Korean J. Soil Sci. Fert. **39**(1), -(2006)

Article

Effect of Chitin Compost on Biological control of Fusarium wilt in Tomato Field

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Biological control by chitinolytic microorganisms is being evaluated as management options for soilborne diseases. Forty kilograms of chitin compost (CTC) and control compost (CC) were amended on tomato plots (15m × 0.5m) 7 d before transplanting to evaluate enzymatic activities and the control of Fusarium wilt. Samples were taken on day 1, 3, 5, and 7, the day 1 corresponded to the 66 d after transplanting, the day on which the initial wilting symptoms occurred in plants of CC treated plots. The chitinase activity in soil of CTC was always higher compared to the control. Pathogenesis related (PR) protein (chitinase, β-1, 3-glucanase and peroxidase) activities in tomato roots in CC increased every day and showed marked differences compared to CTC. Wilting symptoms (96 d after transplanting) were reduced by 25% in CTC compared to the control. Protection of tomato plant may be correlated with the high levels of soil enzyme activities resulting from the chitin compost.

Key words : *Fusarium oxysporum* f. sp. *lycopersici,* chitinase, β-1, 3-glucanase and peroxidase activities

Introduction

To meet the increasing demands for fresh vegetables, continuous monocropping of vegetables are being carried out throughout the year and caused an increase of plant pathogens in soil. *Fusarium oxysporum* f. sp. *lycopersici*, which causes Fusarium wilt is one of the most common pathogens in tomato and economically limits the quality of production (Gao et al., 1998). Once the field is infected by the pathogen, its' quick dispersal *via* the irrigation system makes eradication mostly impossible (Alabouvette et al., 1998). Synthetic chemicals have long been used to reduce the incidence of plant diseases. However, ecological damage and environmental pollution resulting from the excessive use of chemicals have prompted a search for alternative methods (Larena and Melgarejo 1996). During the last decades, biologically active compounds produced by several microorganisms have been evaluated as alternatives to chemicals and are gaining great attention (Chet 1987; Cotxarrera et al., 2002; Larena and Melgarejo 1996; Larkin et al., 1996). However, a great part of these investigations were done *in vitro* and the effectiveness to suppress plant diseases was evident only at the early stage of plant growth or gave insufficient levels to control them (Chet 1987). Field application of these agents often failed to suppress pathogen infection because of their inability to compete with the indigenous microorganisms (Carolina et al., 2001).

It is well known that the cell wall of *Fusarium oxysporum* f. sp. *lycopersici* is composed of chitin that can be degraded by chitinase (Bartnicki-Garcia 1968; Schoffelmeer et al., 1999). Lee et al. (1997) also reported that purified chitinase inhibited growth of *F. oxysporum*. Furthermore, the amended chitin could serve as a substrate for chitinolytic bacteria to increase their populations and keep the soil with high chitinase activity long enough to maintain the protective function until harvest (Nopakarn et al., 2002). It is possible that the chitinolytic bacteria could reduce pathogenic fungi population through cell wall degradation by chitinase.

Production of PR-proteins (chitinase, β-1,3-glucanase and peroxidase) in plants are known to be an important protective response to pathogenic factors (Caruso et al., 1999). Increased chitinase and β-1,3-glucanase activities

Received : January 16. 2006 Accepted : January 30. 2006
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were observed in pepper (Kim and Hwang 1994) and tomato (Lawrence et al., 1996) due to inoculation of *Phytophthora capsici* and *Alternaria solani* respectively. Upon *F. culmorum* infection, peroxidase activity increased significantly in wheat (Caruso et al., 1999). However, several reports suggested that most plants infected with soil borne pathogens are severely damaged even though PR proteins were higher than in non-infected plants (Beffa et al., 1996; Hoffland et al., 1995; Zheng et al., 2004). It is questionable that increased PR-proteins can contribute to disease reduction.

The present study was designed to investigate the effect of chitin compost with crab shell substrate on chitinolytic activities against phytopathogens in the rhizosphere. We also explain whether increased chitinolytic activities could alleviate Fusarium wilt and demonstrate the relationships between increased chitinolytic activities in the rhizosphere and PR-proteins in plant root.

Materials and methods

Plant material and culture condition Tomato (*Lycopersicon esculentum Mill.*) seedlings were obtained from seeds sown in 30 mm diameter plastic cell plug trays filled with bed soil (Bio bed soil , Heongnong Seed Co.) and transplanted to the field 4 week after sowing. The field was heavily infested with *Fusarium oxysporum* f. sp. *lycopersici* due to continuous tomato cultivation on a large-scale and eight times of Fusarium wilt had been recovered during the last 10 years. No fumigant or fungicide had been applied in any part of the greenhouse at least 2 years prior to the experiment. Among the 53 plots in the green house, 10 plots in the middle of the green house were chosen for experiment (each 5 plots for amendment with chitin compost and control compost respectively). Chitin compost (CTC) or control compost (CC) was applied seven days before transplanting at 10 kg/plot and seventy-five tomato plants were transplanted to each plot (15 × 0.5 m). Throughout the experiment, temperature was maintained at 25-35℃ and 60-70% relative humidity and mineral fertilizers (N : P : K = 20 : 5.9: 12.8 g L^{-1}) were applied at every 10 d by per 10a through a nozzle expand.

Preparation of the composts Chitin compost was prepared by mixing 10% crab shell, 20% vermiculite, 40% rice straw, and 30% rice bran with an aliquot of coastal soil (99.9:0.1 w/w) which harboured chitinase-producing bacteria (10^6 g^{-1} soil). No crab shell was added to CC. Composting was carried out in covered 100- *l* jars for one year with 30 min aeration per week maintaining the moisture content between 45 and 60%.

Soil chitinase activity Chitinase (EC 3.2.1.14) activity was determined using the modified method of Trotta et al. (1996) by measuring the amount of the reducing end group, N-acetyl glucosamine (NAG), produced from colloidal chitin (Yedidia et al., 2000). Soil adhering to roots was shaken off and 1 g of soil was mixed with 0.25 ml of toluene, 4 ml of 50 mM NaOAc buffer (pH 5.0) and 1 ml of 0.5% colloidal chitin in a test tube and kept at 37℃ for 2 h. After this period, 1 ml 0.5 M of $CaCl_2$ and 4 ml of 0.5 M NaOH were added, and mixed thoroughly. The mixture was centrifuged at 1000 g for 20 min to yield a soil-free supernatant then filtered through Whatman No 2 filter paper. A 1.0 ml quantity of Schales' reagent (0.5M sodium carbonate and 1.5 mM potassium ferricyanide) was added to 0.75 ml of the filtrate, and then the reaction was stopped by heating in boiling water for 15 min. Chitinase activity was calculated by measuring NAG concentration at 420 nm, in conjunction with data from a NAG standard curve. One unit of chitinase activity was defined as the amount of enzyme that liberated 1 mol of NAG h^{-1} at 37℃.

Plant sampling and enzyme extract Sixty-six days after transplanting (DAT), CC plants showed wilting symptoms and plant samples were randomly taken at intervals of 1, 3, 5 and 7d after wilting was first noted. At each date, three tomato plants were harvested to evaluate PR-protein activities. Tomato roots were gently washed under running tap water and air dried. About 2 g of fresh roots were ground in a mortar with pestle under liquid nitrogen. The ground sample was homogenized with 2.5 ml of 100 mM K-PO_4 buffer solution (pH 7.0) containing 2mM of EDTA, 1% PVP (MW 40,000) and 1mM phenylmethyl sulfonyl fluoride ($C_7H_7FO_2S$), and centrifuged at 14,000 g at 4℃ for 20 min. Supernatant was collected and kept at -20℃.

Pathogenesis-related proteins activity β-1,3-Glucanase activity (EC 3.2.1.6) was assayed by measuring the amount of the reducing end group, glucose, produced from laminarin (Yedidia et al., 2000). The assay mixture consisted of 100 μl supernatant, 25 μl

10mg/ml laminarin, and 375 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 waterbath for 5 min. Glucose concentration was determined spectrophotometrically at 550 nm and β-1,3-glucanase activity was calculated utilizing a standard curve. One unit β-1,3-glucanase activity was defined as the amount of enzyme that liberated 1 mol of laminarin h^{-1} at 37℃.

Chitinase activity (EC 3.2.1.14) was assayed by measuring the amount of the reducing end group, NAG produced from colloidal chitin (Lingappa and Lockwood 1962). The assay mixture consisted of 0.5 ml of supernatant, 0.5 ml of 1.0% colloidal chitin in 0.1M sodium acetate buffer (pH 5.5). After incubation at 37℃ for 2 h, 200 μl of 1N NaOH was added, and the reaction was stopped by heating in boiling waterbath for 15 min. After centrifugation at 10,000 g for 10 min, 750 μl supernatant was mixed with 1 ml of Schales' reagent. Chitinase activity was calculated by measuring NAG concentration at 420 nm. One unit of chitinase activity was defined as the amount of enzyme that liberated 1 μmol of NAG h^{-1} at 37℃.

Peroxidase activity (POD; EC 1.11.1.7) was determined using the method of Chance and Maehly (1995). The reaction mixture contained 50 μl of 20 mM guaiacol, 2.8 ml of 10 mM phosphate buffer (pH 7.0), and 0.1 ml supernatant. Addition of 20 μl of 40 mM H_2O_2 initiated the reaction. The formation of tetraguaiacol was determined spectrophotometrically, by measuring absorbance at 470 nm. One unit of peroxidase activity was described as the amount of enzyme required for the formation of 1 μmol of tetraguaiacol per min.

Evaluation of disease incident rate From the 66 DAT, tomato plants showing wilting symptoms were counted and the incident rate was defined as the percentage of the wilted plants to the total tomato plants in each treatment.

Statistical analysis Measurement of PR-proteins activity was repeated 9 times and the treatment effects were determined by analysis of variance (one-way ANOVA) according to the general linear model procedure of the Statistical Analysis System 8.1. Means were separated with Tukey's Studentized Range Test at $p \leq 0.05$.

Results

Rhizosphere chitinase activity Changes of soil chitinase activity measured in the rhizosphere in CTC and CC are shown in Fig. 1. Activities of chitinase at 5 and 7 d were a little bit higher compared to those on days 1 and 3 in CTC and CC. Chitinase activity in CTC always had higher values compared to CC throughout the 7 d period. The chitinase activity in CC remained constant up to 3 days and slightly increased thereafter.

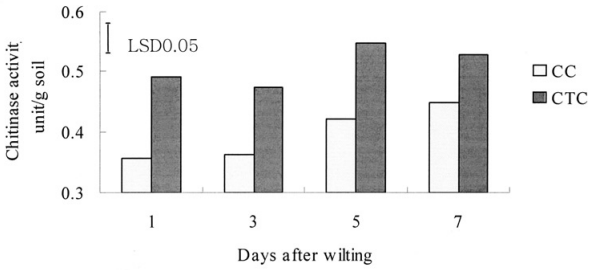

Fig. 1. Changes in chitinase activity in rhizosphere amended with CTC and CC. Day 1 correspond to 66 d after transplanting. Each value is derived from the means of nine replicates per plot.

PR-proteins in plant roots The changes of PR-protein activity in tomato roots are shown in Figs. 2, 3 and 4. Roots from the CC had almost constant chitinase and β-1, 3-glucanase activities until 3 d and thereafter markedly increased. Increased chitinase activity in CTC was noticed at 5th and 7th days after wilting and β-1,3-glucanase activity remained constant at all observations. At 7 d, activities of chitinase and β-1,3-glucanase in CC were around 30 and 20 unit g^{-1} fresh mass (Figs. 2 and 3). In CC peroxidase activity was significantly higher and showed 2 fold increment at 7 d, compared to CTC (Fig. 4).

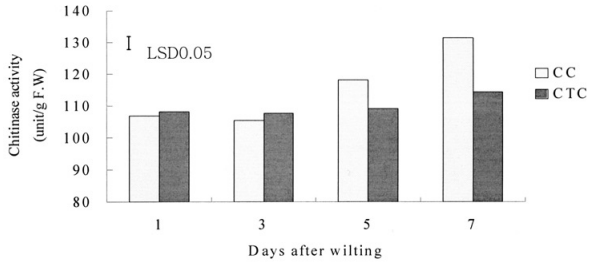

Fig. 2. Changes in chitinase activity in tomato roots transplanted to CTC and CC. Day 1 correspond to 66 d after transplanting. Each value is derived from the means of nine replicates per plot.

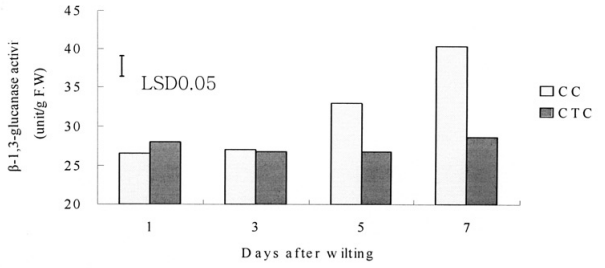

Fig. 3. Changes in β-1,3-glucanase activity in tomato roots transplanted to CTC and CC. Day 1 correspond to 66 d after transplanting. Each value is derived from the means of nine replicates per plot.

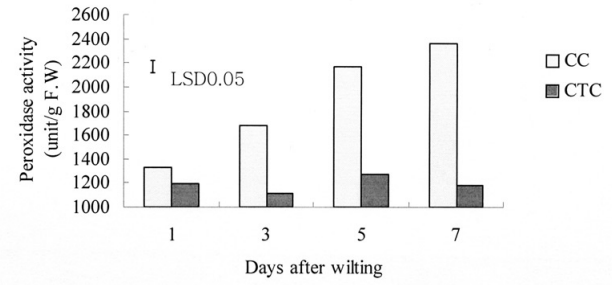

Fig. 4. Changes in peroxidase activity in tomato roots transplanted to chitin CTC and CC. Day 1 correspond to 66 d after transplanting. Each value is derived from the means of nine replicates per plot.

Incidence rate of Fusarium wilt in tomato plant During the investigation, the older and bottom leaves of tomato changed from green to yellow, followed by wilting, browning, and defoliation in CC, while plants in CTC appeared to be normal (data not shown). Incidence rate of Fusarium wilt of leaves of CC increased constantly and reached maximum (28%) after 30 d (96 DAT). However, no incidence of Fusarium wilt in CTC occurred until 25 d. At the end of the experiment, incidence rate of Fusarium wilt in CTC was reduced by 25% compared to CC (Fig. 5). At 96 DAT, the leaves of plants in CC plots were yellow and wilted and reached an advanced stage of decay. However, plants in CTC appeared to be normal.

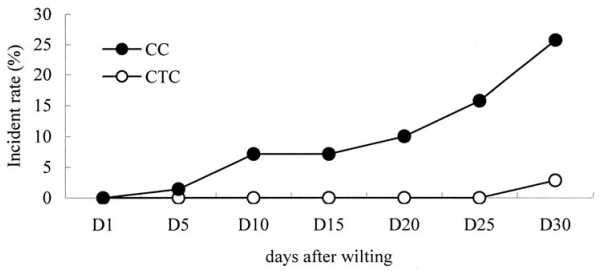

Fig. 5. Fusarium wilt incidence rate in tomato plants transplanted to CTC and CC. Day 1 correspond to 66 d after transplanting.

Discussion Symptoms of Fusarium wilt were visible on a large scale during the tomato culture period in the CC amended plots. Few of the tomato plants growing in the CTC amended plots showed symptoms of *Fusarium oxysporum* f. sp. *lycopersici* induced Fusarium wilt.

Soil amended with CTC showed higher chitinase activity compared to CC amended soil. This result indicates that the increased chitinase activities derived from the CTC amendment may be an important factor in protecting tomato plants from *Fusarium oxysporum* f. sp. *lycopersici* infection. As reported elsewhere, disease control in amended soil may be the cumulative result of complex interactions between host-plant and pathogen. However, the most important factor in control of soil-borne disease is the adaptation of the antagonistic microorganisms to the soil environment and keeping them in a superior position. Mian et al. (1982) reported that the addition of chitin to soil stimulated the growth of bacterial species with chitinolytic properties. Crab shell in chitin compost could stimulate the growth of chitinolytic bacteria, increasing soil chitinase activity. The hydrolytic enzyme chitinase catalyzes the degradation of chitin abundantly present in the cell wall of many filamentous fungi and is thought to be capable of inhibiting fungal growth in planta (Wessels and Sietsma 1981). Chitinase produced from *Bacillus* sp.739 (Melent'ev et al. 2001) and *Paenibacillus illinoisensis* KJA-424 (Jung et al., 2003) destructed the fungal cell wall ultimately mycelia.

Other explanations might be derived from the production of antibiotic compounds by the increased number of several species of microorganisms in the rhizosphere amended with CTC. Chitinase produced by *Bacillus subtilis* and *P. illinoisensis* were mainly isolated from the CTC and released 3-methylbutyric acid, 2-methylbutyric acid, and methyl 2-hydroxy, 3-phenylpropanoate with strong antifungal activity (Lee 2003). Wang et al. (2002) reported that *B. subtilis* W113 and W118 were isolated from soil that exerted antifungal activity in the presence of chitin and had concluded that chitin is an essential element for induction of antifungal activity. Strohl (1997) reported that the compost amended with shrimp shell containing chitin polymer promoted the proliferation of Gram-positive bacteria, a group of bacteria producing more than 70% of known antibiotics. Several reports established that numerous Gram-positive

bacteria could act as biological tools against Oomycetes and product of antibiotics is an important asset for most of these biocontrol agents (Emmert and Handelsman 1999; Toussaint et al., 1997).

On the other hand, the accumulation of PR-proteins is thought to play a role in pathogen-induced plant defense responses (Lawrence et al., 1996). Most plants contain relatively low levels of constitutive chitinase and produce higher levels of the enzyme when they are subjected to attack from phytopathogens (Byrne et al., 2001). In this study, PR-protein (chitinase, β-1,3-glucanase and peroxidase) activity in tomato roots in CC increased and showed a marked difference compared to the CTC (Figs. 2, 3 and 4). These results were consistent with Yedidia et al. (2000), who reported that peroxidase, chitinase, and β-1, 3-glucanase activities increased in the roots of cucumber seedlings pre-treated with *Trichoderma harzianum* strain T-203, whereas there were no significant changes in control plants treated with either water or autoclaved *T. harzianum*. Several reports have 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., 2004). Accumulation of PR-proteins may be regarded as an indicator of plant response to infection by pathogens. Minor changes in PR-protein activities in CTC applied tomato confirmed that CTC could protect plants from pathogen infection.

All the enzymatic activity may be responsible for the suppression of Fusarium wilt in tomato associated with high level of soil chitinase activity secreted by chitinolytic microorganisms in CTC. We also suggest that the synthesis of antibiotics may also play a role in the suppression of Fusarium wilt, although further evidence is needed.

Acknowledgement

This study was supported by a grant from BioGreen 21 program, Rural Development Administration, and Technology Development Program for Agriculture and Forestry, Ministry of Agriculture and Forestry, Republic of Korea.

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키틴퇴비를 이용한 토마토의 *Fusarium* 시들음병의 생물학적 제어

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최근 들어서 생물학적 제어 방법의 하나로써 키틴분해 미생물을 이용한 제어 수단이 식물병제어에 일정한 효과가 있는 것으로 보고되고 있다. *Fusarium* 시들음병을 억제하기 위하여 40kg의 키틴퇴비를 면적이 7.5m^2 인 토양에 정식 7일전 처리하였으며 토마토가 시들음병 증세를 보이기 시작하는 날(정식 후 66일)로부터 시작하여 4번에 걸쳐 시료를 채취하였다. 키틴퇴비 처리구(CTC)의 근권토양의 키틴효소와 β-1,3-glucan 효소 활성은 일반퇴비 처리구 (CC) 토양 보다 항상 높은 값을 나타냈다. 그러나 식물체 뿌리에서 측정된 chitinase, β-1,3-glucanase, peroxidase과 같은 병 관련 효소들은 CNC에서 실험기간동안 증가 추이를 보였다. 실험의 마지막 단계인 정식 후 96일째에는 CTC의 토마토는 CC 와 비교 할 때 25% 낮은 치사율을 나타냈다.