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, 1H NMR, and 13C 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 eco-systems.

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 antagonists 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. Daeung (seeds from Jeil Seed and Agricultural Products Co., Jeung Byong-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 (95%). Ethanol extracts, dried overnight, and placed in 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.

**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 again with 0.1 M phosphate buffer solution three times for 15 min per wash. The specimens were then fixed in 2% OsO₄, in 0.2 M phosphate buffer (pH 7.2) at 4°C for 1 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 30%, 70%, 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, 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.

**Mass Spectrometry, 1H-NMR, and 13C-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 1H-NMR and 13C-NMR, respectively.

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

Seeds of the radish cultivar Daeung were sown in plastic containers (11×9×4.5 cm) containing a soil mixture (organic matter:soil=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* inoculum, 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
CONTROL OF RHIZOCTONIA WITH TRICHTODERMA AND PEONY ROOT BARK

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

<table>
<thead>
<tr>
<th>Species</th>
<th>Plant parts used</th>
<th>T. harzianum</th>
<th>R. solani AG2-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anethum graveolens</td>
<td>Fruit</td>
<td>4.5±0.1</td>
<td>4.5±0.0</td>
</tr>
<tr>
<td>Cinnamomum cassia</td>
<td>Stem bark</td>
<td>4.2±0.2</td>
<td>4.4±0.0</td>
</tr>
<tr>
<td>Cnidium officinale</td>
<td>Rhizome</td>
<td>4.2±0.1</td>
<td>4.4±0.0</td>
</tr>
<tr>
<td>Coptis chinensis</td>
<td>Rhizome</td>
<td>4.5±0.0</td>
<td>4.5±0.0</td>
</tr>
<tr>
<td>Paeonia suffruticosa</td>
<td>Root bark</td>
<td>4.4±0.2</td>
<td>4.5±0.0</td>
</tr>
<tr>
<td>Phellodendron amurense</td>
<td>Stem bark</td>
<td>4.5±0.0</td>
<td>4.5±0.1</td>
</tr>
</tbody>
</table>

Data are means ± standard deviations of 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. 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 hyphal cells shortened in length (L) and swelled (S).

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.
structure, which is identical to paeonol. $^1$H-NMR and $^{13}$C-NMR spectra were recorded with CD$_3$OD as the solvent, and the chemical shift $\delta$ (ppm) was measured and summarized in Table 2. $^1$H NMR of the antifungal material showed two methyl 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$_9$H$_{10}$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>
<thead>
<tr>
<th>No.</th>
<th>Chemical shifts (ppm)</th>
<th>$^1$H</th>
<th>$^{13}$C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>115.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>166.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>6.40 (s)</td>
<td>101.72</td>
<td>167.72</td>
</tr>
<tr>
<td>4</td>
<td>6.48 (d, J=8.2 Hz)</td>
<td>108.32</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>7.77 (d, J=8.2 Hz)</td>
<td>133.98</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>204.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>2.54 (s)</td>
<td>26.32</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>3.83 (s)</td>
<td>56.09</td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Effect of medicinal plant materials on controlling radish damping-off caused by Rhizoctonia solani AG2-1.

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Disease incidence (%)</th>
<th>Control value (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paeonia suffruticosa</td>
<td>2.3±2.5</td>
<td>95.1*</td>
</tr>
<tr>
<td>Cinnamomum cassia</td>
<td>8.1±3.6</td>
<td>82.9</td>
</tr>
<tr>
<td>Coptis chinensis</td>
<td>16.7±1.8</td>
<td>64.6abc</td>
</tr>
<tr>
<td>Crinum officinale</td>
<td>16.9±3.1</td>
<td>64.2abc</td>
</tr>
<tr>
<td>Phellodendron amurense</td>
<td>32.7±9.6</td>
<td>30.9d</td>
</tr>
<tr>
<td>Anethum graveolens</td>
<td>34.0±7.4</td>
<td>28.9d</td>
</tr>
<tr>
<td>Untreated (control)</td>
<td>47.3±2.8</td>
<td>0.0f</td>
</tr>
</tbody>
</table>

*1% soil amendment (w/v).
Data are means ± standard deviations of two trials with five replications per treatment.
*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 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.

<table>
<thead>
<tr>
<th>Treatment concentration (%)</th>
<th>Disease incidence (%)</th>
<th>Control value (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRB</td>
<td>0.0</td>
<td>90.9±15.8</td>
</tr>
<tr>
<td>T. harzianum</td>
<td>0.0</td>
<td>90.9±15.8</td>
</tr>
<tr>
<td>0.05</td>
<td>0.2</td>
<td>53.4±4.8</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>46.3±5.4</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>44.1±8.7</td>
</tr>
<tr>
<td>0.08</td>
<td>0.0</td>
<td>42.5±1.7</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>23.3±12.6</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>13.2±4.5</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>11.2±6.2</td>
</tr>
<tr>
<td>0.1</td>
<td>0.0</td>
<td>41.2±10.6</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>26.9±7.6</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>12.3±3.1</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>14.9±10.7</td>
</tr>
</tbody>
</table>

LSD_{0.05} = 12.9

Data are means ± standard deviations of five replications.
*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.

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 crop 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 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.
(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 antagonistic effect of PRB or its extract with other soilborne pathogens, 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]. 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