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Integration of Biological and Chemical Methods for the Control of Pepper Gray Mold Rot Under Commercial Greenhouse Conditions

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Integration of microbial antagonists with fungicides was tried to control the gray mold caused by *Botrytis cinerea* on pepper in greenhouse conditions and to reduce fungicide uses. All of the selected bacterial antagonists, *Bacillus amyloliquefaciens* BL3, *Paenibacillus polymyxa* BL4, and *Pseudomonas putida* Cha94, completely inhibited the conidial germination of *B. cinerea* until 30 days after treatment. However, bacterial colonization on pepper phylloplane was poor in BL4, while the other bacterial isolates and the fungal antagonist *Trichoderma harzianum* TM colonized well on the phylloplane, maintaining the population density of 10^4-10^5 cfu/g until 15 days after microbial treatments. Out of 13 kinds of selected fungicides used for gray mold diseases, polyoxin B and BKF 1995 showed the most discriminatory activity on the fungal growth between *B. cinerea* and TM. TM grew readily on the media containing those fungicides, while *B. cinerea* showed poor or no mycelial growth on them. The selected fungicides and antagonists alone reduced incidence of gray mold on pepper, showing disease indices of about 2.4 to 3.0, while it was increased up to 4.2 in the untreated control. Alternate treatments with the antagonists and 2-fold diluted fungicides inhibited the disease incidence as much as the antagonists or fungicides alone, and reduced the secondary inoculum more than the single treatments. This suggests that integration of antagonists and fungicides may be an efficient way to reduce fungicide sprays with reliable control efficacy of the disease. However, there was not much difference in the early and mid-term disease progress among the treatments and the untreated control, probably due to extremely favorable environmental conditions for the disease development in this experiment.

Keywords : alternate treatment, *Botrytis cinerea*, integrated control, pepper, secondary inoculum.

For the successful biological control of foliar disease, biocontrol agents introduced into fields or greenhouses should compete with other microorganisms and establish an active population on the phylloplane (Elad et al., 1994; Elad and Shtienberg, 1996). It is suggested that microorganisms isolated from the phylloplane of a specific crop may be adapted well to the crop environments, and thus controlling diseases more efficiently than those isolated from other plant species (Aylor, 1998). Such plant-associated microorganisms may become more efficient biocontrol agents because of their capacity for adaptation to the environmental conditions (Blakeman and Fokkema, 1982; Cook, 1993). Redmond et al. (1987) reported that fungi and bacteria isolated from rose petals were potential biocontrol agents of Botrytis blight, a serious disease of greenhouse-grown roses (Kohl et al., 1995).

Several bacteria such as *Erwinia* sp., *Corynebacterium* sp., *Pseudomonas syringe*, *Bacillus* spp. and *Serratia marcescens*, and yeast isolates *Auerobasidium pullulans* and *Candida saitoana* were found to control gray mold in rose, cyclamen, apple and pear (Kohl et al., 1998; Mari et al., 1996; Redmond et al., 1993). Isolates of filamentous fungi *Ulocladium atrum*, *Gliocladium roseum*, *Cladosporium herbarum*, *Trichoderma harzianum*, and *T. viride* were reported to be effective in suppressing *B. cinerea* on grape or in greenhouse crops (El-Ghaouth et al., 1998; Haran et al., 1996; Jackson et al., 1997; Kohl et al., 1998; O'Neill et al., 1996; Zimand et al., 1996). Most of the isolates mentioned above inhibited the sporulation of the pathogen on infected lesions. Especially, effective control of *B. cinerea* by isolates of *Trichoderma* sp. and *Gliocladium* sp. has been reported in tomato, cucumber, strawberry and grape (Elad and Shitenberg, 1996; Elad et al., 1993; Sutton et al., 1997).

In field and greenhouse trials, use of biological agents has generally resulted in significant but inconsistent and only partial disease control because of their insufficient adaptation to such conditions as low nutrient availability, UV radiation, low temperature and relative humidity (Leibinger et al., 1997). This aspects hinder biocontrol agents themselves to be used as sole measures for disease management.

One way to improve the reliability of biocontrol agents is to integrate them with chemical fungicides (Shitenberg and

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Elda, 1997). Integration of biological and chemical control methods for gray mold diseases has been investigated in several studies, for example, by Harman et al. (1996) for bunch rot of grape caused by *B. cinerea* and by Elad et al. (1993) for cucumber gray mold under commercial greenhouse conditions. However, there still remain some major problems in the integration of chemical and biological control agents, most of which may be derived from the fact that the living biological control agents are influenced by the environmental conditions. It is difficult to determine their application time and intervals for the maximum control efficacy, although alternating sprays of chemical and biological agents largely improve the control efficacy and may reduce chemical sprays. The environmental conditions significantly affects on them, but only few researchers have examined the feasibility of using weather forecasts as a predictive system for biological controls (Vincelli and Lorbeer, 1989). Shtienberg and Elad (1997) found it an effective approach to consider future weather as a basis for integration of biological and chemical control measures.

We have attempted to develop an integrated control strategy against *B. cinerea* epidemics in the greenhouse, considering our previous results in the forecasting system of the initial disease incidence of pepper gray mold rot monitored by the air-borne conidia and based on threshold environmental conditions (unpublished). The objectives of this study were to determine the effects of fungicides and biological agents and to evaluate the alternate applications of both agents in reduced concentrations to establish an integrated control measure for gray mold rot of pepper in the greenhouse.

MAterials and Methods

Antagonistic bacteria and fungus. Several epiphytic bacteria and fungi were isolated and identified. Bacterial strains resistant to rifampicin were selected from parent cultures by plating cell suspension on Luria-Bertani agar (LBA) medium containing 50 ppm rifampicin. The bacterial isolates identified were *Bacillus amyloliquefacience* BL3, *Paenibacillus polymyxa* BL4, *Pseudomonas putida* Cha94 and *Trichoderma harzianum* TM. BL3 and BL4 were cultured on LB, Cha94 on King's B (KB), and TM on PDA media, respectively.

Antifungal activity of the antagonistic microbes. Suppression of conidial germination of *B. cinerea* by the bacterial antagonists were examined. The bacterial strains were grown for 24 hr, and three loops of about 10 μl each were taken from the resulting colonies, diluted in 0.1 M $MgSO_4$ to the concentration of 10^6 cfu/ml. The fungal conidia were suspended in sterile distilled water (SDW) containing 0.05% Tween 80 to the concentration of 10^6 conidia/ml. The conidial suspension of the pathogen was mixed with the equal volume of each bacterial suspension, and dropped on a hole slide glass, incubated at 20°C for 33 hr, and observed under a light microscope during the incubation period.

Survival and colonization of the antagonists. To monitor survival and colonization of introduced isolates on flowers and leaves of pepper plants, greenhouse trials were conducted in 1997 using a commercial pepper cultivar Nogkwang in Gumgok near Chinju, Korea. The experimental field consisted of a total of 12 plots, each of which 25 pepper plants were planted. Each antagonist (10^8 cfu/ml) was applied to the phylloplane of pepper plants on January 14, 1997 with a compressed-air sprayer. Treatments in the greenhouse were arranged in a completely randomized block design with three replicate plots per treatment. One gram of treated flowers and leaves for each was cut from the pepper plant and transferred into 250 ml flasks containing 10 ml of 0.1 M $MgSO_4$ solution, and shaken at 150 rpm for 30 min followed by heat treatment at 85°C for 30 min (only for BL3 and BL4). Aliquots with 0.1 ml of 10-fold dilutions were plated on PDA containing 50 μg/L rifampicin for BL3 and BL4 on KBA containing 50 μg/L rifampicin and 100 μg/L cycloheximide (Sigma Co., St. Louis, MO., USA) for Cha94, and on *Trichoderma*-selective media for TM. After incubation at 28°C for 2 days, bacterial and fungal colonies grown on the media were counted. All antagonistic populations were retrieved for 6 times at 2 to 3-day intervals in 15 days.

Fungicidal resistance of antagonists. Growth of *B. cinerea* and TM on the media containing fungicides was examined were examined to select appropriate fungicides that can be used together with to the fungal antagonist. To screen fungicidal sensitivity of the pathogen and TM, each of 8 mm mycelial discs of the fungi was placed on the center of agar media containing each fungicide, and mycelial growths (diameters of fungal colonies) were measured after 30, 125 and 50, 150 hr of incubation, respectively. Fungicide-free media were used as untreated controls.

Monitoring the secondary inoculum by air-borne conidia. In the greenhouse study, we aimed to relate the incidence of gray mold on pepper plants to the population density of air-borne inoculum and environmental factors affecting the susceptibility of host in the greenhouse. For this, air-borne conidia released were sampled by the spore-trapping device.

Evaluation of single treatments of fungicides or antagonistic microbes and their alternate treatments. Field trials were conducted in a commercial greenhouse in Gumgok near Chinju from November 1997 to February 1998 using pepper cultivar Nogkwang. Polyoxin B, BKF1995 (Korea Bayer Co., Seoul, Korea) and biocontrol agents (Cha94, BL3 and TM) were used for this experiment. The pepper plants were sprayed until run-off with each antagonist (10^8 cfu/ml) or fungicide at the recommended concentration or with a 2-fold diluted fungicide and an antagonist in alternation. Each treatment consisted of three replicates arranged in a randomized block design. Each plot consisted of 25 pepper plants. Spray applications were made as many as seven times at 10-day intervals from December 4, 1997 to February 5, 1998. After 5 days of treatment, suppressive effects against disease progress of the gray mold were evaluated for antagonists, fungicides, and both in alternation. To determine reproduction of the pathogen in the treatments, the secondary inoculum was monitored by sucking the air-borne conidia within each plot by means

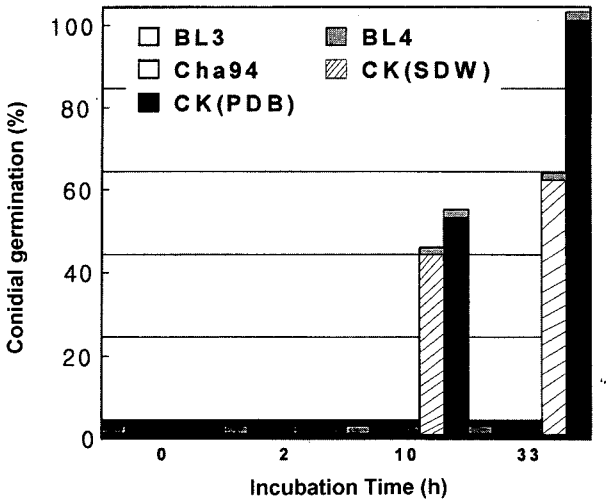

Fig. 1. Effect of antagonistic bacteria on the conidial germination of *Botrytis cinerea in vitro*. The conidial suspension of the pathogen was mixed with an equal volume of each bacterial suspension of *Bacillus amyloliquefacience* BL3 (BL3), *Paenibacillus polymyxa* BL4 (BL4) and *Pseudomonas putida* Cha94 (Cha94) or sterile distilled water (SDW) or potato dextrose broth (PDB), and incubated at 20°C for 33 hr.

of spore-trapping device, and enumerated with a haemacytometer under a light microscope.

Results

Inhibition of conidial germination by the bacterial antagonists and their epiphytic survival. The three bacterial strains, BL3, BL4 and Cha94, completely suppressed spore germination of the gray mold fungus at 20°C until 33 hr after treatment (Fig. 1). In sterile distilled water or in potato dextrose broth (PDB) conidia of the pathogen were readily germinated, expecially almost 100% in PDB after 33 h of incubation. On the other hand, BL3 and Cha94, and the fungal antagonist TM maintained their populations of 10^4-10^5 cfu/g on flower and leaf surfaces until 15 days after treatment, but the population of BL4 was sharply declined to 10^2 cfu/g (Fig. 2).

Selectivity of fungicides between the pathogen and antagonists. Out of 13 kinds of selected fungicides used for gray mold diseases, polyoxin B and BKF1995 showed the most discriminatory activity on the fungal growth between *B. cinerea* and TM (Fig. 3). TM grew readily on the media containing those fungicides, while *B. cinerea* showed poor or no mycelial growth on them. The other fungicides, except carboxamide+benzimidazole and benzimi-

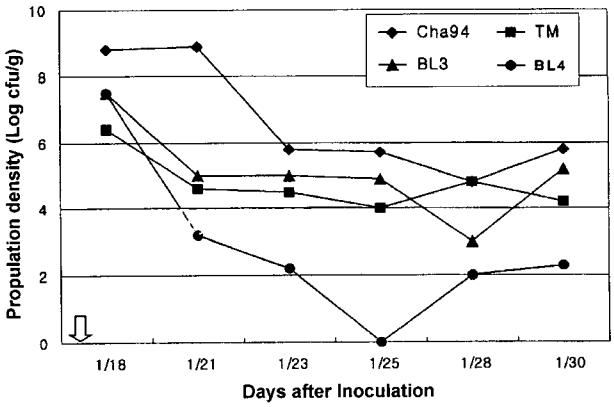

Fig. 2. Population densities of antagonistic microorganisms on petals and leaves of pepper. Arrow indicates the date of microbial application. (on Jan. 14) (sprayed at the concentration of 10^8 cfu/ml with suspension of *Bacillus amyloliquefacience* BL3 (BL3), *Paenibacillus polymyxa* BL4 (BL4), *Pseudomonas putida* Cha94 (Cha94), and *Trichoderma harzianum* TM (TM).

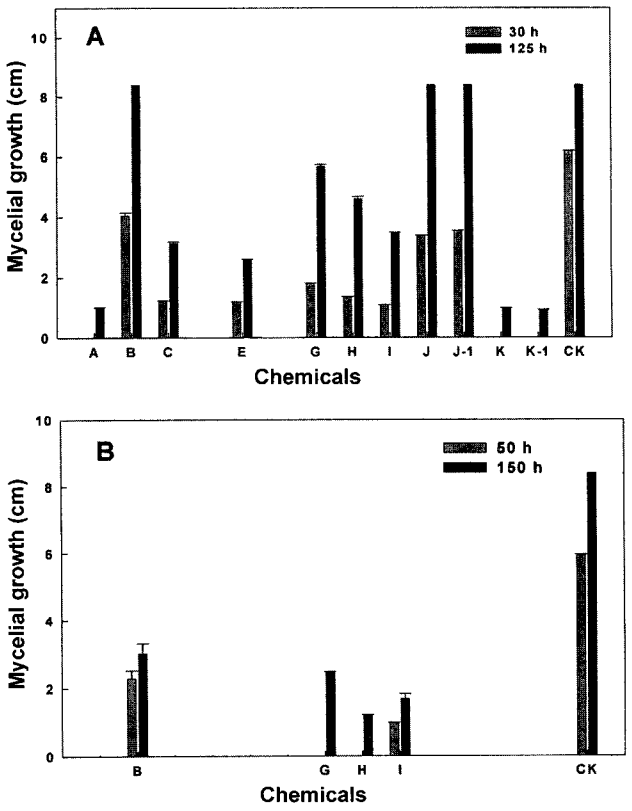

Fig. 3. Evaluation of mycelium growth of *Trichoderma harzianum* TM (A) and *B. cinerea* (B) on various fungicidal media. Mycelial plugs of *T. harzianum* and *B. cinerea* of 8 mm in diameter were inoculated on fungicidal media, and colony growths were measured after 30 and 125 hr, 50 and 150 hr of incubation, respectively. Arrows indicate the time (62 hr for TM and 70 hr for *B. cinerea*) when the colonies grew maximum to the margin of Petri plates in fungicide free media. A, Tebuconazole + Tolyfluanid; B, Polyoxin B; C, Dicarboximide; D, Carboxamide + Benzimidazole; E, Dichlofluanid; F, Benzimidazole; G, Pyrimidiamine + Phenyl pyrrole; H, Pyrimidiamine; I, Ditroanilin; J, BKF 1995; J-1, 2-fold diluted solution of BKF1995; K, BKF1995 + Tebuconazole; K-1, 2-fold diluted solution of K.

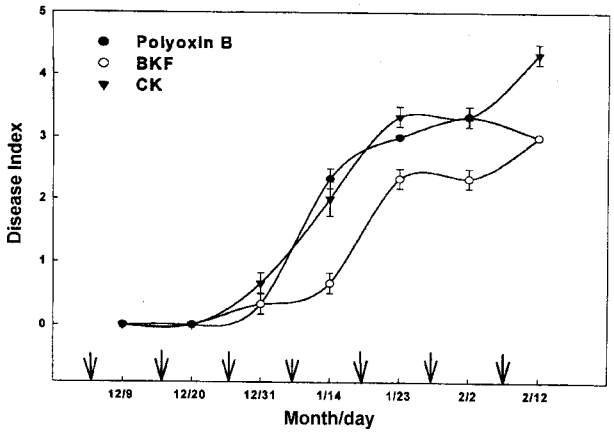

Fig. 4. Effects of fungicides polyoxin B and BKF 1995 (BKF) on the control of gray mold. Arrows indicate fungicidal application dates. Disease indecencies were examined 5 days after each treatment, and the resulting data were converted to Botrytis disease indices ranging from 0 to 5 for none to 100% disease incidences. Vertical bars represent the values of least significant difference at p = 0.05.

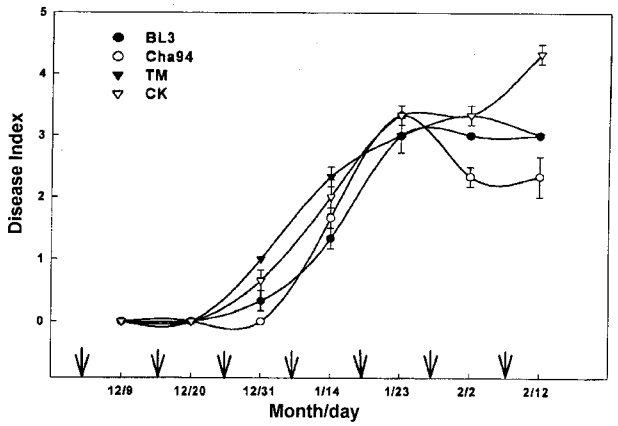

Fig. 5. Effects of antagonists Bacillus amyloliquefaciens BL3, Pseudomonas putida Cha 94, and Trichoderma harzianum TM on the control of the gray mold of pepper. Microbial suspensions (10^8 spores/ml) were sprayed on pepper plants until run-off (arrows indicate the application dates). Vertical bars represent the values of least significant difference at p = 0.05.

dazole that completely inhibited mycelial growth of both fungi, also had medium or weak discriminatory fungicidal activity against the pathogen. In a preliminary study, the bacterial antagonists also grew well on the media containing those fungicides, and thus, we selected polyoxin B and BKF 1995 to be integrated to biological treatments because of their fungicidal discrepancy.

Efficacies of fungicides and antagonists in disease suppression. The disease incidence in the plots treated with the fungicides, polyoxin B and BKF was 3.0 of disease index, while it was 4.2 in the untreated plots at the end of the experiment (Fig. 4), indicating that fungicide treatments reduced the disease significantly. In both treated and untreated plots, the disease increased rapidly during January, and in this period the disease progressed more rapidly in polyoxin B than BKF 1995. Thus, in the point of disease accumulation, BKF 1995 was relatively more efficient than polyoxin B as the early and mid-term disease progress was retarded relatively more by the former fungicide.

Disease incidence was also significantly reduced by the microbial treatments, showing disease indices of 2.4, 3.0 and 3.0 by Cha94, BL3 and TM, respectively, while that of the control was 4.2 as mentioned above (Fig. 5). However, there was not much difference in the early and mid-term disease progress among the microbial treatments and the control. This might be due to the fact that the inoculum density was high (about 10^3 spores/0.5 m^3) and environmental conditions were very favorable with high RH and optimum temperature (100% RH and 14-17°C) at the infection periods (data not shown). In these conditions, the efficacies of biological controls may be reduced substantially.

Suppressive effect of alternate treatments of fungicides and antagonists. Disease development in alternate treatments with 2-fold diluted fungicides and antagonists significantly reduced disease incidence, in which disease indices were 2.0 to 3.2 as compared with 4.4 in the control treatment at the end of the experiment (Fig. 6). Fungal reproduction, estimated by the number of secondary inoculum (conidia) of *B. cinerea,* was significantly lower in the experimental plots sprayed with both fungicides (2-fold diluted) and the biocontrol fungus (TM) in alternation than for those treated with fungicides or antagonist alone. Spore density of *B. cinerea* reproduced in each plot was about 10^2 conidia/0.5 m^3 in the plots of the alternate treatments, while

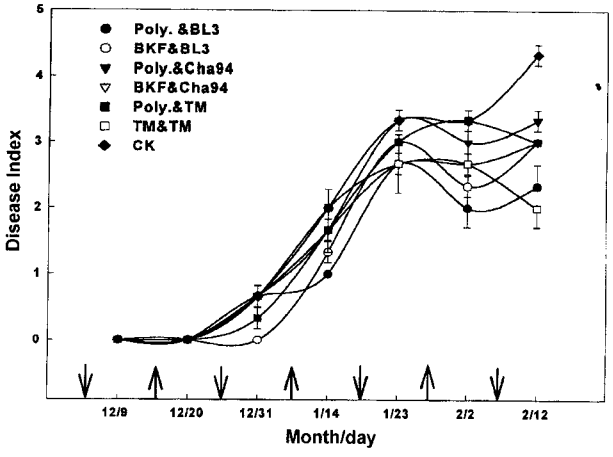

Fig. 6. Effects of alternate treatments of fungicides and microbial antagonists on the control of the gray mold of pepper. A pair of a 2-fold diluted fungicide and an antagonist were sprayed alternatively (application dates are indicated by ↓ for fungicides and ↑ for antagonists). Vertical bars represent the values of least significant difference at p = 0.05.

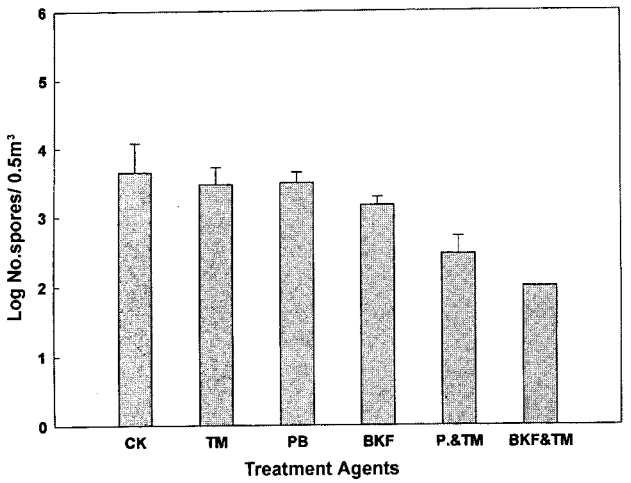

Fig. 7. Reproduction of *B. cinerea* as affected by the treatment of fungicides, antagonists or their alternation examined by the secondary inocula (conidia) by sucking air-borne conidia above the corresponding experimental plots by means of spore-trapping device. TM, *Trichoderma harzianum* TM; Poly. & TM, Polyoxin B & TM.

it was about 10^3 conidia/0.5 m^3 in the plots treated with chemicals or antagonists alone, having no significant difference compared with the untreated control (Fig. 7).

Discussion

Until now, most biological control studies have been conducted by laboratory plate assays to examine antibiosis or mycoparasitism on a specific medium (Papavizas, 1985). Generally, the *in vitro* results have not been matched well with the ability to reduce or prevent diseases in greenhouse or field conditions (Lewis and Papavizas, 1985). This is probably because besides antibiosis and mycoparasitism, survival and activity of microbial antagonists on host plants are importantly involved in the efficacy of biological control. The introduced biocontrol agents in fields or greenhouses must compete with other microorganisms and establish an active population on the phylloplane to function (Elad et al., 1994; Elad and Shtienberg, 1996). In this respect, plant-associated microorganisms may be better biocontrol agents because they are already closely associated with and adapted to the plant or plant part as well as the particular environmental conditions in which they must function (Blakeman and Fokkema, 1982; Cook, 1993). In our study, the isolates of Cha94, BL3 and TM survived and colonized on floral and foliar canopies of pepper for 15 days at concentration of 10^4-10^5 cfu/ml in the commercial greenhouse. Such a high level of colonizing ability on the ecological niches should be a valuable component for successful biological control.

Currently avaliable biocontrol agents may not provide reliable disease suppression because of environmental variations associated with disease development (Leibinger et al., 1997). Consequently, biocontrol agents do not always meet a commercial standard for disease management. There are many ways to solve this problem. Integration of chemical and biological control agents may be one way, providing a method of not only reducing chemical use but also coping more effectively with the development of pathogen population, especially fungicide-resistant pathogens (Elad et al., 1993; Harman et al., 1996; Shitenberg and Elda, 1997). Harman et al. (1996) reported that an alternation of biological and chemical agents may result in acceptable levels of disease control with reduced levels of pesticide usage. Elad et al. (1993) also achieved up to 90% control using *T. harzianum* in alternation with fungicides in controlling cucumber gray mold in most experiments under commercial greenhouse conditions. In our study, polyoxin B and BKF were selected for integration to TM, Cha94 and BL3 as the fungicides showed weak fungicidal activity to the antagonists relative to the pathogen, *B. cinerea*. As results, the alternate treatments of both agents achieved similar control value of pepper gray mold rot to single treatments even when the concentrations of the fungicides were reduced by a half. The secondary inoculum was less in alternate treatments than in other treatments. Therefore, the alternate treatments of both fungicides and antagonists may be a reliable method for reducing the build-up of secondary inoculum in experimental plots, which would result in less secondary infection. Therefore, this study demonstrated that the alternate use of biological and chemical control measures may practically prevent the disease to the acceptable level, ant that it may reduce pesticide usage.

Occurrence of gray mold diseases caused by *B. cinerea* is highly dependent on climatic conditions. Gross et al. (1998) suggested that leaf wetness is perhaps the environmental variable that permits an infection event, but temperature determines the rapidity and extent of that event. The colonization and subsequent sporulation of *B. cinerea* on necrotrophic leaf tissue results not only in a high inoculum potential within the canopy of individual plants, but also an increase in the spore load within the greenhouse (Kohl et al., 1998). The environmental conditions may also influence on the efficacy of the biocontrol agents. Elad et al. (1993) reported that the calculations of relationships between microclimatic conditions and disease control achieved by *T. harzianum* revealed a negative effect of wetness and a positive effect of temperatures above 20°C. O'Neill et al. (1996) reported that the optimum temperature for wound infection of *B. cinerea* was around 15°C and that for growth of *T. harzianum* in their study was 26°C. In our study, disease incidence rapidly increased despite the application of fungicides and biocontrol agents after initial infec-

tion of pepper plants. The climatic conditions were 14-17°C and 90-100% RH for prolonged periods of time during our experiment (unpublished data). This indicates that the environmental conditions were extremely favorable to the pathogen and may be unfavorable to the host and antagonists, in which any control measures may be minimized in their activity.

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