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Article

Biocontrol of Damping-Off (*Rhizoctonia solani*) in Cucumber by *Trichoderma asperellum* T-5

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A fungal strain of *Trichoderma* having strong chitinolytic activity was isolated from field soil enriched with crabshell for several years. Based on 5.8S rRNA, partial 18S, 28S rRNA genes, ITS1, ITS2 sequence analysis and morphological characteristics, the fungus was identified as *Trichoderma asperellum* and named as *Trichoderma asperellum* T-5 (TaT-5). The fungus released lytic enzymes such as chitinase and β-1, 3-glucanse, and produced six antifungal substances in chitin broth medium. To demonstrate the protective effect of TaT-5 against damping off in cucumber plant caused by *Rhizoctonia solani*, TaT-5 culture broth (TA), chitin medium (CM) and distilled water (DW) were applied to each pot at 10 days after sowing, respectively. Then, the homogenized hyphae of *R. solani* were infected to each pot at 1 week after TaT-5 inoculation. During experimental period, fresh weight of shoot and root in cucumber plant more increased at TA treatment compared to other treatments. PR-proteins (β-1, 3-glucanase and chitinase) activities in cucumber leaves markedly increased at CM and DW treatments, but the activity slightly increased and then decreased at TA treatment at 3 days after infection of *R. solani.* The activity of PR-proteins activities in cucumber roots at all treatments decreased with time where the degree of decrement was more alleviated at TA treatment than CM and DW. These results suggest that the lytic enzymes (chitinase and β-1, 3-lucanse) and antifungal substances produced by TaT-5 can reduce the pathogenic attack by *R. solani* in cucumber plants.

Keywords : *Trichoderma* sp., chitinase, β-1, 3-lucanse, antifungal substance

Introduction

Cucumber is one of the most popular vegetables in Korea. However, continuous monocropping has caused a lot of diseases in cucumber plants. Especially, damping-off caused by *Rhizoctonia solani* is regarded as one of serious diseases. To control pathogens, large amount of chemical substances were used, which caused various environmental problems such as chemical residue in soil, toxicity and increase in resistant pathogens. Biological control, the uses of microorganisms or their metabolites against plant diseases, offers an attractive for the management of plant diseases without the negative impact of synthetic chemical control (Wang et al., 2002).

In recent years, the antagonistic microorganisms such as *Bacillus* sp., *Paenibacillus* sp., *Pseudomonas* sp., and *Trichoderma* sp. were extensively studied as biocontrol agents (Lee et al., 2005; Jung et al., 2003; Hoffland et al., 1996; Yedidia et al., 2000). Among these antagonistic microorganisms, genus Trichoderma has been widely used owing to induction of systemic acquired resistance in plant, plant growth promotion, antagonistic activity against a wide range of plant pathogenic fungi, and cell wall degrading enzymes against many plant pathogens (Enkerli et al., 1999; Inbar et al., 1994; Paulitz et al., 2001; Lorito, 1998).

Genus *Trichoderma* has been known to release lytic enzymes such as chitinase, β-1, 3-glucanase (Sanz et al., 2004; Nampoothiri et al., 2004). Therefore, these lytic enzymes have been considered to be effective compounds for biological control of plant pathogens because of their ability in degrading fungi cell walls (Singh et al., 1999). Russell et al. (1994) reported that culture filtrates containing β-1, 3-glucanase and chitinase was capable of degrading *R. solani* mycelium. Inbar et al. (1995) reported that the induction of chitinolytic enzymes in the biocontrol agent *Trichoderma harzianum* during parasitism on *Sclerotium rolfsii*. Viterbo et al. (2002) described that endochitinase CHIT36 recombinant

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protein from the yeast *Pichia pastoris* was active against different phytopathogens, conforming the importance of this endochitinase in the mycoparasitic activity of *Trichoderma* antagonistic strains.

Benitez et al. (2004) described that the most *Trichoderma* strains produce volatile and non volatile toxic metabolites that impede colonization by antagonized microorganism ; among these metabolites, the production of harzianic acid, alamethicins, tricholin, peptaibols, antibiotics, 6-penthyl-α-pyrone, massoilactone, viridian, gliovirin, glisoprenins, heptelidic acid and others have been described. Ghisalberti et al. (1991) reported antibiotic metabolites in 7 strains of *Trichoderma*. Dennis et al. (1971) reported that 6-n- phntyl-2H-pyran-2 one inhibited growth of *Rhizoctonia solani* by only 1 day exposure. Endo et al. (1985) reported that heptelidic acid was an antifungal sesquiterpene lactone obtained from a strain of *T. Koningii* and *T. viride*, which was active against *R. solani* and anaerobic bacteria such as *Bacteroides fragilis*. Okuda et al. (1982) reported that liquid cultures of *T. hamatum* produced Isonitrin A, which was broadly active against certain Gram-positive and negative bacteria, yeasts, and pathogenic fungi.

The combination of hydrolytic enzymes and antibiotics resulted in a higher level of antagonism than that obtained by either mechanism alone (Benitez et al., 2004). Synergetic effects between an endochitinase and gliotoxin from *T. harzianum,* and between hydrolytic enzymes and peptaibols on conidial germination of *B. cinerea* were well known (Howell, 2003). Benitez et al. (2004) revealed that β-1, 3-glucanases strongly inhibit spore germination or the growth of pathogens by synergistic cooperation with chitinases and antibiotics.

Production of pathogen-related (PR) proteins in plants are known to be an important protective response to pathogenic factors (Mohammadi et al., 2002; El-Shora, 2002). Previous studies indicated a transient increase of PR-protein such as chitinase and β-1, 3-glucanases, interpreting as an early defense response from the plant to the invading fungus (Pozo et al., 2002; Caruso et al., 1999). Pozo et al. (2002) reported that PR proteins were accumulated in tomato roots infected with *Phytophtora parasitica* and chitinase were accumulated in cucumber plants inoculated with *T. harzianum* (Yedidia et al., 1999). These enzymes have been correlated with defense against pathogens in several plants, such as tobacco, tomato, cabbage, cucumber and rice (Tuzun, 2001; Zhang et al., 1996; Nandakumar et al., 2001; Silva et al., 2004). These enzyme production was changed by infection of pathogen or inoculation of plant growth promoting rhizobacteria (PGPR) and plant growth promoting fungi (PGPF) such as *Trichoderma* sp. (Lee et al., 2005; Chen et al., 2000; Yedidia et al., 2000).

The purposes of the research are to investigate lytic enzymes and antifungal substances released by *T. asperellum* T-5 (TaT-5) and to explain changes of PR-proteins and lignification-related enzymes induced in cucumber plants in response to TaT-5 and *R. solan* causing damping off.

Materials and Methods

Microorganism isolation Soils were collected from the field enriched with crabshell in Yeonggwang, Korea. Soils were serially diluted with sterile water until a rate of 10^{-5}, inoculated on chitin agar medium containing colloidal chitin 0.5% ; Na_2HPO_4 0.2% ; KH_2PO_4 0.1% : NaCl 0.05% ; 0.1% ; NH_4Cl 0.1% ; $MgSO_4 \cdot 7H_2O$ 0.05% ; $CaCl_2 \cdot 2H_2O$ 0.05% ; Yeast Extract 0.05% ; Agar 2% and then incubated at 26℃ for 5 days. Ten strains of *Trichoderma* were isolated by morphological peculiarity and selected by serially plating onto the chitin agar medium. An active strain was identified using 5.8S rRNA, partial 18S, 28S rRNA genes, ITS1, ITS2 sequence analysis and morphological characteristics.

Enzyme assay The isolated fungus was cultured in chitin broth medium (pH 5) at 26℃ for 9 days on the 170 rpm shaking incubator. During shaking incubation, samples were taken at 1, 2, 3, 5, 7, 9 days. Each sample was centrifuged at 8,000 rpm for 5 min and then the supernatant was used for analyze enzyme activities.

The isolated fungus was cultured in laminarin broth medium containing laminarin 0.5% ; Na_2HPO_4 0.2% ; KH_2PO_4 0.1% : NaCl 0.05% ; 0.1% ; NH_4Cl 0.1% ; $MgSO_4 \cdot 7H_2O$ 0.05% ; $CaCl_2 \cdot 2H_2O$ 0.05% ; Yeast Extract 0.05% ; Agar 2% (pH 5) at 26℃ for 9 days on the 170 rpm shaking incubator. During shaking incubation, samples were taken at 1, 2, 3, 5, 7, 9 days. Each sample was centrifuged at 8,000 rpm for 5 min and then the supernatant was used for analyze enzyme activities.

Identification of produced antifungal compound by *Trichoderma asperellum* Fungus was cultured at 26℃ for 5 days in the chitin broth (pH 5.0) and then centrifuged at 6,000 rpm for 15 min. 16 L supernatant

was adjusted to pH 3.0 with 1N HCl and mixed with ethylacetate (EtOAc). Antifungal activity was found on the EtOAc phase, and it was concentrated using a rotary evaporator with vacuum (Bűchi, Switzerland). The concentrated EtOAc fraction was separated to the water soluble phase and the insoluble (methanol soluble) phase. The antifungal material of water soluble phase was extracted by n-butanol. Antifungal activity was detected on the soluble and insoluble phase, respectively. Each phase was purified using the Diaion HP-20 ion exchange chromatography (Mitsubishi chemical Co., Japan) and it was eluted with stepwise gradient of water methanol (100:0, 50:50, 30:70, 0:100, v/v). Each fraction of the elute was concentrated using a evaporator, and the antifungal activity of each fraction against *R. solani* was conformed using the paper disk agar diffusion method. The 70% methanol and 100% methanol elute, which had strong antifungal activity, was purified using sephadex LH-20 column chromatography (25-100μ, SIGMA, Sweden) and it was eluted two times with methanol at a flow rate of 0.2ml min^{-1}. The 3ml of each fraction was harvested using a fraction collector (GILSON FC203B Fraction collector), and all the fractions were confirmed by the paper disk-agar diffusion method for antifungal activity. The antifungal activity fraction was performed to high performance liquid chromatography (HPLC) using a C_{18} reversed-phase column (Symmetry Prep C_{18} 7.8 X 300 mm, waters). The HPLC was performed with 80% acetonitrile at a flow rate 0.7ml min 1 using a SHIMADZU system. The eluate of each peak was collected at 210nm by a SPD-10A UV-VIS detector (Shimadzu) and each fraction was performed to the paper disk agar diffusion method for antifungal activity.

Plant growth and inoculation Cucumber (*Cucumis sativus* L.) plants were grown in a growing mixture (bed soil : vermiculite, 1 : 4, v : v). Cucumber seedlings were grown at 27℃ in an artificially illuminated room with a 16 hrs (hours) photoperiod. Plants were harvested at 0, 1, 2, 3, 5, 7, 8, 9, 11 and 13 days after inoculation *T. asperellum* T-5 (TA). Each harvested Plants was then carefully washed in running tap water, and fresh weight of shoots and roots was measured. *T. asperellum* T-5 was cultured at 26℃ in chitin medium as described above except agar for 5 days. The TA was treated with 20 ml of culture solution at 1 week after sprout. Other treatments were prepared with same volume of sterilized diluted water (DW) and sterilized chitin medium (CM) instead of cultures solution, respectively. The 5 mm in diameter of *R. solani* plugs which taken from a PDA medium was inoculated to the 250 ml Erlenmeyer flasks containing 100 ml of sterile PDB medium. The flasks were incubated without shaking in the dark at 26℃ for 1 week. Then the mycelia on the surface of medium were homogenized in the sterile water and poured into the soil surrounding cucumber.

Enzyme extract in plant Plant sample was divided into shoots and roots. The sample was washed under running tap water and dried gently and then ground with a mortar and pestle under liquid nitrogen. The ground sample was homogenized with 3 ml of 10mM KH_2PO_4 buffer (pH 7.0) (Yedidia et al., 2000). The homogenate was centrifuged at 12,000 g for 10 min at 4 , and the supernatant was collected and kept at -20℃ until enzyme analysis.

Detection of pathogenesis related proteins activity Chitinase activity was assayed by measuring the amount of the reducing end group of produced N-acetyl glucosamine (NAG) from colloidal chitin (Lingappa et al., 1962). The assay mixture consisted of 0.05 ml of supernatant, 0. 5 ml of 0.5% colloidal chitin and 0.45 ml 50mM sodium acetate buffer (pH 5.0). After incubation at 37℃ for 1 h, 200 μl of 1 N NaOH was added and heated 15 min in boiling water for the reaction stop. After centrifugation at 10000 g for 5 min, 750 μl of supernatant was mixed with 1 ml of Schales' reagent (0.5 M sodium carbonate and 1.5 mM potassium ferricyanide). Chitinase activity was calculated by measuring O.D. at 420 nm, and these data were compared with data from a NAG standard curve for NAG concentration calculation. One unit of chitinase activity was defined as the amount of enzyme that liberated 1 μmol of NAG per h at 37℃.

β-1, 3-glucanase activity was assayed by measuring the amount of the reducing end group of produced glucose from laminarin (Yedidia et al., 2000). The assay mixture consisted of 50 μl supernatant, 50 μl of 10 mg ml^{-1} laminarin, and 400 μl of 50 mM sodium acetate buffer (pH 5.0). After incubation at 37℃ for 1 h, 1.5 ml of DNS was added, and the reaction stopped by heating in boiling water for 5 min. Glucose concentration was calculated by measuring O.D. at 550 nm, and these data were compared with data from a glucose standard curve for glucose concentration calculation. One unit of β-1, 3-glucanase activity was defined as the amount of enzyme

that liberated 1 mol of laminarin per hr at 37℃.

Results

Selection of antagonistic fungus. *Trichoderma* sp. T-5 having antifungal activity was isolated from enriched field with crabshell in Bubsung-Myun, Yeonggwang, Korea. Antifungal activity of *Trichoderma* sp. T-5 against *R. solani* was shown in Fig. 1.

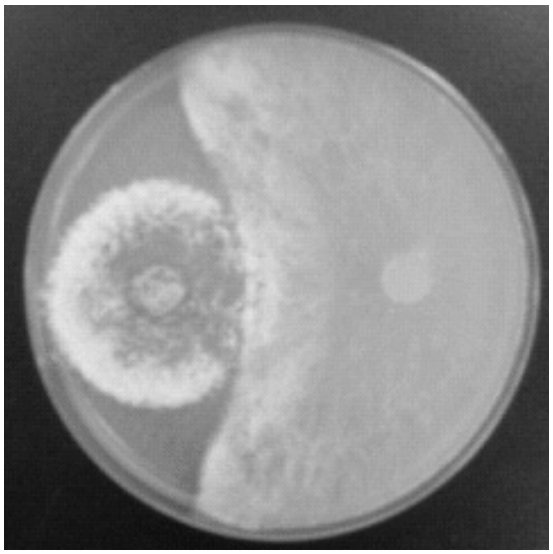

Fig. 1. Inhibition zone of *Trichoderma* sp. T-5 against *R. solani* on PDA medium at 26℃.

Identification of *Trichoderma* sp. T-5 Morphological characteristics of *Trichoderma* sp. T-5 was shown in Fig. 2 and 3. According to the characteristics such as production of chlamydospores in a short time and the formation of warted condial wall which was reported by Lieckfeldt et al. (1999), the fungus was indentified as *Trichoderma asperellum*. Based on 5.8S rRNA and partial 18S and 28S rRNA genes, ITS1, ITS2 sequences, the fungus was identified as *Trichoderma asperellum* with 100% similarity to AJ230668.1 through BLASTN searching.

Enzyme activity of *Trichoderma* sp. T-5

Cell wall degradation enzyme activity of *Trichoderma* sp. T-5 was examined during 9 days of saking culture at 26℃. Chitinase activity rapidly increased at 2 day, slowly increased till 7 day and then decreased at 9 day as shown

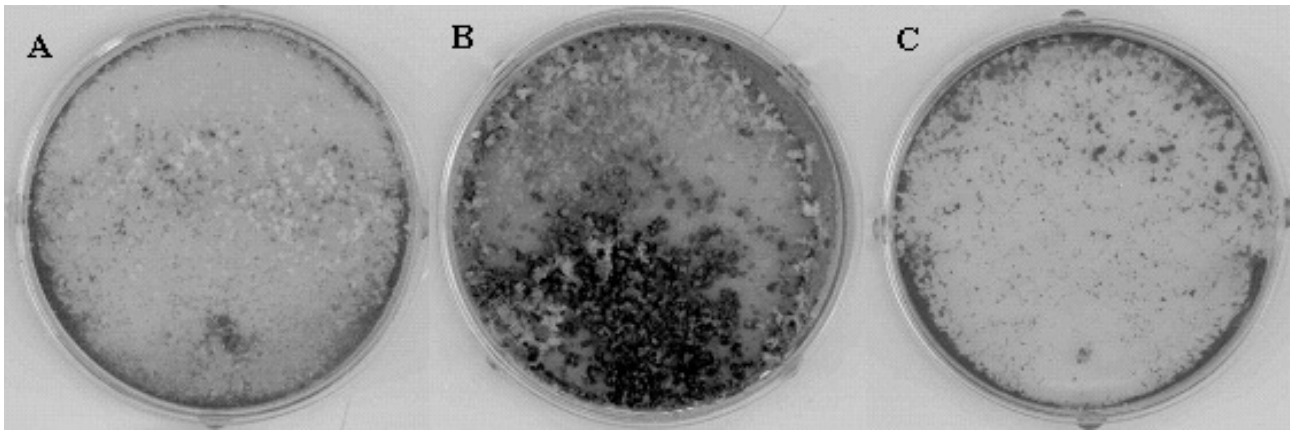

Fig. 2. Colony formation of *Trichoderma* sp. T-5 on the Potato Dextrose Agar (A), Oatmeal Agar (B) and Malt Extract Agar (C) after 7 days inoculation at 25℃.

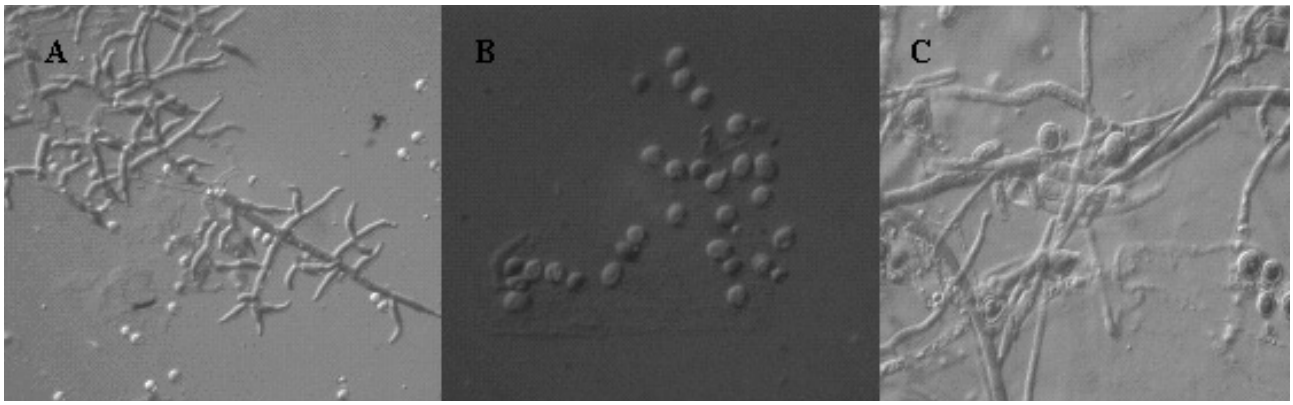

Fig. 3. Micrographs of *Trichoderma* sp. T-5 conidiophores and phialides (A), conidia (B), chlamydospores (C).

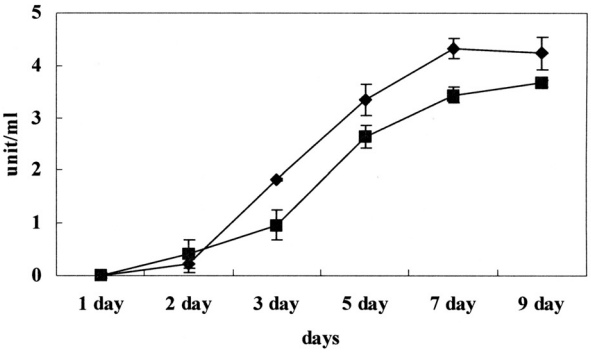

Fig. 4. Production of chitinase (-◆-) and β-1, 3 glucanase (-■-) by *T. asperellum* T-5 in chitin broth medium and laminarin medium at 26℃, respectively. Mean values were 3 replicates.

in Fig. 6. β-1, 3-glucanase activity rapidly increased at 3 day, slowly increased till 9 day as shown in Fig. 4.

HPLC spectrum of antifungal substances produced by *T. asperellum* T-5 The fractions were further purified by Diaion HP 20 column, silica gel column and sephadex LH-20 column. And then the fractions purified by HPLC, resulted in six fractions, t_R 22.3, 23.9, 25.6, 28.8, 30.2 and 37.3 (Fig. 5). Each fraction inhibited *R. solani* growth on the PDA medium as shown in Fig. 6.

Plant growth The changes in plant fresh weight were shown in Fig. 7. Interestingly, inoculation with *T. asperellum* T-5 increased the growth of cucumber over no-inoculum. Fresh weights of shoot and root were highest in TA plants.

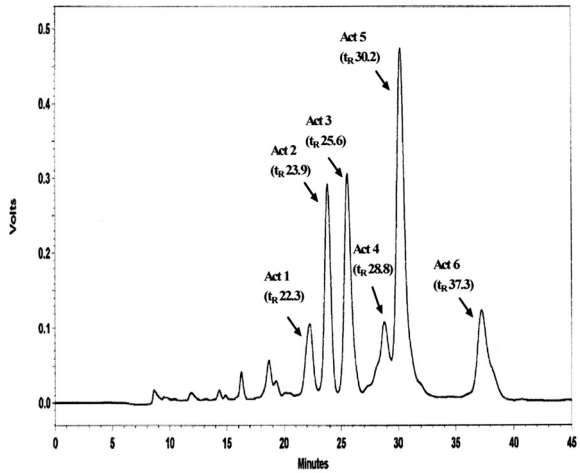

Fig. 5. HPLC spectrum of the crude extract after sephadex LH -20 column chromatography, using a C_{18} reversed phase column (Symmetry Prep C_{18} 7.8 X 300 mm, Waters) and 80% acetonitrile at 0.7ml/min.

PR-proteins in cucumber plant PR-protein activities in each treated plants were investigated as shown in Fig. 8 and 9. Chitinase activity in leaves at TA plants rapidly increased 3 days after inoculation *T. asperellum* T-5 (Fig. 8-A). In roots, chitinase activity on TA plants decreased 3 days after inoculation *T. asperellum* T-5 (Fig. 8-B). Chitinase activities in cucumber leaves markedly increased at CM and DW, but the activity slightly increased and then decreased at TA at 9 days after inoculation of *T. asperellum* T-5 (2 days after infection of *R. solani* hyphae) (Fig. 8-A). Chitinase activities in cucumber roots at all treatments decreased with time

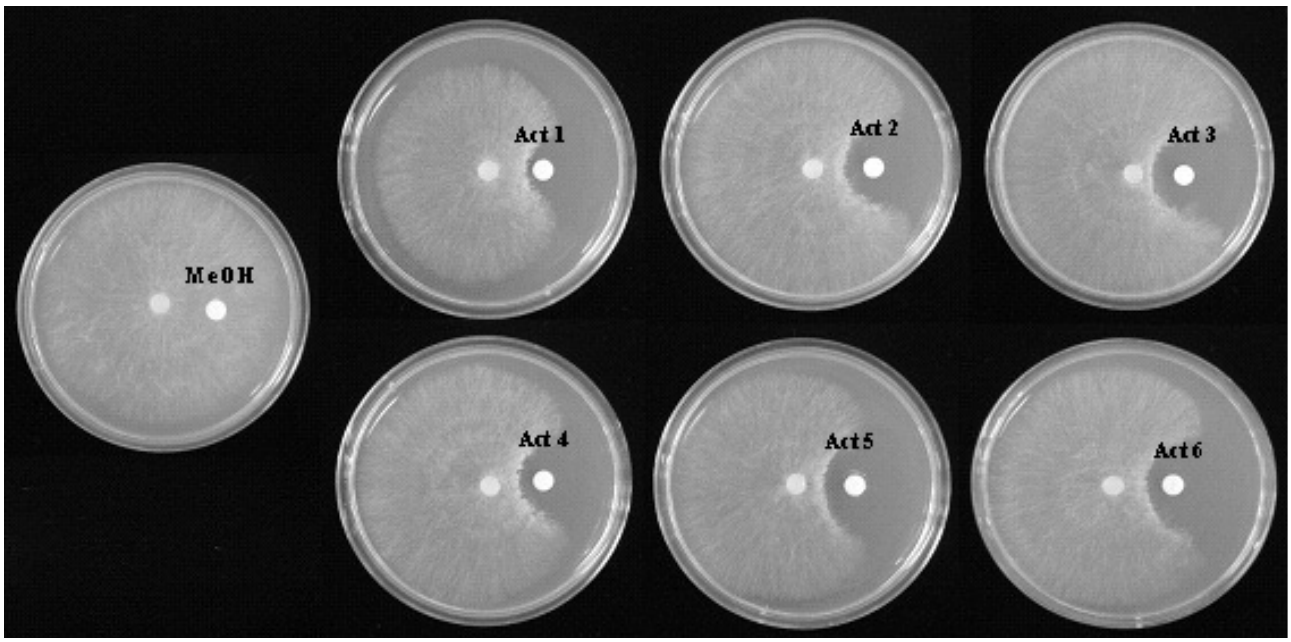

Fig. 6. Inhibitory activity of each fraction obtained from HPLC against *R. solani* was shown on the PDA medium. Methanol was loaded such as control.

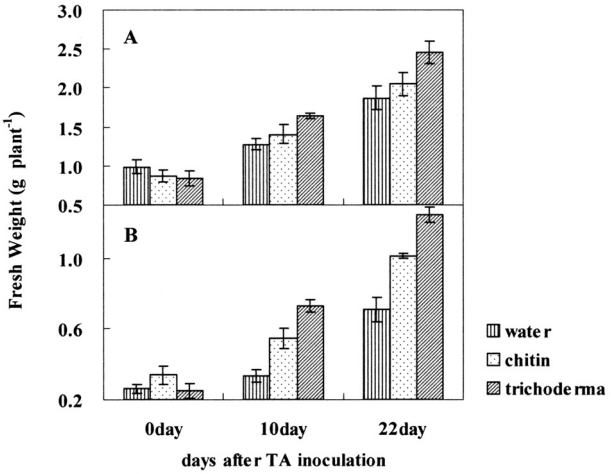

Fig. 7. Changes in fresh weight in cucumber shoot (A) and root (B) as influenced by diluted water (DW), chitin medium (CM) and *T. asperellum* T-5 culture broth (TA). Mean values were 3 replicates. Bars represent standard error.

where the degree of decrement was more reduced at TA than CM and DW (Fig. 8-B).

β-1, 3-glucanse activity in leaves at TA plants increased 3 days after inoculation *T. asperellum* T-5 (Fig. 9-A). In roots, β-1, 3-glucanse activity on TA plants decreased 3 days after inoculation *T. asperellum* T-5 (Fig. 9-B). β-1, 3-glucanse activities in cucumber leaves markedly increased at CM and DW, but the activity slightly increased and then decreased at TA at 9 days after inoculation of *T. asperellum* T-5 (2 days after infection of

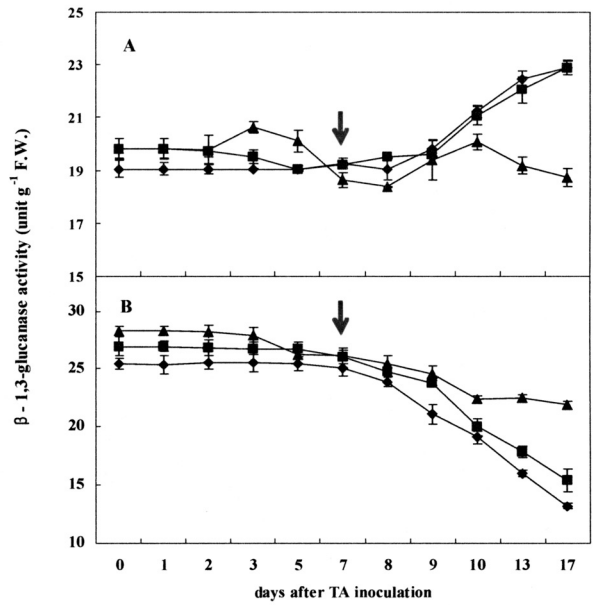

Fig. 9. Changes in β-1, 3 glucanase activity in cucumber shoot (A) and root (B) as influenced by diluted water (DW) (-◆-), chitin medium (CM) (-■-) and *T. asperellum* T-5 culture broth (TA) (-▲-). *R. solani* infection day was shown arrow (↓). Mean values were 3 replicates. Bars represent standard error.

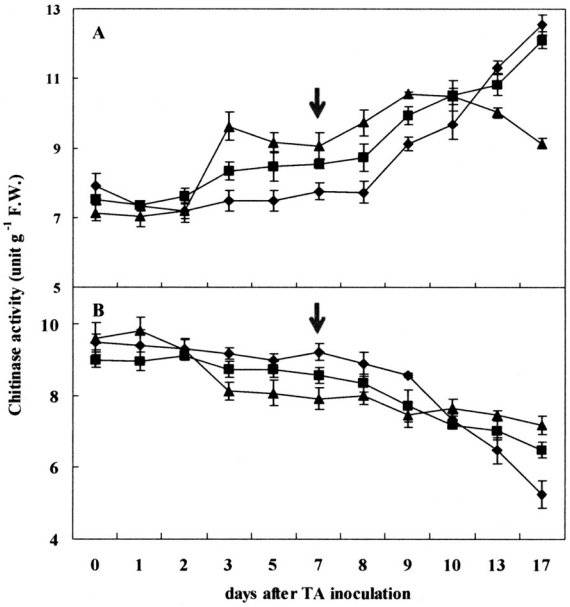

Fig. 8. Changes in chitinase activity in cucumber shoot (A) and root (B) as influenced by diluted water (DW) (-◆-), chitin medium (CM) (-■-) and *T. asperellum* T-5 culture broth (TA) (-▲-). *R. solani* infection day was shown arrow (↓). Mean values were 3 replicates. Bars represent standard error.

R. solani hyphae) (Fig. 9-A). β-1, 3-glucanse activities in cucumber roots at all treatments decreased with time where the degree of decrement was more reduced at TA than at CM and DW (Fig. 9-B).

Discussion

A fungal strain of *Trichoderma, Trichoderma asperellum* T-5 (TaT-5) with strong antifungal activity was isolated from field soil enriched with crabshell (Fig. 2, 3). This fungus released chitinase and β-1, 3-glucanase (Fig. 4). There have been many previous reports regarding the productions of cell-wall degradation enzymes by *Trichoderma* species and their antifungal activities.

Bara et al. (2003) reported that *T. asperellum* produced two extracellular β-1, 3-glucanases upon induction with cell wall from *R. solani*. Nampoothiri et al. (2004) have found optimal condition for production of extracellular chitinase by *T. harzianum* and the produced enzyme inhibited growth of *Aspergillus niger, A. ficuum, A. oryzae, Rhizopus oligosporus* and *Mucor racemosus*. Viterbo et al. (2001) reported that culture filtrates containing secreted CHIT36 as the sole chitinolytic enzyme completely inhibited the germination of *Botrytis cinerea* conidia. Also, several studies reported biological control of pathogens using lytic enzymes released by

bacteria, which degrade the cell wall of *Fusarium* sp. and *Pythium* sp. (Mitchell et al., 1961) and *R. solani* (Ordentlich et al., 1988; Jung et al., 2003). As shown in Fig. 2 and 6, chitinase and β-1, 3-glucanse produced by TA T-5 should inhibit pathogen growth through degradation of fungi cell wall.

Genus *Trichoderma* has been known to produce antifungal antibiotics in many studies (Benitez et al., 2004; Ghisalberti et al., 1991; Howell, 1998; Claydon et al., 1987). Howell (1998) reported that strains of *T. virens* with the best efficiency as biocontrol agents are able to produce gliovirin. Ghisalberti et al. (1991) reported that 6-n-pentenyl-2H-pyran-2 one showed potent inhibitory properties against a wide range of fungi and considerably reduced the rate of damping-off in lettuce seedlings by *R. solani*. Claydon et al. (1987) reported that 6 pentenyl pyrone isolated from *T. viride* exhibited some inhibitory properties towards *Phytophthora cinnamomi*. In this experiment, we found six antifungal compounds produced by TaT-5 in chitin broth medium (Fig. 5). The each compound has antifungal activity against *R. solani* (Fig. 6). According to our results, we suggest that TaT-5 have synergism effect against *R. solani* by antibiotics and lytic enzymes. The synergism effect has been reported by previous studies. Howell (2003) reported that synergism occurred in all cases, but the level depended on the antifungal activity of the enzymes. Suarez et al. (2004) concluded that a serine protease of 28-kDa with trypsin isolated from *Trichoderma* strain 2413 reduced the number of hatched eggs of root-knot nematodes and showed synergistic effects with other proteins produced during antagonistic activity of the strain. Rey et al. (2001) reported that *Trichoderma* strain 2413 has extracellular enzymes and α-pyrone, which strongly protected grape against *B. cinerea*.

TA T-5 showed plant growth promoting effect (Fig. 7). Shoots and roots fresh weights of the TA were more rapid increment than other treatments. Monte (2001) explained that several *Trichoderma* strains had abilities to promote plant weight, size, and foliar surface area. Kleifeld et al. (1992) reported that the fungus *T. harzianum* which was applied to pathogen-free soil, induced an increase in emergence of seedlings, plant height, leaf area and dry weight.

Most plants contain relatively low levels of constitutive enzymes and only begin to produce higher levels when subject to phytopathogens or other factors (Yedidia et al., 2000; Zhao et al., 2005). In this study, PR-proteins at the CM and the DW showed nearly no change in cucumber leaves until *R. solani* infection and then sharply increased. Interestingly, the increment of PR proteins in the TA was turned to decrement from 2 days after *R. solani* infection (Fig. 8A and 9A). PR-proteins activities in cucumber roots decreased at all treatment during the experimental period. However, the decrease was alleviated in TA compared to CM and the DW (Fig. 8B and 9 B). These results were widely consistent with the several previous reports. Yedidia et al. (1999) found that activities of PR proteins increased in cucumber seedlings following *T. harzianum* application. Lawrence et al. (1996) also reported that chitinase and β-1, 3-glucanase in leaves of susceptible tomato increased initially after inoculation with *Alternaria solani*. Moreover, these results are consistent with our previous results (Kim, 2006; Zheng et al., 2005) that PR proteins activities of pepper plants decreased in root after infection of P. capsici, while they increased in leaves. Our previous results (Lee et al., 2005; Kim, 2006; Zheng et al., 2005) reported that the change of PR proteins in pepper plants were detected in only plants infected with *P. capsici*. However, plants infected with P. capsici had damage and even died. Several reports revealed that even though PR-proteins increased to protect plants from disease at the early stage, most of plants died (Lawrence et al., 1996; Zheng et al., 2005). Accumulation of PR-proteins may be regarded as an indicator of plants response to infection by pathogens. A minimal change in the pathogenesis related protein activities in cucumber plant at TA was regarded as the cucumber plant response to the TA T-5 inoculation.

These results are consistent with our previous reports (Lee et al., 2005; Kim, 2006; Jung et al., 2004) regarding the change of PR-proteins in plants were not prerequisite for plant protection from disease, suggesting that the induction of those enzymes may be regarded as indicators of plants response to infection by pathogens.

In conclusion, minor changes in levels of PR-proteins in TA may be responsible for the suppression of damping-off in cucumber associated with produced lytic enzymes and strong antibiotic compounds from TaT-5. Although the suppression activity of TaT-5 against damping off is not complete, the use of TaT-5 as a biocontrol agent may supplement or minimize that of synthetic chemicals.

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References

Benitez, T., Rincon, A.M., Limon, M.C. and Codon, A.C. 2004. Biocontrol mechanisms of *Trichoderma* strains. Int. Microbial. 7, 249-260.

Bara, M.T.F., Lima, A.L. and Ulhoa, C.J. 2003. Purification and characterization of an exo-β-1, 3-glucanase produced by *Trichoderma asperellum*. FEMS Microbiol. Lett. 219, 81-85.

Chen, C., Belanger, R.R., Benhamou, N., and Paulitz, T.C. 2000. Defense enzymes induced in cucumber roots by treatment with plant growth promoting rhizobacteria (PGPR) and *Pythium aphanidermatum*. Physiol. Mol. Plant Pathol. 56, 13-23.

Caruso, C., Chilosi, G., Caporale, C., Leonardi, L., Bertini, L., Magro, P., and Buonocore, V. 1999. Induction of pathogenesis related proteins in germinating wheat seeds infected with *Fusarium culmorum*. Plant Sci. 140, 107-120.

Claydon, N., Allan, M., Hanson, J.R., and Avent, A.G. 1987. Antifungal alkyl pyrones of *Trichoderma harzianum*. Transactions of the British Mycological Soc. 88, 503-513.

Dennis, C., and Webster, J. 1971. Antagonistic properties of species groups of *Trichoderma*. Ⅱ. Production of volatile antibiotics. Transactions of the British Mycological Soc. 57, 41-48.

El Shora, H.M. 2002. Properties of phenylalanine ammonia lyase from marrow cotyledons. Plant Sci. 162, 1-7.

Enkerli, J., Felix, G., and Boller, T. 1999. Elicitor activity of fungal xylanase does not depend on enzymatic activity. Plant Physiol. 121, 391-398.

Endo, A., Hasumi, K., Sakai, K., and Kanbe, T. 1985. Specific inhibition of glyceraldehydes-3-phosphate dehydrogenase by koningic acid (heptelidic acid). J. Antibiot. 38, 920-925.

Ghisalberti, E.L., and Sivasithamparam, K. 1991. Antifungal antibiotics produced by *Trichoderma* spp. Soil Biol. Biochem. 23, 1011-1020.

Howell, C.R. 1998. The role of antibiosis in biocontrol. In: Harman GE, Kubicek CP (eds) *Trichoderma & Gliocladium,* vol. 2. Taylor & Francis, Padstow, pp 173-184.

Howell, C.R. 2003. Mechanisms Employed by *Trichoderma* species in the Biological Control of Plant Diseases: The History and Evolution of Current Concepts. Plant dis. 87, 4-10.

Hoffland, E. Hakulinen, J., and Pelt, J. 1996. Comparison of systemic resistance induced by avirulent and nonpathogenic *Pseudomonas* species. Phytopathology 86, 757-762.

Inbar, J. Abramski, M. Coen, D., and Chet, I. 1994. Plant growth enhancement and disease control by *Trichoderma harzianum* in vegetable seedlings grown under commercial conditions. Eur. J. Plant Pathol. 100, 337-346.

Inbar, J., and Chet, I. 1995. The role of recognition in the induction of specific chitinases during mycoparasitism by *Trichoderma harzianum*. Microbiology 141, 2823-.2829

Jung, W.J., An, K.N., Jin, Y.L., Park, R.D., Lim, K.T., Kim, K.Y., and Kim, T.H. 2003. Biological control of damping-off caused by *Rhizoctonia solani* using chitinase producing *Paenibacillus illinoisensis* KJA 424. Soil Biol. Biochem. 35, 1261-1264.

Jung, W.J., Jin, Y.L., Kim, Y.C., Kim, K.Y., Park, R.D., and Kim, T.H. 2004. Inoculation of *Paenibacillus illinoisensis* alleviates root mortality, activates of lignification-related enzymes, and induction of the isozymes in pepper plants infected by *Phytophthora capsici*. Biol. Control 30, 645-652.

Kim, S.J. 2006. Biocontrol of lite Blight (Phytophthora capsici) in pepper by chitin broth containing multitude of chitinolytic bacteria. A Master's Thesis, College of Agriculture and Life Sciences, Chonnam National University, Gwangju, Republic of Korea.

Kleifeld, O., and Chet, I. 1992. *Trichoderma harzianum* interaction with plants and effect on growth response. Plant Soil 144, 267-272.

Lee, H.J., Park, K.H., Shim, J.H., Park, R.D., Kim, Y.W., Cho, J.Y., Hwangbo, H., Kim, Y.C., Cha, G.S., Krishnan, H.B., and Kim, K.Y. 2005. Quantitative Changes of plant defense enzymes in biocontrol of pepper (*Capsicium annuum* L.) late blight by antagonistic *Bacillus subtillus* HJ927. J. Microbiol. Biotechnol. 15, 1073-1079.

Lingappa, Y., and Lockwood, J.L. 1962. Chitin media for selectived culture of actinomycetes. Phytopathology 52, 317-323.

Lorito, M. 1998. Chitinolytic enzymes and their genes. In *Trichoderma* and *Gliocladium* (Kubicek CP & Harman GE, eds), pp. 73-99. Taylor & Francis, London

Lawrence, C.B., Joosten, M.H.A.J., and Tuzun, S. 1996. Differential induction of athogenesis-related proteins in tomato by *Alternaria solani* and the association of a basic chitinase isozyme with resistance. Physiol. Mol. Plant P. 48, 361-377.

Lieckfeldt, E., Samuels, G.J., Nirenberg, H.I., and Petrini, O. 1999. A morphological and molecular perspective of Trichoderma wiride : Is it one or two species?. Appl. Environ. Microb. 65, 2418-2428.

Mohammadi, M., and Kazemi, H. 2002. Change in peroxidase and polyphenol oxidase acrivities in susceptible and resistant wheat heads inoculated with *Fusarium graminearum* and induced resistance. Plant Sci. 162, 491-498.

Mitchell, R., and Alexander, M. 1961. The mycolytic phenomenon and biological control of *Fusarium* in soil. Nature 190, 109-110.

Monte, E. 2001. Understanding *Trichoderma* : between biotechnology and microbial ecology. Int. Microbiol. 4, 1-4.

Nampoothiri, K.M., Baiju, T.V., Sandhya, C., Sabu, A., Szakacs, G., and Pandey, A. 2004. Process optimization for antifungal chitinase production by *Trichoderma harzianum*. Process Biochem. 39, 1583-1590.

Nandakumar, R., Babu, S., Wiswanathan, R., Raguchander, T., and Samiyappan, R. 2001. Induction of systemic resistance in rice against sheath blight disease by *Pseudomonas fluorescens*. Soil Biol. Biochem. 33, 603-612.

Okuda, T., Fujiwara, A., and Fujiwara, M. 1982. Correlation

between species of *Trichoderma* and production patterns of isonitrile antibiotics. Agr. Biol. Chem. 46, 1811-1822.

Ordentlich, A., Elad, Y., and Chet, I. 1988. The role of chitinase of *Serratia marcescens* in biocontrol of *Sclerotium rolfsii*. Phytopathology 78, 84-88.

Paulitz, T., and Belanger, R. 2001. Biological control in greenhouse systems. Annu. Rev. Phytopathol. 39, 103-133.

Pozo, M.J., Cordier, C., Dumas Gaudot, E., Gianinazzi, S., Barea, J.M., and Azcon Aguilar, C. 2002. Localized versus systemic effect of arbscular mycorrhizal fungi on defense responses to *Phytophthora* infection in tomato plants. J. Exp. Bot. 53, 525-534.

Rey, M., Delgado jarana, J., and Benitez, T. 2001. Improved antifungal activity of a mutant of *Trichoderma harzianum* CECT 2413 which produces more extracellular proteins. Appl. Microbiol. Biot. 55, 604-608.

Russell, J., Tweddell, Suha, H., Jabaji Hare, and Pierre, M. Charest. 1994. Production of chitinases and β-1, 3-glucanses by *Stachybotrys elegans*, a Mycoparasite of *Rhizoctonia solani*. Appl. Environ. Microb. 60, 489-495.

Sanz, L., Montero, M., Redondo, J., Liobell, A., and Monte, E. 2005. Expression of an β-1, 3-glucanase during mycoparasitic interaction of *Trichoderma asperellum*. FEBS J. 272, 493-499.

Singh, P.P., Shin, Y.C., Park, C.S., and Chung, Y.R. 1999. Biological control of *Fusarium* wilt of cucumber by chitinolytic bacteria. Phytopathology 89, 92-99.

Silva, H.S.A., Romeiro, R.S., Macagnan, D., Halfeld Vieira, B.A., Pereira, M.C.B., and Mounteer, A. 2004. Rhizobacterial induction of systemic resistance in tomato plants : non specific protection and increase in enzyme activities. Biol. control 29, 288-295.

Suarez, B., Rey, M., Castillo, P., Monte, E., and Liobell, A. 2004. Isolation and characterization of PRA1, a trypsin like protease from the biocontrol agent *Trichoderma harzianum* CECT 2413 displaying nematicidal activity. Appl. Microbiol. Biot. 65, 46-55.

Tuzun, S. 2001. The relationship between pathogen-induced systemic resistance (ISR) and multigenic (horizontal) resistance in plants. Eur. J. of Plant Pathol. 107, 85-93.

Viterbo, A., Haran, S., Friesem, Ramot, O., and Chet, I. 2001. Antifungal activity of a novel endochitinase gene (chit36) from *Trichoderma harzianum* Rifai TM. FEMS Microbiol. Lett. 200, 169-174.

Viterbo, A., Montero, M., Ramot, O., Friesem, D., Monte, E., Llobell, A., and Chet, I. 2002. Expression regulation of the endochitinase chit36 from *Trichoderma asperellum* (*T. harzianum* T-203). Curr. Genet. 42, 114-122.

Wang, S.L., Shih, I.L., Wang, C.H., Tseng, K.C., Chang, W.T., Ywu, Y.K., Ro, J.J., and Wang, C.L. 2002. Production of antifungal compounds from chitin by *Bacillus substilits*. Enzyme Microb. Tech. 31, 321-328.

Yedidia, I., Benhamou, N., and Chet, I. 1999. Induction of defense responses in cucumber plants (*Cucumis sativus* L.) by the biocontrol agent *Trichoderma harzianum*. Appl. Environ. Microb. 65, 1061-1070.

Yedidia, I., Benhamou, N., Kapulnik, Y., and Chet, I. 2000. Induction and accumulation of PR proteins activity during early stages of root colonization by the mycoparasite *Trichoderma harzianum* strain T-203. Plant Physiol. Biochem. 38, 863-873.

Zhang, W., Dick, W.A., and Hoitink, H.A.J. 1996. Compost-induced systemic acquired resistance in cucumber to *Pythium* root rot and anthracnose. Phytopathology 86, 1066-1070.

Zhao, H.C., Zhao, H., Wang, B.C., and Wang, J.B. 2005. Effect of local stress induction on resistance-related enzymes in cucumber seeding. Colloids and Surfaces B: Biointerfaces 43, 37-42.

Zheng, H.Z., Kim, Y.W., Lee, H.J., Park, R.D., Jung, W.J., Kim, Y.C., Lee, S.H., Kim, T.H., and Kim, K.Y. 2004. Quantitative changes in PR proteins and antioxidative enzymes in response to *Glomus intraradices* and *Phytophthora capsici* in pepper. J. Microbiol. Biotechn. 14, 553.

Trichoderma asperellum T-5를 이용한 오이 모잘록병(*Rhizoctonia solani*)의 생물학적 제어

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몇 년동안 게껍질이 풍부하게 있었던 밭토양에서 강한 키틴분해능력을 가진 *Trichoderma* 곰팡이를 분리하였다. 분리된 곰팡이의 5.8S rRNA, partial 18S, 28S rRNA genes, ITS1, ITS2 sequence 분석과 형태학적 특징을 살펴본 결과 Trichoderma asperellum으로 동정되었고, 이를 *Trichoderma asperellum* T-5 (TaT-5)로 명명하였다. 이 곰팡이는 chitianse와 β-1, 3-glucanase같은 lytic enzyme을 분비하며, 키틴배지 상에서 6가지의 항 곰팡이성 물질을 생산했다. *R. solani*가 원인인 오이의 모잘록병에 대해 TaT 5의 방제효과를 보기 위해서 TaT-5 배양액(TA), chitin medium(CM), 증류수(DW)를 씨를 심은 후 10일 째에 각 pot에 관주했다. 그리고 관주 1주일 후에 *R. solani*의 균사를 갈아서 각 pot에 주었다. 실험기간 동안에 오이의 지상부와 지하부 생체중은 다른 처리구에 비하여 TA 처리구가 더 많이 증가하였다. 오이 잎에서 PR-protein (chitianse, β-1, 3-glucanase)활성은 *R. solani* 감염 후 CM과 DW에서 증가를 보였고, TA 처리구에서는 증가하다가 감소하는 경향을 보였다. 뿌리에서는 모든 처리구가 감소하는 경향을 보였지만 TA 처리구가 CM과 DW 처리구보다 감소하는 정도가 적었다. 오이의 잎과 뿌리에서 lignification related enzyme(POD, PPO, PAL)활성은 R. solani 감염 초기에는 증가하다가 점점 감소하는 경향을 보였다. 이러한 결과들은 TaT-5에 의하여 생산된 lytic enzymes와 항 곰팡이성 물질들이 오이에 *R. solani*의 공격을 줄여준다고 생각된다.