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Article

Suppression of Clubroot Formation in Chinese Cabbage by the Chitin Compost and Broth

Rong De Jin, Tae Ho Han[1], Yong Woong Kim and Kil Yong Kim*

Division of Applied BioScience and Biotechnology, and Environmental-Friendly Agriculture Research Center, Chonnam National University, Gwangju 500-757, Korea

[1]Institute of Agricultural Science and Technology, Chonnam National University, Gwangju 500-757, Korea

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Chitin compost and broth were used to suppress clubroot. Individual cabbage seedlings were transplanted into pots (3500 m*l*) containing a mixture of 3% chitin compost and 50 m*l* of chitin broth (T1) or the same quantity control compost and control compost broth (T2). The media in each pot was then infected with *Plasmodiophora brassicae*. Samples were taken at 6, 7 and 8 weeks after transplanting. The population of chitinase producing bacteria in T1 was consistently larger than that observed in T2. Chitinase activity in the T1 rhizosphere was two-fold greater than that of T2 at each time point observed. Shoot dry weight, leaf number and leaf area in T1 were enhanced 20%, 10% and 12% relative to those seen in T2, respectively. The disease index and root mortality at 8 weeks after transplanting were reduced by 50% and 25% in T1 compared to T2, respectively. Results presented in this study are strongly indicative that chitin compost and broth suppress clubroot in Chinese cabbage.

Key words: *chitin compost, chitin broth, clubroot, disease index.*

As the basic ingredient in Gimchi, Chinese cabbage is the most popular vegetable in Korea.[1)] Clubroot disease caused by *Plasmodiophora brassicae*, a soil-borne fungal pathogen often causes significant yield loss, especially under mono-culture. Since first being observed in Kangwon province in 1926, clubroot has become a serious soil borne disease in other parts of Korea. In 1999, the area damaged by clubroot was over 700 hectares where *P. brassicae* had survived for several years.[2)] When susceptible roots are near the spore, it germinates and a "zoospore" is released that can swim through the soil moisture to the plant root hairs. It infects the root cells and multiplies to form yet more zoospores. These either swim through the soil to infect more roots or go deeper into the roots. Inside the roots they form amoeba-like cells called 'plasmodia'. They multiply and induce plant hormones that cause the gall. The roots are destroyed, crops fail to reach maturity and the soil is contaminated with spores that infest future crops.[3)]

Currently, the disease is controlled by the use of chemical fungicides. However, the possibility of fungicide residues in food and the overall environmental impact of their usage has become an increasingly important issue.[4)] The foregoing concerns provide an impetus for discovery of innocuous yet efficacious methods of disease control. Although some clubroot-resistant cultivars of Chinese cabbage have been developed, most have not been widely accepted. Recent years, researchers have tried to introduce biological methods for control of clubroot.[4-7)]

The enzyme chitinase has taken a center stage in the investigation of biological control of fungal pathogens. This enzyme has the ability to lyse fungal cell walls[8,9)] containing chitin as the major component such as the spore walls of *P. brassicae*[10)]. Lee *et al.* reported that purified chitinase inhibited growth of *F. oxysporum*.[11)] Furthermore, the enhanced level of chitin could serve as a substrate for chitinolytic bacteria increasing their population and supporting a high soil chitinase activity long enough to maintain the protective function until harvest.[12)] The spore walls of *P. brassicae* may be the target of potential enzymatic degradation and thus a possible means of clubroot control. Generally, a large population of chitinase producing bacteria was found in the soil that had been enriched with chitin.[12)] Several reports revealed that chitinase producing bacteria not only promote plant growth but also generate many commercial products such as chitinase, antibiotics, and essential growth factors.[9,13)]

The present study was designed to investigate the population of chitinase producing bacteria and exogenous enzyme activities in soil amended with chitin compost and broth harboring a multitude of chitinase producing bacteria and to ascertain whether this method is efficacious in alleviating the deleterious effect of *P. brassicae* on Chinese cabbage.

Materials and Methods

Preparation of chitin compost and control compost. Chitin compost was prepared by mixing 10% crab shell, 20%

*Corresponding author
Phone: 82-62-530-2138; Fax: 82-62-530-2139
E-mail: kimkil@chonnam.ac.kr

vermiculite, 40% rice straw, and 30% rice bran with an aliquot of coastal soil (99.9 : 0.1 w/w), which harbored chitinase-producing bacteria (10^6 g^{-1} soil). Control compost was devoid of crab shell. Both media were composted in covered 100-liter jars for one year with weekly aeration to maintain the moisture content between 45 and 60%.

Preparation of chitin broth and control broth. Chitin broth contained 4 kg of chitin compost with an additional 2 kg of crab shell suspended in 500 *l* of tap water, while control broth contained 4 kg of control compost and 2 kg of glucose per 500 *l*. The broths were incubated at room temperature with continuous aeration for 5 days.

Plant growth conditions. Chinese cabbage seeds (*Brassica pekinensis*) were sown in pots (50 mm diameter) containing prepared medium (Bio bed soil I, Heong Nong Seed Co. South Korea) and grown in a glasshouse for 2 weeks. Three days prior to transplanting, individual pots filled with 4 kg of a steam-sterilized field soil were inoculated with 3% chitin compost and 50 m*l* chitin broth (T1) or 3% control compost and 50 m*l* control broth (T2). Each treatment was replicated 18 times and the experiment was conducted in randomized blocks on the glasshouse bench. Throughout the experiment, the plants were grown at 25-35 and 50-60% relative humidity and water or mineral fertilizers (20 : 5.9 : 12.8 g l^{-1}) were applied to the base of each pot when required.

Pathogen inoculation. Resting spores of *P. brassicae* were obtained from clubroot galls collected from Chinese cabbage plants grown in the field at Haenam, Chonnam, Korea. The galls were washed with tap water and stored at −70°C. After maceration using sterile deionized water the galls were filtered and the filtrates centrifuged at 300 rpm for 10 min. The collected pellets were re-suspended in sterile water and the suspension was centrifuged 3 more times and kept at 4°C until use. Two weeks after transplanting, resting spores of *P. brassicae* were inoculated into soil around the roots at a concentration of 3×10^6 spores g^{-1} dried soil.

Plant sampling. Chinese cabbage plants were taken randomly at 6, 7 and 8 weeks after transplanting. At each harvest time, six Chinese cabbage plants were taken from each treatment for investigation. Roots were washed carefully under running tap water and disease symptoms (disease index) were assessed. Approximately 250 mg of root tissues were collected individually from the three Chinese cabbage plants roots for root mortality assay and the last three Chinese cabbage were divided into roots and shoots to estimate the dry weight in an incubator at 70°C for 2 days.

Soil chitinase activity. Soil chitinase (EC 3.2.1.14) activity was determined using the modified method of Trotta *et al.*[14] by measuring the amount of the reducing end group, *N*-acetyl glucosamine (NAG), produced from colloidal chitin.[15] Soil adhering to roots was collected and a one gram aliquot was mixed with 0.25 m*l* of toluene, 4 m*l* of 50 mM NaOAc buffer (pH 5.0) and 1 m*l* of 0.5% colloidal chitin in a test tube and kept at 37°C for 2 hours. After this period, 1 m*l* 0.5 M of $CaCl_2$ and 4 m*l* of 0.5 M NaOH were added, and mixed thoroughly. The mixture was centrifuged at 1,000 *g* for 20 min to yield a soil-free supernatant then filtered through Whatman No. 2 filter paper. A 1.0 m*l* quantity of Schales' reagent (0.5 M sodium carbonate and 1.5 mM potassium ferricyanide) was added to 0.75 m*l* 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 μmol of NAG per hour at 37°C.

Microbial populations. Microbial population of the rhizosphere was enumerated by the soil dilution plate method.[16] Soil samples were incubated in a basal medium containing 1% colloidal chitin as the sole carbon source. The composition of the basal medium was 2 g Na_2HPO_4, 0.5 g KNO_3, 1 g KH_2PO_4, 1 g NH_4Cl, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g $CaCl_2 \cdot 2H_2O$, 0.1 g yeast extract per *l*, and 1% colloidal chitin, and agar 2%. After seven days incubation at 3°C, pellucid zones appeared around colonies of chitinase producing bacteria.

Root mortality assay. Root mortality was measured by the method of Liu and Huang.[17] Fresh root (0.5 g) was incubated in 5 m*l* of 50 mM phosphate buffer (pH 7.4) containing 0.6% 2,3,5-triphenyltetrazolium chloride for 24 hr in the dark at 30°C. Roots were then rinsed thoroughly with distilled water. Formazan was extracted from the roots twice with 95% ethanol at 70°C for 4 hr. Extracted solution was measured at 490 nm. A standard curve was developed using different proportions of living and autoclaved roots. Root mortality was expressed as percentage of dead root fresh weight of total root fresh weight.

Disease severity assess. Roots of Chinese cabbage plants were classified into four categories (0-3) according to following criteria. Category 0: not clubbed; Category 1: only lateral roots clubbed; Category 2: less than half of tap root clubbed; Category 3: more than half of tap root clubbed.

Disease index for each treatment at each sampling time was calculated by the method described by Yang and Kim.[18]

$$\text{Disease Index} = (1 \times n_1 + 2 \times n_2 + 3 \times n_3)/(3 \times N) \times 100.$$

N: total number of plants; n_1: number of plants classified as category 1; n_2: number of plants classified as category 2; n_3: number of plants classified as category 3;

Statistical analysis. Data (except Root Mortality and Diseases index) were statistically analyzed according to standard procedures including SAS general linear model (GLM), least-significant difference (LSD), and regression procedures (SAS Institute, Cary, NC). Unless otherwise stated, all differences noted in the text were significant at the 5% level of probability.

Results

Microbial activities in the rhizosphere. Changes in soil chitinase activity and population of chitinase producing bacteria in the rhizosphere of T1 and T2 are shown in Table 1

Table 1. Changes in the population of Chitinase Producing Bacteria and chitinase activity in soil amended with chitin compost and broth (T1) or control compost and broth (T2) at 6, 7, and 8 weeks after transplanting

Treatment	weeks	Chitinase producing bacteria	Chitinase activity
		log CFU g^{-1} dry soil	Unit/g dry soil
T1	6	7.99 ± 0.11a	1.76 ± 0.12a
	7	7.91 ± 0.09a	1.81 ± 0.08a
	8	7.50 ± 0.23a	1.90 ± 0.09a
T2	6	6.30 ± 0.42a	0.72 ± 0.18a
	7	6.00 ± 0.37a	0.79 ± 0.11a
	8	6.33 ± 0.16a	0.68 ± 0.05a

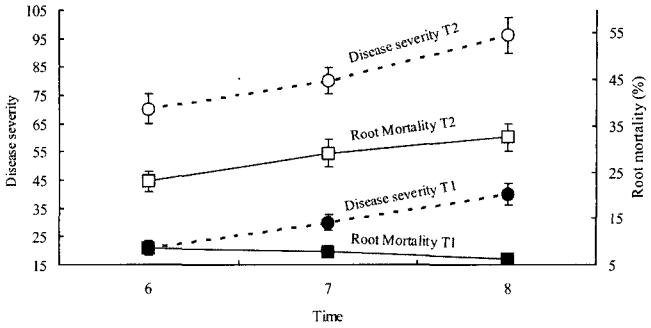

Fig. 1. Changes of Root Mortality and Disease index in Chinese cabbage plants amended with chitin compost and broth (T1) or control compost and broth (T2) at 6, 7, and 8 weeks after transplanting. Mean value of 3 replicates. Bars represent standard error.

and 2 respectively. In T1, activities of chitinase had higher values and increased gradually compared to T2 throughout the experimental period. At each time point, chitinase activities in T1 was 2 times higher than T2 (Table 1). The population density of the chitinase producing bacteria in the rhizosphere of plants grown in T1 was approximately 100 times higher than T2 as seen in Table 1.

Chinese cabbage plants growth. An increase in the shoot dry weight, number of leaves and leaf area of the Chinese cabbage plants was observed over the eight-week duration of the trial in both treatments and the values in T1 were always higher than T2 (Table 2).

Root mortality and Disease severity. Throughout the investigation, amendment of chitin compost and broth reduced disease index and root mortality compared to the control compost and broth treatment (Fig. 1). Disease index of T2 increased constantly with the highest incidence (92%) 8 weeks after transplanting. At 6, 7 and 8 weeks after transplanting, the disease index in T1 was markedly reduced by 70%, 75% and 50% compared to the T2 respectively (Fig. 1). Root mortality was 25% less in plants growing in T1 than in those from T2 (Fig. 1). Furthermore, clubroot symptoms were significantly suppressed in plants growing in T1 especially at 8 weeks after transplanting. Clubroot symptoms were reduced by 50% in T1 compared to T2 (Fig. 2).

Discussion

The research was designed to establish whether increased chitinase production by the chitinase producing bacteria could inhibit the development of clubroot and promote Chinese cabbage growth. During the investigation, disease index in T1 was markedly reduced compared to T2 and root mortality in T2 showed 3 times higher value compared to T1. The population levels of chitinase producing bacteria and chitinase activity in the rhizosphere of plants grown in T1 revealed higher values compared to T2 at each time point.

Results from this study indicate that increased chitinolytic microbial activities derived from soil amended with chitin compost and chitin broth may be the main factor in reducing *P. brassicae*.

In the last decades, researchers have tried to inhibit of clubroot in Chinsese cabbage and other diseases in plants in many ways including soil amendments using chitin or/and chitinolytic.[4-7,19] Amendments using these antagonists has often failed to suppress plant disease successfully because of their inability to compete with the indigenous microorganisms and to survive long periods.[20] Accordingly, an important factor in the control of soil-borne disease using antagonistic microorganisms is that they successfully compete and flourish

Table 2. Changes of shoot dry weights, number of leaves and Leaf Area in Chinese cabbage plants amended with chitin compost and broth (T1) or control compost and broth (T2) at 6, 7, and 8 weeks after transplanting

Treatment	Weeks	Shoot dry weight	Number of leaves	Leaf area
		Gram		cm^2
T1	6	27.67 ± 3.26a	29.33 ± 1.53a	4400.9 ± 181.2a
	7	32.31 ± 3.91a	30.67 ± 0.58a	4838.6 ± 201.4a
	8	36.05 ± 3.41a	33.33 ± 2.65a	5079.9 ± 119.9a
T2	6	23.91 ± 1.75a	24.00 ± 1.00a	3588.1 ± 98.9a
	7	25.83 ± 2.06a	26.00 ± 1.00a	4156.3 ± 132.3a
	8	27.41 ± 2.11a	32.00 ± 2.60a	4335.2 ± 191.8a

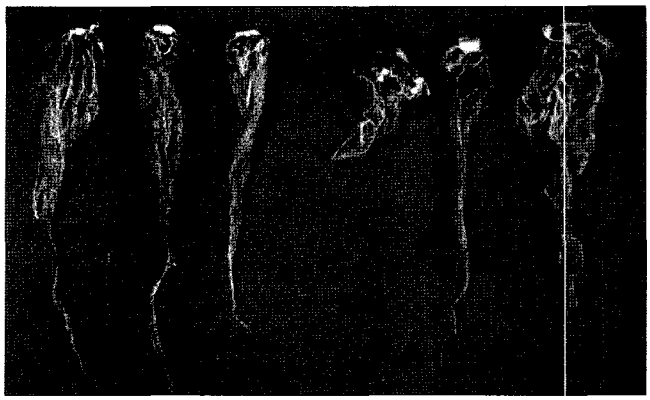

Fig. 2. Clubroot symptoms in Chinese cabbage as influenced by T1 and T2 at 8 weeks after transplanting.

in the respective soil environment. Chang *et al.* reported that the chitinase produced by *Bacillus cereus* with shrimp and crab shell powder as carbon source inhibited the growth of *F. oxysporum, F. solani* and *P. ultimum* on PDA medium.[9] Melent'ev *et al.* reported that chitinase produced from *Bacillus* sp. 739 contributed to mycelium destruction and vacuolization.[21] Furthermore, in a previous report, we provided evidence that the cell wall of *Rhizoctonia solani* was degraded by chitinase-producing *Paenibacillus illinoisensis* KJA-424 and that chitinolytic *B. subtilis* HJ927 inhibited the growth of *Phytophthora capsici*.[8, 11]

Mian *et al.* reported that addition of chitin to soil stimulated the growth of bacterial species with chitinolytic properties.[12] The chitin compost and broth used in this study contained large populations of chitinase producing bacteria. Additionally Crab shell in chitin compost stimulated the growth of chitinolytic bacteria in the rhizosphere effectively protecting Chinese cabbage from clubroot formation. In our previous study, amendment of chitin compost protected pepper from late blight and root-knot nematode.[19, 22]

In summery, our results show that the use of chitin compost and broth could effectively inhibit development of clubroot and thus provide an alternative to synthetic chemicals for control of *P. brassicae* in Chinese cabbage. The chitinase produced by chitinolytic microorganisms may be an important factor in the inhibition of clubroot in Chinese cabbage although other unknown factors may also contribute to suppression. Currently, research is continuing into the mechanisms of action, the conditions under which they are effective and ways to improve their consistency and level of effectiveness.

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