22.5
0.05	0.0	87.8 e	8.4
	0.3	54.3 cd	37.1
	0.6	41.2 c	57.0
0.1	0.0	73.7 de	23.1
	0.3	40.1 c	58.2
	0.6	12.5 b	87.3
Control		0.0 a	–

^a Treatment with CSB (*Cinnamomum loureirii*) powder and TH (*T. harzianum*) at various concentrations in potted soil.

^b Means within columns followed by the same letters are not significantly different at $P = 0.05$ by Duncan's multiple range test.

effect of 79.4%, whereas applying mycelia plus culture medium and its culture filtrate suppressed disease incidence moderately with control effects of 55.1% and 49.0%, respectively. When the washed mycelia of *T. harzianum* and other materials were heat-treated, their control efficacy decreased drastically to between 15.9% and 23.8%, indicating that the antagonistic activity of *T. harzianum* may be related to biological factors.

Effect of individual and combined treatments of CSB and *T. harzianum* on radish damping-off. Applying CSB and *T. harzianum* individually or in combination reduced the severity of radish damping-off caused by *R. solani* AG 2-1 at all doses tested, but was more effective when two were combined than when either treatment was used alone (Table 5). When CSB and *T. harzianum* were applied separately at dose concentrations of 0.05-0.1% and 0.3-0.6% (W/W), it resulted in 8.4-23.1% and 6.4-22.5% disease control, respectively. In contrast, the combined treatments of CSB powder and *T. harzianum* at various dose concentrations greatly increased the control efficacy, with disease control values of 37.1-87.3%.

Effect of the CSB amendment on *T. harzianum* growth in rice-bran peat medium. The population of *T. harzianum* in rice-bran peat medium with or without CSB powder grew continuously until 24 days after inoculation (Fig. 5). The *T. harzianum* populations at 5 days after inoculation were 4.5, 4.6, and 4.6 log CFU/g medium containing CSB powder at a rate of 1.0%, 0.1%, and 0.0% (control), respectively, showing that population growth of *T. harzianum* in

Fig. 5. Effect of *Cinnamomum loureirii* stem bark (CSB) amendment doses (YG1 = 1% CSB powder, YG0.1 = 0.1% CSB powder) on growth of *Trichoderma harzianum* in rice-bran peat medium.

Table 6. Effect of formulated *Trichoderma harzianum* in peat medium on *Rhizoctonia* damping-off of radish

Treatment ^a	Disease incidence (%)	Control effect (%)
Control	0.0 a ^b	—
RS	100.0 d	0.0
Peat	100.0 d	0.0
TH + 0.00% CSB	77.1 c	22.9
TH + 0.01% CSB	47.5 b	52.5
TH + 0.1% CSB	19.4 b	80.6

^a Control (uninoculated), RS (inoculated with *R. solani* AG 2-1 only at a 0.03 ratio, w/w); peat (0.1% (w/w) peat powder only), TH (*T. harzianum* cultured for 14 days in rice-bran peat medium amended with 0.0%, 0.01%, and 0.1% (w/w) CSB powder).

^b Means followed by the same letters within the column are not significantly different at $P = 0.05$ by Duncan's multiple range test.

rice-bran peat medium was not affected by the CSB amendment at the tested concentrations (Fig. 5).

Effect of a formulated biocontrol agent on radish damping-off. *T. harzianum* in rice-bran peat medium amended with CSB powder efficiently reduced disease severity of *Rhizoctonia* radish damping-off. *T. harzianum* culture without a CSB amendment had a control effect of 22.9%, but the control effect increased to 52.5% and 80.6% after adding 0.05% and 0.1% CSB to the *T. harzianum* treatments, respectively (Table 6).

Discussion

Among the 55 medicinal plant materials tested for antifungal activity against *R. solani* AG 2-1, six of them, namely CSB, EFB, PR, CR, ZF, and MSB, had moderate to strong antifungal activity against *R. solani* AG 2-1. In particular, EFB and CSB were highly antagonistic toward

R. solani AG 2-1, but EFB also inhibited mycelia growth of *T. harzianum* significantly. This antifungal nature of the plant materials was more antagonistic to the pathogen than to the biocontrol agent, which may have enhanced control efficacy when these materials were applied together with the biocontrol agent for pathogen control. The antifungal activity of *E. caryophyllata* essential oil has been demonstrated on several food-borne fungi (Lopez et al., 2005; Ranasinghe et al., 2002; Velluti et al., 2004), and one of its ingredient, eugenol, displays acaricidal activity against *Dermatophagoides* spp. (Kim et al., 2003; Lee and Kim, 2006). CSB has shown antifungal activity against *Alternaria alternata* (Kim et al., 1996) and acaricidal activity against *Dermatophagoides* spp. (Kim, 2002). Its antimicrobial and antifungal properties have drawn great attention from several researchers (Cheng et al., 2006; Kim et al., 2004; Lee et al., 2005), but so far, no synergistic effects of these materials with microbial biocontrol agents had been examined.

The synergistic effect of antifungal activity was tested with a combined treatment of *T. harzianum* and EFB or CSB in an *in vitro* system. The combined treatment of EFB and *T. harzianum* did not effectively enhance the control efficacy of *Rhizoctonia* damping-off of radish, compared to the use of either treatment alone. In contrast, control efficacy was greatly enhanced by the combined treatment of CSB and *T. harzianum* in various concentrations, compared to the use of either treatment alone. The combined treatment of CSB and *T. harzianum* enhanced the control efficacy more than the sum of single treatments alone in pot experiments, and compared to the combined treatments of FEB and *T. harzianum*, probably because of large differential antibiosis. CSB may also enhance the competitiveness of the biocontrol agent over the pathogen, thus increasing control efficacy due to the differential antibiosis.

The CSB ethanol extract induced hyphal swelling and rugged morphology of *R. solani* AG 2-1 hyphal cells, of which the mode of action may involve the release of volatile antimicrobial substances such as cinnamaldehyde (Kim et al., 1996). *T. harzianum* hyphae grew along those of *R. solani* AG 2-1 and formed branches that coiled around and penetrated the pathogen hyphal cells. Considering these microscopic aspects and that the control efficacy of *T. harzianum* mycelia washed in distilled water to remove antibiotic substances was the highest among all treatments, the major mode of action for controlling *R. solani* AG 2-1 may be mycoparasitism. Mycoparasitism is the chief mechanism for the biocontrol of *Sclerotinia sclerotiorum* by *T. harzianum* (Jacob et al., 1996). The major mode of action for the combined treatment activity of CSB and *T. harzianum* may involve severe destruction of pathogen hyphae by the initial antagonistic effect of CSB and the subsequent hyperparasitism of *T. harzianum*, which may

further damage and weaken hyphae. Because of these synergistic effects in addition to the differential antibiosis, combined treatments may further enhance the efficacy of controlling *R. solani* AG 2-1 than either single treatment alone.

The amendment of 1.0% (w/w) CSB powder in rice-bran peat medium did not inhibit the *T. harzianum* growth rate as compared to the control medium without CSB powder. Furthermore, this formulation significantly enhanced control efficacy. These results suggest that CSB can be added to proper media to mass produce the biocontrol agent, which can be directly used to produce the biocontrol formulation and be applied to the soil without an additional CSB amendment to improve control efficacy. This biocontrol method would reduce the amount of biocontrol agent needed and increase the convenience of application.

Acknowledgments

This study was conducted with the support of the "Research Cooperating Program for Agricultural Science & Technology Development (Project No. OA-20060501036009)," RDA, Republic of Korea.

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