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Development of effective environmentally-friendly approaches to control Alternaria blight and anthracnose diseases of Korean ginseng

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Abstract In ginseng fields, Alternaria blight, caused by *Alternaria panax*, and anthracnose, caused by *Colletotrichum gloeosporoides*, have become serious problems in Korea, and control of these diseases relies mainly on intensive applications of fungicides. In an effort to develop an effective environmentally-friendly control system, we utilized preventative control approaches including rain shelter shading plates and the removal of dead plants, as well as a curative control approach, such as the application of microbial agents. In the presence of rain shelter shading plates, the occurrence of Alternaria blight and anthracnose decreased significantly compared to that seen with polyethylene shading nets. In addition, the eradication of dead ginseng plants, which harboured abundant spores of the pathogens, significantly reduced the incidence of both diseases. In fields with rain shelter shading plates and in which dead plants were eradicated, four applications of a bioformulated product containing chitinolytic bacterial strains in a simple medium containing chitin provided control similar in effect to that observed with the application of the fungicide under low disease pressure. The efficacy of the bioformulated product was decreased slightly under severe disease pressure. These findings indicate that integration of the three disease management measures might constitute a new effective and environmentally-friendly system for the control of Alternaria blight and anthracnose in Korean ginseng fields.

Keywords Biological control · Chitinolytic bacteria · Field sanitation · Rain shading shelter plate

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Introduction

Korean ginseng, *Panax ginseng* C.A. Meyer, is cultivated in Asian countries including Korea, Japan, and China, whereas American ginseng, *Panax quinquefolius* L., is cultivated in the United States, Canada, and recently in Europe. Ginseng is an herbaceous perennial plant, and its roots are highly valued for medicinal and nutritional uses (Protor 1996). Ginseng is cultivated under artificial shade, and requires a

4–6 year cultivation period prior to harvest (Sivakumar et al. 2005). Disease control remains the principal issue in the commercial cultivation of ginseng, as dense plantings, continuous cultivation practices, and low light conditions exacerbate fungal diseases which are the principal factors causing significant economic losses of ginseng (Ohh 1981).

The major fungal diseases of ginseng limiting production in Korea are anthracnose (*Colletotrichum gloeosporoides*), damping-off (*Pythium debaryanum*, *Pythium ultimum*, and *Rhizoctonia solani*), Phytophthora blight (*Phytophthora cactorum*), gray mold (*Botrytis cinerea*), and Alternaria blight (*Alternaria panax*) (Kim et al. 2007; Ohh 1981). Alternaria blight and gray mold are also important foliar diseases in North American ginseng fields (Punja 1997; Putnam and du Toit 2003; Quayyum et al. 2003; 2005) Symptoms of Alternaria blight on ginseng appear first on the stems in the spring, progressing to leaves and fruits by secondary inoculum production in the early summer (Kim et al. 1990). Anthracnose disease symptoms are generally observed on leaves and fruits in late spring (McPartland and Hosoya 1998). Weather conditions influence the severity of both diseases, which tend to be extremely destructive during the rainy season (Kim et al. 2007; Ohh 1981). Farmers have adopted a strategy of repeated spraying of fungicides in an effort to prevent these diseases (Kang et al. 2007). Multiple applications of pesticides may foster environmental contamination, create pesticide residues, and may result in unfavourable conditions for beneficial microbial communities. Until now, no effective methods have been developed to address the pesticide residue problem. Thus, organic cultivation practices have been considered as a possible strategy for the production of pesticide-free ginseng, using ecologically-based and/or biological control methods.

Several biocontrol approaches have been developed as an alternative to fungicide applications. *Bacillus subtilis* QST713 has been shown to have the potential to control Alternaria leaf blight in ginseng fields (Li et al. 2008). However, compared to fungicides, biocontrol products are unstable and microbial agents are highly dependent on environmental conditions (Ojiambo and Scherm 2006). In addition, biocontrol approaches have generally proved ineffective in controlling plant diseases under severe disease pressure (Boland 1997). Thus, fungicide applications combined with biocontrol methods have been considered as a method that might prove more beneficial in restricting plant diseases, while avoiding or ameliorating some of the more problematic issues associated with current methods (Favel 2005). With regard to mixed or alternative fungicide applications, *Enterobacter aerogenes* B8 and *Bacillus cereus* GB01-complex were identified as having potential as components of a biocontrol method to reduce the necessity of fungicide application in ginseng fields (Li et al. 1997).

No effective control methods have yet been introduced for the inhibition of Alternaria blight and anthracnose in ginseng without the use of fungicides. In organic systems, it is appropriate to develop integrated disease-control strategies that involve a combination of ecological and biological control methods, including the removal of infected plants, manipulation of the environment to make it unfavourable for disease development, and the use of effective biocontrol agents. As a cultural control method, the use of the rain shelter systems (Kim et al. 1990) has been shown to significantly decrease occurrence of Alternaria blight in ginseng. Previously, we developed a formulated biocontrol product based on a cost-effective growth medium containing chitin; this product contained a combination of three chitinolytic bacteria with profound inhibitory activity against *Phytophthora capsici*, in addition to *Rhizoctonia solani*, *Fusarium oxysporum*, and *Fusarium solani*. Applications of the formulated product were sufficient for the suppression of Phytophthora blight in peppers under both greenhouse and field conditions (Kim et al. 2008).

The principal objective of this study was to test an integrated control method involving the use of shading plates, removal of dead plants, and application of an effective biocontrol product under field conditions. Our results indicated that the integrated control methods could be utilized effectively in the control of Alternaria blight and anthracnose in ginseng fields.

Materials and methods

Test of antagonistic activity and compatibility of the biocontrol bacteria with ginseng

Alternaria panax, cause of Alternaria blight, and *Colletotrichum gloeosporiodes*, cause of anthracnose in ginseng, were acquired from the Ginseng Research division of the National Institute of Horticultural and

Herbal Science (Suwon, Korea). These pathogens were grown on potato dextrose agar (PDA, Difco, Ditroit, MI) at 28°C. The chitinolytic *Chromobacterium* strain C-61, *Serratia plymuthica* C-1, and *Lysobacter enzymogenes* C-3 were previously isolated from soil in Korea (Kim et al. 2008). The bacteria were transferred to the edges of PDA plates and agar disks (0.5 cm in diameter) harbouring mycelia of each fungus were positioned in the centers of the plates. The antagonistic activity of the bacteria was assessed by determining the extent of inhibition of mycelial growth. The bacterial strains were grown on nutrient agar (NA, Difco, Detroit, MI) or nutrient broth (NB, Difco, Detroit, MI) at 28°C, and were stored at −70°C in NB containing 20% glycerol.

L. enzymogenes strain C-3, *Chromobactererium* sp. strain C-61, and *S. plymuthica* strain C-1 were tested for their effects on emergence of ginseng seeds and phytotoxicity on leaves. *S. plymuthica* C-1 and *Chromobacterium* sp. C-61 were cultivated for 12 h and *L. enzymogenes* strain C-3 was cultivated for 36 h in nutrient broth with a rotary shaker (180 rpm) at 28°C. Twenty ginseng seeds were planted in sterile soil (sandy loam) in a plastic box (43cm×26cm×15cm), and drenched with 200 ml of each bacterial suspension (ca 10^8 cells ml^{-1}). Control seeds were drenched with 200 ml of distilled water. After the plastic boxes were incubated in a shaded greenhouse at 25°C for 30 days, the emergence of seedling was evaluated. Each bacterial suspension (ca 10^8 cfu ml^{-1}) was sprayed on the surfaces of the two-month-old ginseng leaves. Control leaves were sprayed with sterile water. The leaves were evaluated for phytotoxicity symptoms after 10 days of incubation in a shaded greenhouse at 25±2°C.

A formulation of chitin amended with chitinolytic antagonists *Lysobacter enzymogenes* strain C-3 and *Chromobacterium* sp. strain C-61 was further investigated as a biocontrol product; *S. plymuthica* C-1 was not used due to the damage it caused to both the seeds and leaves of ginseng. The formulated microbial product was prepared by a method described in a previous study (Kim et al. 2008). In brief, each bacterial strain was cultivated for 24 h in nutrient broth on a rotary shaker (28°C, 180 rpm) and 100 ml of the bacterial suspension was added to 500 l of chitin minimal medium [0.6 g $(NH_4)_2SO_4$, 0.8 g KH_2PO_4, 0.6 g K_2HPO_4, 0.04 g $MgSO_4$ $7H_2O$ and 0.2 g crude chitin l^{-1} distilled water]. The microbial agents including the chitin medium were cultivated for an additional 10 days at 28°C. Finally, the cultivated bacterial suspension, a viable count of 9×10^8–1×10^9 cfu ml^{-1}, was utilized for the control of Alternaria blight and anthracnose.

Field studies

Field experiments were conducted in conventional ginseng fields located at Haenam in Jeonnam province, which is located in the South-east region of Korea. All experiments were conducted with three replications in a randomized block design. Each plot was 1,000 m^2 containing initially 22 plants m^{-2}. The ginseng was cultivated using standard farming practices without any applications of fungicide or insecticide unless indicated in the experimental details section. For assessments of disease incidence of Alternaria blight and anthracnose, three hundred leaves were collected randomly from each plot at defined time points.

1. Effect of different shading materials on disease incidence

To examine the effects of different shading materials on occurrence of the diseases the ginsengs used were 3 years old in 2005 and 4 years old in 2006. Incidence of Alternaria blight and anthracnose was compared under rain shelter shading plates (Samwoo Steel Co., Paju, Korea) which reflect sunlight and prevent rain and polyethylene mesh shade nets (Samwoo Steel Co., Paju, Korea) in the ginseng field (Fig. 1). Azoxystrobin SC (20% ai) was sprayed four times onto the cultivated three-year-old ginseng plants on June 3rd, June 12th, July 4th, and July 25th of 2005, and on four-year-old ginseng plants on June 1st, June 15th, July 3rd, and July 24th of 2006. The incidence of Alternaria blight and anthracnose was determined at defined time points.

2. Effect of removal of dead plants on disease incidence

The ginsengs were 5 years old in 2005, and the same ginseng plants were used again in 2006. Dead plants of five-year-old ginseng (year 2005) and six-year-old ginseng (year 2006) grown under rain shelter shading plates were immediately removed and discarded from June to August, whereas the dead plants were left in place in the control plot. In both plots,

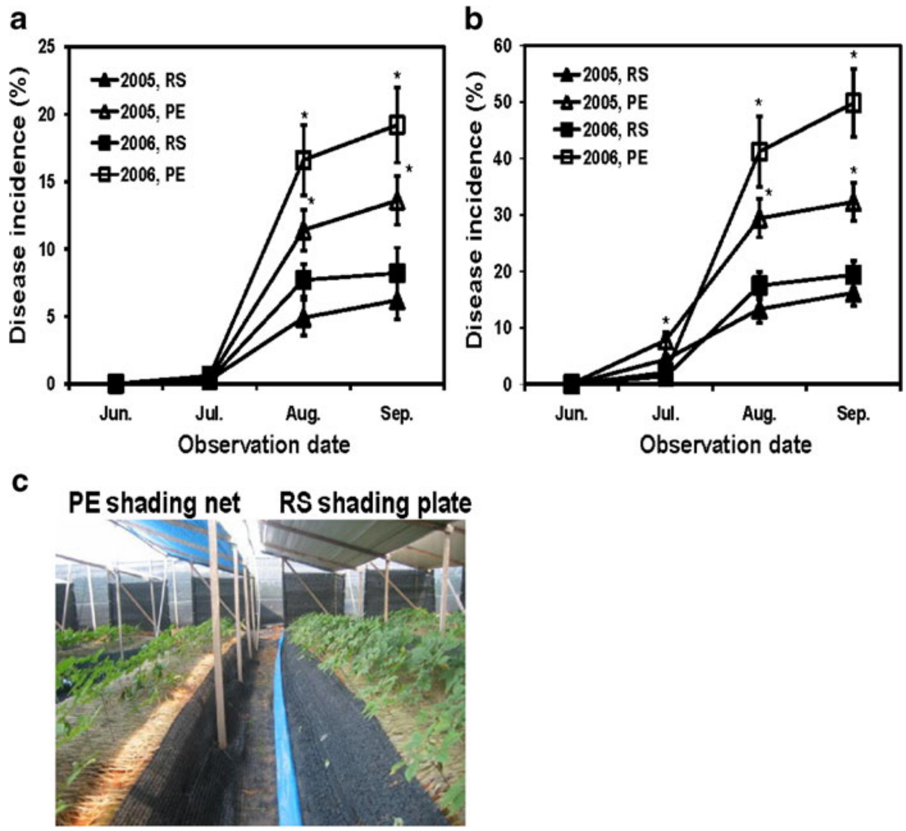

Fig. 1 Effect of rain shelter (RS) on disease incidence of ginseng anthracnose (**a**) and Alternaria blight (**b**) compared with polyethylene shading net (PE) in 2005 and 2006. All plots received four applications of Azoxystrobin SC (20% ai). * indicates a significant difference using Student's T-test at $P<0.05$ between treatments tested 2005 or 2006. Each treatment was replicated in three plots for each year. (**c**) Rain shelter plates and shading nets used in ginseng cultivation

Azoxystrobin SC (20% ai) was sprayed four times as described above. The incidence of Alternaria blight and anthracnose was determined on September 5th.

To confirm the role of the removed materials as an inoculum source, conidia and conidiophores of *Alternaria* and acervuli and setae of *Colletotrichum* were observed microscopically. Conidia of *Alternaria* and acervuli of *Colletotrichum* on dead stems were transferred to 10 μl of sterile water on a water agar plate using a finely drawn glass needle, then streaked using an inoculation loop. A single spore was transferred to PDA agar containing 10 μg/ml of ampicillin and incubated at 25°C in darkness. The spores of *Alternaria* and *Colletotrichum* were formed on V-8 juice agar with 14 days of incubation at 22°C with a 12 h light/ 12 h dark photocycle and on PDA by 14 days of incubation at 25°C in continuous darkness, respectively. The shape, size, and number of septa of 100 conidia randomly selected from the dead stems and from the cultured plates were recorded and compared with known strains.

The pathogenicity of three representative isolates of each genus was tested on leaves of two-month-old ginseng seedlings grown in a shaded greenhouse. The leaves were sprayed with 70% ethanol and washed immediately with an excess of sterilized water. The aqueous conidial suspension (10^5 conidia ml^{-1}) of each pathogen was sprayed onto the surfaces of the two-month-old ginseng leaves. Control leaves were sprayed with sterile water. The inoculated plants were enclosed in plastic bags for 3 days in a shaded greenhouse at 25±2°C. Seven days after opening, leaf symptoms were evaluated.

3. Biocontrol effect of the formulated microbial product

The efficacy of the microbial agents was determined in field plots with a rain shelter shading plate and eradication of dead plants. The formulated bacterial product (300 l /1,000 m^{-2}) was sprayed four times on five-year-old ginseng on June 3rd, June 12th, July 4th, and July 25th of 2005 and on six-year-old ginseng on June 1st, June 15th, July 3rd, and July 24th of 2006. Azoxystrobin SC (20% ai) and chitin minimal medium were used separately as controls, applied four times on the same schedule and in the same field. The incidence of Alternaria blight and anthracnose was assessed on August 5th. The number of Alternaria blight and anthracnose symptoms on the leaves was counted.

Statistical analysis

Data were analyzed by ANOVA using SPSS 12.OK for Windows software (SPSS Institute, Republic of Korea). The significance of the effects of bacterial treatment was evaluated by Duncan's multiple range test ($P=0.05$). The significance of the effect of the eradication of dead ginseng plants from the ginseng field or different shading nets were determined by Student T-test ($P<0.05$).

Results

Effect of rain shelter shading plates on incidence of Alternaria blight and anthracnose

The severity of both diseases was higher in 2006 than in 2005. The incidence of Alternaria blight was greater than the incidence of anthracnose in both years. Occurrence of both diseases increased dramatically from early July to early August. The incidences of both diseases were decreased by 50–60% by rain shelters in comparison to growth in plots with shading by polyethylene nets (Fig. 1a, b, and c).

Effect of dead plant removal on Alternaria blight and anthracnose

Abundant conidiophores and conidia of *Alternaria panax* (Fig. 2b and c) and numerous acervuli and setae of *Collectotrichum gloesporiodes* (Fig. 2d and e) were detected on the surfaces of the dead stems in the examined field (Fig. 2a). Both pathogens were detected in different sites of the dead stems. Three isolates of each pathogen, selected through single spore isolations, were pathogenic on young ginseng leaves causing typical symptoms of blight and anthracnose by artificial inoculation (data not shown). These findings establish that such infected plants could act as a source of secondary inoculum.

Removal of dead ginseng plants from the rain sheltered field plots resulted in a reduction in the incidence of both diseases over 2 years of field studies. The incidence of anthracnose decreased by 27% in 2005 and 31% in 2006 (Fig. 3a) and the incidence of Alternaria blight decreased by 36% in 2005 and 37% in 2006 (Fig. 3b).

Effect of application of microbial agents on Alternaria blight and anthracnose

The chitinolytic antagonists *S. plymuthica* and *L. enzymogenesis* inhibited mycelial growth of *A. panax* and *C. gloeosporioides*, while *Chromobacterium* sp. strain C-61 inhibited the mycelial growth of *A. panax* (Fig. 4). *S. plymuthica* was not selected for further studies due to its observed inhibitory effect on ginseng seed germination and because it caused leaf spots on healthy ginseng plants (data not shown). Thus, *L. enzymogenesis* and *Chromobacterium* sp. strain C-61 were utilized in the preparation of the chitin-based microbial product (Kim et al. 2008).

The efficacy of the formulated microbial product was compared with that of a fungicide treatment in the field, coupled with the removal of dead ginseng plants under the rain shelter shading plates. The incidence of anthracnose and Alternaria blight reached levels of 30–75% (2005) and 70–100% (2006) in early August in untreated control plots. Application of the formulated product resulted in a reduction in the incidence of both diseases similar to that of fungicide application in 2005, but slightly lower than that noted with fungicide treatment in 2006 (Fig. 5).

Discussion

The shading materials in ginseng are crucial to the quality and yield of ginseng roots (Lee 2007). Rice straw thatch shading, which had been previously used in Korean ginseng fields, was replaced with a polyethylene (PE) shading net, due to convenience of installation. However, PE shading was associated with several defects owing to high field temperatures and substantial rain permeability as compared with the thatch shading (Kim et al. 1990). Recently, the rain shelter (RS) shading plates, which can reflect sunlight and prevent rain-permeation, were developed to resolve the deficiencies of PE shading. This study revealed that the occurrences of anthracnose and

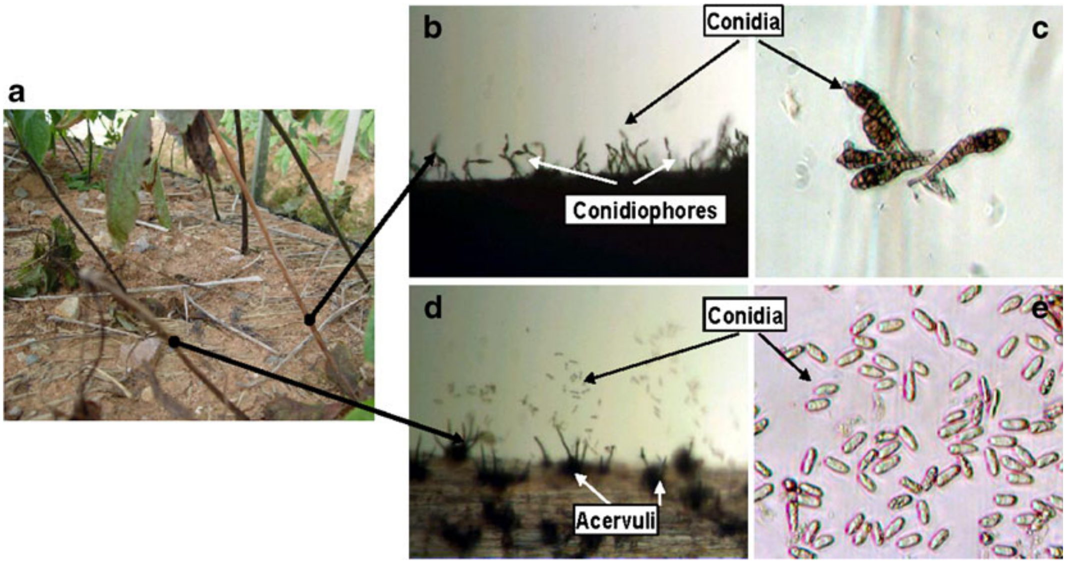

Fig. 2 Fungi observed on dead stems (**a**) collected from ginseng plots. Conidiophores (×40, **b**) and conidia (×400, **c**) of *A. panax*, Acervuli (×40, **d**) and conidia (×400, **e**) of *C. gloesporiodes*

Alternaria blight were reduced significantly under RS shading conditions as compared to PE shading conditions. It can be speculated that the lower disease incidence of Alternaria blight and anthracnose in RS shading as reported in this study might result from the higher survival rates, higher yields, and superior quality of ginseng grown in RS shading as compared to PE shading (Lee 2007).

In order to control foliar diseases in Korean ginseng, a 10-day interval application method for fungicide spraying has been suggested (Kang et al. 2007). Alternaria blight and anthracnose in ginseng have been reported to occur from June to early September in Korea (Ohh 1981), which suggests that fungicides should be applied more than nine times in the field, depending on the prevailing weather conditions. A recent study showed that seven repetitions of fungicide spraying resulted in control values of 78.8% for Alternaria blight and 70.5% for anthracnose, under PE shading conditions (Kim et al. 2007). In this study, similar levels of control of Alternaria blight and anthracnose were achieved by four applications of

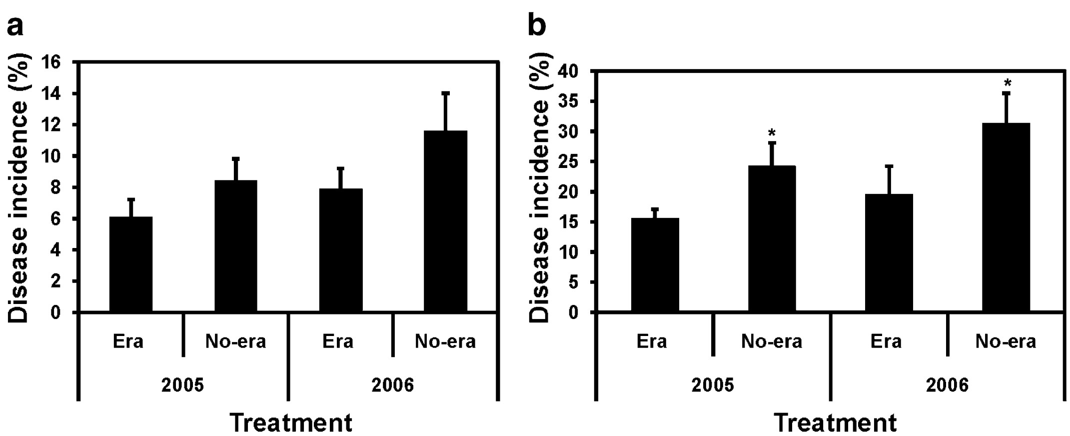

Fig. 3 Incidence of ginseng anthracnose (**a**) and Alternaria blight (**b**) by removal of dead ginseng plants and application of four Azosystrobin SC (20% ai) sprays. Dead five-year-old ginseng (year 2005) and six-year-old ginseng (year 2006) plants under rain shelter shading plate fields were immediately discarded (Era) from June to August, while the dead plants were left in the fields for control plot (No-era). * indicates a significant difference between treatments in the same year based on Student's T-test at $P<0.05$

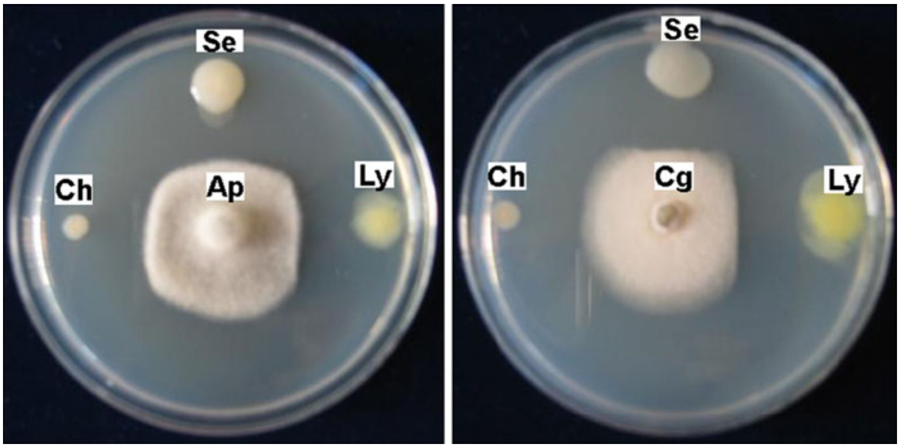

Fig. 4 Inhibitory effect of chitinolytic bacteria against the ginseng pathogens, *A. panax* (Ap) and *C. gloeosporiodes* (Cg), on potato dextrose agar plates. Se, *Serratia plymuthica*; Ch, *Chromobaterium* sp. C-61; Ly, *Lysobacter enzymogenes* C-3

fungicide coupled with the replacement of shading plates with RS (Fig. 1). The field longevity of the rain shelters makes this practice more cost effective.

We found abundant inocula of *A. panax* and *C. gloeosporoides* in the dead plants, which can function as the primary source of inoculum for severe disease incidence in the field. The results of this study showed that the mere removal of dead plants results in a reduction in the occurrence of the diseases. In conventional Korean ginseng fields, weeds are removed twice during the growing season, but the dead plants are left untouched in most fields. Our findings indicate that dead plants should be removed along with the weeds, as the dead plants are the primary source of the spread of Alternaria blight and anthracnose in the field.

Bacillus subtilis QST713 (BS QST713), which is effective in inhibiting powdery mildew, has also proved effective in the control of Alternaria blight in ginseng (Li et al. 2008). The formulated product used here gave control similar in efficacy to fungicide use against Alternaria blight and anthracnose of ginseng under RS shading conditions coupled with the eradication of dead plants. The efficacy of the biocontrol product was similar to that of fungicide applications in 2005, but slightly less effective than fungicide treatments in 2006 when disease pressure was higher. This suggests that the control efficacy of the formulated product may be reduced under severe disease occurrence conditions as has been observed before in other systems (Boland 1997). In order to achieve a completely environmentally-friendly control system, we

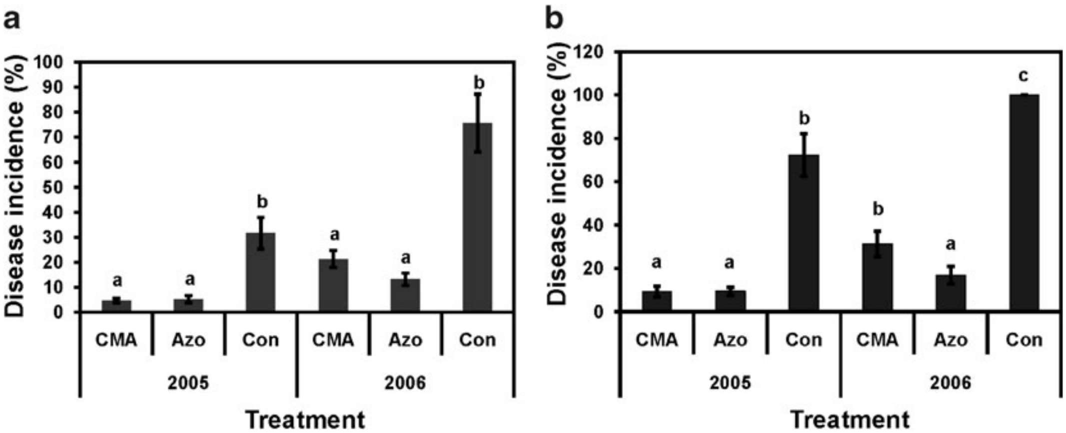

Fig. 5 Efficacy of the formulated microbial product of a combination of two chitinolytic bacteria (CMA) and a chemical fungicide, Azoxystrobin (Azo) on anthracnose (**a**) and Alternaria leaf blight in ginseng (**b**). This study was conducted using rain shelter shading plates combined with the removal of dead plants in the field. Differences in letters indicate the differences between treatments on the basis of Duncan's multiple range test at $P=0.05$

will need to establish optimal application timings and intervals for the application of the formulated product under severe disease pressure. Alternaria blight and anthracnose in ginseng occur frequently in Korea from mid to late July (Kim et al. 1990; Ohh 1981). In our study, the incidence of the diseases increased dramatically in July even though fungicide was applied four times from early June to late July (Fig. 1). The control efficacy of the formulated product was almost equal to that of the fungicide, even under severe disease pressure and might be equal if the formulated product is applied one more time in mid July.

This study illustrates the possibility of developing an environmentally-friendly control using RS shading, combined with the eradication of dead plants and use of a formulated microbial product against Alternaria blight and anthracnose of ginseng. Additionally, the installation of RS shading and the eradication of the dead plants do not require inordinate expense or physical labour as compared with the traditional cultivation practices. The formulated microbial agents can be made at low cost by the farmers themselves (Kim et al. 2008). Therefore, this control system will be useful for many ginseng growers, organic farmers in particular.

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