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Effect of GlycinecinA on the Control of Bacterial Leaf Spot of Red Pepper and Bacterial Leaf Blight of Rice

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***Xanthomonas axonopodis* pv. *glycines* 8ra produces a bacteriocin called glycinecinA, which specifically inhibits the growth of bacteria belonging to *Xanthomonas* species. GlycinecinA was produced by culturing *Escherichia coli* DH5 containing biosynthetic genes for glycinecinA, and was tested for its control effect against *X. vesicatoria* on red pepper and *X. oryzae* pv. *oryzae* on rice. The bacteriocin activity was much higher in the cell extract than in the supernatant. It reached a maximum level at the stationary phase, was maintained up to 2 months at room temperature and approximately 10 months at 4°C. The optimum concentration of glycinecinA for the control in the greenhouse and in the field was 12,800 AU/ml. In this study, the activity of glycinecinA on rice and red pepper leaves continued for 7-8 days, during which the pathogen populations remained at low levels. Bacterial leaf spot of red pepper and bacterial leaf blight of rice were significantly reduced by the bacteriocin treatments. The control efficacy was as high as, or even higher than, the chemical treatment of copper hydroxide. These results suggest that the bacteriocin is a potential control agent for bacterial diseases.**

Keywords : bacteriocin, bacterial leaf blight of rice, bacterial leaf spot of red pepper, glycinecinA.

Bacteriocins are defined as non-replicating antimicrobial compounds produced by bacteria that have specific inhibitory effect on other closely related bacteria. Colicins, which are antibiotic-like compounds produced by *Escherichia coli*, were first discovered as bacteriocins (Gratia, 1925). The specificity and chemical composition of these compounds distinguish them from classical antibiotics such as streptomycin, a low molecular weight, broad-spectrum glycoside. The primary criterion for identifying bacteriocins is that antagonism is specific, and not due to non-specific compounds such as hydrogen peroxide and lactic acid.

Bacteriocinogeny, or the ability to produce bacteriocins, is widespread. Species in over 30 bacterial genera including gram-positive bacteria such as *Corynebacterium* spp., *Lactobacillus* spp., and many plant pathogens have been reported to produce antimicrobial substances (Vidaver, 1978).

Bacteriocins are used for food preservation (Ray and Daeschel, 1992) and biological control (Kerr and Htay, 1974). Nisin produced by *Lactobacillus lactis* subsp. *lactis* is active against almost all gram-positive bacteria. Agrocin84 produced by *Agrobacterium radiobacter* strain K84 for biological control is used as an effective biological control agent against *A. tumefaciens* (Kerr and Htay, 1974). Only few bacteriocins have been found in phytopathogenic bacteria and have been characterized. Antagonism between bacterial strains due to bacteriocins has been found in *Agrobacterium radiobacter* (Kerr and Htay, 1974), *Clavibacter michiganense* (Echandi, 1976; Gross and Vidaver, 1979), *Erwinia carotovora* (Cooksey, 1990; Gross and Vidaver, 1979), *E. chrysanthemi* (Echandi and Moyer, 1979), *E. herbicola* (Lee and Cho, 1996; Vanneste et al., 1992; Wodzinski et al., 1994), *Pseudomonas syringae* (Smidt and Vidaver, 1982), *Ralstonia solanacearum* (Chen, 1984; Yi and Son, 1993), *Rhizobium japonicum* (Gross and Vidaver, 1978), *Xanthomonas oryzae* pv. *oryzae* (Mew et al., 1982; Xu and Gonzalez, 1991), and *X. axonopodis* pv. *glycines* (Ahn and Cho, 1996). Majority of bacteriocins of plant pathogenic bacteria are macromolecular compounds, which are either heat-sensitive or trypsin-resistant, or both. Another major group of bacteriocins contains low molecular compounds, while others are sensitive to both heat and proteolytic enzymes, resistant to both heat and enzymes, or shows differential sensitivity to trypsin and proteinase.

Fett et al. (1987) reported that *X. axonopodis* pv. *glycines* produces a bacteriocin only on solid medium, and named it as glycinecin comparing to the glycin produced by *P. syringae* pv. *glycinea*. Glycinecin is inhibitory against *Xanthomonas* species, which include many important plant pathogenic bacteria. In previous studies (Woo, 1997; Woo et al., 1998), it was revealed that bacteriocin was produced

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by *X. axonopodis* pv. *glycines* 8ra in liquid culture medium, and had inhibitory effects against *Xanthomonas* species. It was sensitive to proteinase K and trypsin, but not inducible by mitomycin C and UV light. Also, glycinecin is heat-resistant and stable at a wide range of pH and is precipitated by 30-60% $(NH_4)_2SO_4$. Glycinecin-coding genes were isolated through the genomic library screening of *X. axonopodis* pv. *glycines* 8ra (Ahn and Cho, 1996). It has been shown that *E. coli* DH5α carrying pBL5 expressed glycinecinA and exerted antibacterial activity (Ahn and Cho, 1996, Heu et al., 2001).

Bacterial leaf blight of rice caused by *X. oryzae* pv. *oryzae* and bacterial leaf spot of red pepper caused by *X. vesicatoria* are widespread diseases found all over the world. Copper-containing compounds have usually been used to control these diseases. However, their heavy use has caused emergence of bactericide-resistant (copper-resistant) strains, lessening the control efficacies of these compounds (Adaskaveg and Hine, 1985; Bender et al., 1990; Burdhead et al., 1994; Cooksey et al., 1990; Marco and Stall, 1983). In addition, their heavy use is detrimental to the environment. Other cultural control measures such as sanitation and crop rotation before cultivating the crops are not always effective in suppressing the diseases because they are very sporadic depending on the environmental conditions. In these respects, biological control is more advantageous over chemical and cultural control methods; it is safe to the environment and can be applied at an appropriate time for the disease control during the growing season.

In this study, glycinecinA was tested for antibiotic activity, shelf life, and control of diseases caused by *X. vesicatoria* and *X. oryzae* pv. *oryzae* in greenhouse and field conditions. The results provide information on the potential and practical use of glycinecinA for the control of the diseases.

Materials and Methods

Bacteria and media. The glycinecinA-producing bacterial strain was *Escherichia coli* DH5α transformed with pBL5, a cosmid clone containing all biosynthetic genes of glycinecinA. *Xanthomonas vesicatoria* YK93-4 was used as an indicator strain for testing bacteriocin activities. *Xanthomonas vesicatoria* Uijongbu1 and *X. oryzae* pv. *oryzae* KXO169 were used to inoculate red pepper and rice plants, respectively. *Escherichia coli* DH5α carrying pBL5 was grown at 37°C, shaken at 200 rpm in Luria-Bertani (LB) broth (Sambrook et al., 1989) or on LB agar plates. *Xanthomonas* isolates were grown in peptone-sucrose broth (PSB) shaken at 200 rpm or on PS agar (PSA) (Shadd, 1988) at 28°C. Nutrient broth (NB) or nutrient agar (NA) was also used for these bacteria.

Measurement of bacteriocin production. *Escherichia coli* DH5α carrying pBL5 was used to produce glycinecinA in liquid media (LB broth). Cell-free supernatant and crude cell extract were prepared as follows. Bacterial cell culture was grown to log phase in LB broth and then sub-cultured into 100 ml of the same medium in a 250 ml flask, incubating at 37°C on a rotary shaker (200 rpm) for 24 h when the bacterial population reached between 5×10^8 cfu/ml and 1×10^9 cfu/ml. The bacterial culture was centrifuged at $6{,}000 \times g$ for 10 min. The supernatant was used for the bacteriocin activity test. The pellet was washed with sterile distilled water and re-suspended with 100 ml of distilled water in a 250-ml flask, and sonicated with a VCX400 high intensity ultrasonic liquid processor (Sonics & Materials Inc., USA). The homogenate was centrifuged at $6{,}000 \times g$ for 10 min, and the supernatant was collected and used as bacterial cell extract for the bacteriocin activity test.

For mass production of glycinecinA, a 5-liter fermenter containing 3 liters of LB broth was inoculated with 30 ml of the overnight culture of *E. coli* DH5α (pBL5), and incubated at 37°C with agitating at 280 rpm. Bacterial cells were harvested 24 h after incubation, and cell extract was collected as mentioned above.

Bacteriocin activity. The activity of bacteriocin prepared from each experiment was measured as described previously (Ahn and Cho, 1996). The titer of the bacteriocin solution, in arbitrary units per ml (AU/ml), was calculated as $(1000/10) \times D$, where D is the dilution factor (Parente et al., 1995).

Stability of glycinecinA in the laboratory. Stability of glycinecinA was tested at room temperature and at 4°C. The glycinecinA activity was measured at 1-week interval with critical dilution assay methods as described in the bacteriocin activity test.

Plant growth, bacteriocin treatment, and pathogen inoculation. Seeds of red pepper (cv. Nogkwang) and rice (cv. Milyang 23) were sown in a flat tray (36 × 50 cm) containing a mixture of steam-sterilized soil and sand amended with the same amount. For red pepper and rice, the bacteriocin was treated 7 weeks (growing green stage) or 4 weeks (4-leaf stage) after sowing, respectively. All treated plants were kept in the greenhouse at 28-35°C under natural light conditions.

For bacteriocin treatment, crude cell extract prepared as above was diluted to various concentrations ranging from 3,200 AU/ml to 25,600 AU/ml, and sprayed 1 day before the pathogen inoculation. Unless mentioned otherwise, the amount of the cell extract solution used was about 50 ml per plant each time. Cultures of *X. vesicatoria* and *X. oryzae* pv. *oryzae* grown in PSB were diluted to 5×10^8 cfu/ml, and sprayed by using a power-driven sprayer. Approximately 50 ml of bacterial suspension was used per plant in all of the inoculation tests, unless mentioned otherwise.

Stability of glycinecinA on red pepper and rice leaves. After the cell extract including the glycinecinA has been sprayed onto the leaves of red pepper and rice, four leaf disks were cut with a cork borer (diameter 6 mm) from each three leaves of three replicate pots, and were placed on dried NA plates. The plates were overlaid with 7 ml of 0.7% water agar containing 0.1 ml of indicator cells ($O.D_{600} = 0.1$). Bacteriocin activity was determined for 1 week by measuring the diameter of inhibition zones.

Population changes of pathogen on red pepper and rice. Bacterial cell extract adjusted to 12,800 AU/ml of glycinecinA was prepared and sprayed onto leaves of red pepper and rice 1 day before pathogen inoculation. Control plants were inoculated with *X. vesicatoria* or *X. oryzae* pv. *oryzae* without glycinecinA treatment. Pathogen populations were monitored daily after inoculation. The leaves were weighed and ground in 2 ml of 0.01 M phosphate buffer solution (pH 7.0), serially diluted, plated on PSA containing rifampicin for isolating *X. vesicatoria* Uijongbu1(rif') and *X. oryzae* pv. *oryzae* KXO169 (rif'). Colony from duplicate plates were counted and converted to colony-forming units (cfu) per gram of fresh leaf and then to log cfu per gram of fresh leaf. Nine individual leaves, three from each of three replicate pots, were collected from each treatment.

Control effects of the bacteriocin in greenhouse. For red pepper plants, treatments were composed of: copper hydroxide application (2 mg/ml, 1 day before inoculation and at 1-week interval), cell extract (12,800 AU/ml glycinecinA) treatments (1 day before inoculation only (1pr); 1 day before and 1 week after inoculation (1pr-1po), 1 day before and two times at intervals of 1 week after inoculation (1pr-2po); and two times of 1 and 7 days before and three times at intervals of 1 week after inoculation (2pr-3po), and control (pathogen inoculation only). Each plant was examined and disease severity index was divided into 6 grades based on symptom severity: 0 = no symptom; 1 = slight; 2 = moderate; 3 = moderately severe; 4 = severe symptoms; and 5 = defoliation.

Rice cultivars, Jinmi and Milyang 23, were inoculated as mentioned above, and blight symptoms were examined 4 weeks after inoculation. The disease severity was divided into none (< 1%), slight (1-5%), moderate (5-25%), moderately severe (26-50%), and severe (> 50%) levels, of which the calculation values were 0, 3, 15, 38, and 75%, respectively (Anonymous, 1995).

Experimental design and treatments in the field. Field experiments were conducted in the fields of the Seoul National University Research Farm located in Suwon, Korea, in 1998 and 1999. Red pepper cv. Nogkwang and rice cvs. Jinmi and Milyang 23 were used in the experiments. Their seedlings were transplanted on May 5, 1998 and 1999, and cultural practices including planting, fertilization, water management and other cultural methods followed the standard commonly practiced around Suwon area.

For red pepper, each treatment consisted of three replicates arranged in a randomized block design. Each plot contained 10 red pepper plants, and a total of 300 plants were planted in the field 5 m × 50 m in size. For rice, a treatment consisted of three replicates arranged in a split plot design using treatment as the main plot and cultivar as subplots. Each plot was 10 m wide and 30 m long in size and consisted of 48 rice plants.

For inoculation, the bacterial suspensions were sprayed by using a power-driven sprayer onto 2-month-old red pepper and rice plants. The cell extract solution (12,800 AU/ml glycinecinA) and copper hydroxide (2 mg/ml) were used as treatments. Chemical treatments were positive controls, and treatments with pathogens only were negative controls. The red pepper plants were sprayed until run-off with glycinecinA or copper hydroxide. Disease severities were examined 3 and 4 weeks after inoculation, following the indexing methods as in the greenhouse experiments.

Data analysis. Analyses of variance (ANOVA) and comparison of treatment means were conducted with SAS PROC GLM (SAS Institute Inc. Cary, NC, USA).

Results

Bacteriocin production. The culture supernatant and cell extract of *E. coli* DH5α (pBL5) grown in LB at 37°C were collected at various times during exponential and stationary growth and death phase, and were tested for bacteriocin activity. Bacteriocin activity of bacterial cell extract was much higher than that of the culture supernatant (Figs. 1 and 2). The bacteriocin activity of the cell extract was approximately 15 times more than that of the culture supernatant 24 h after incubation (Fig. 1). The bacteriocin activity in the supernatant increased slowly but continuously, while that of the cell extract increased rapidly up to a stationary phase, then suddenly decreased 60 h after incubation (Fig. 2). The bacterial population reached a plateau 16-40 h after incubation, but the bacteriocin activity of the cell extract exhibited a maximum level 40-60 h after incubation.

Stability of glycinecinA. The bacteriocin activity remained constant at least 2 months at room temperature, and 10 months at 4°C (Fig. 3). At room temperature, the bacteriocin activity decreased sharply after the constantly active period.

Optimum concentrations of glycinecinA for the control of bacterial diseases in red pepper and rice. Incidences of bacterial diseases on red pepper and rice treated with gly-

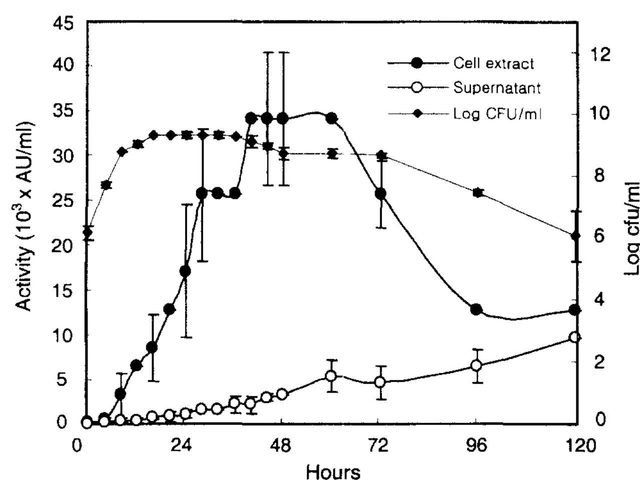

Fig. 1. Growth and glycinecinA production patterns of *Escherichia coli* DH5α (pBL5) strain. *E. coli* DH5α carrying pBL5 for glycinecinA genes was used to produce glycinecinA in LB broth. The bacteriocin activities of cell-free supernatant and crude cell extract were measured by the critical dilution method as described in the Materials and Methods.

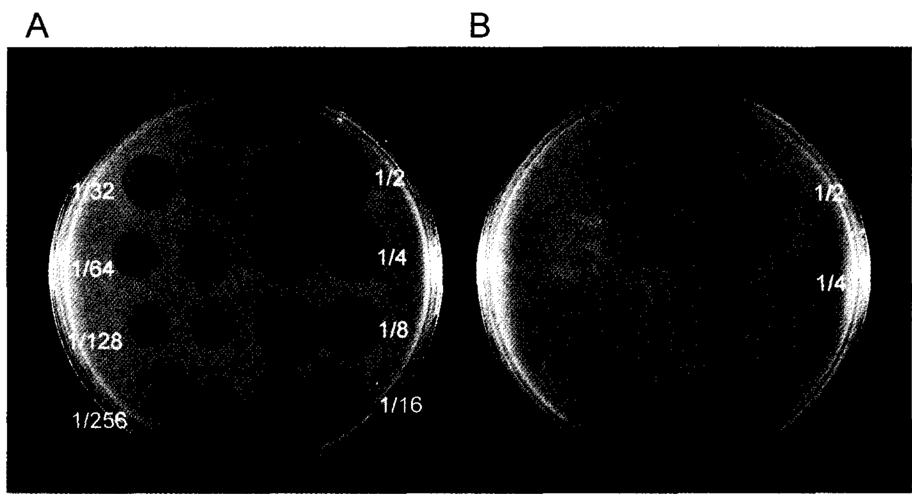

Fig. 2. Comparison of bacteriocin activity between cell extract and culture supernatant of *Escherichia coli* DH5α (pBL5) against the indicator strain of *Xanthomonas vesicatoria* YK93-4. Numbers are dilutions of the cell extract or culture supernatant. **A**: Cell extract, **B**: Culture supernatant.

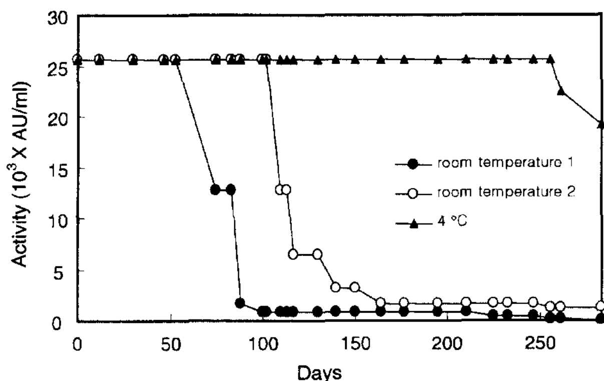

Fig. 3. Stability of glycinecinA at room temperature and at 4°C. Activity of glycinecinA was measured at 1-week interval with critical dilution assay methods as in the bacteriocin activity test.

cinecinA at different concentrations were examined 4 weeks after inoculation. The bacterial diseases significantly decreased at bacteriocin concentrations of 3,200 AU/ml or more, and were suppressed as much as, or even more than, the chemical treatment at concentrations of 12,800 and 25,600 AU/ml (Fig. 4). At these concentrations, disease index was about 0.2 in red pepper and disease rate was about 12%, while those of the controls were approximately 3.1 and 43%, respectively. Therefore, the concentration of glycinecinA was adjusted to 12,800 AU/ml in greenhouse and field experiments.

Stability of glycinecinA activity on red pepper and rice leaves. On red pepper leaves, bacteriocin activity continued for 8 days (Fig. 5). The activity was maintained for 6 days

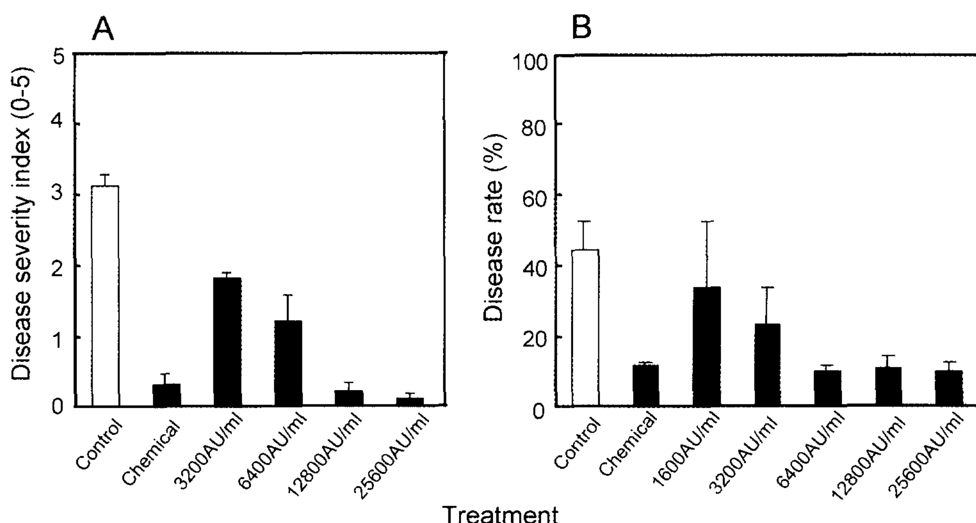

Fig. 4. The efficient glycinecinA concentration was evaluated using various concentrations ranging from 3,200 AU/ml to 25,600 AU/ml. **A**: bacterial leaf spot of red pepper, **B**: bacterial leaf blight of rice.

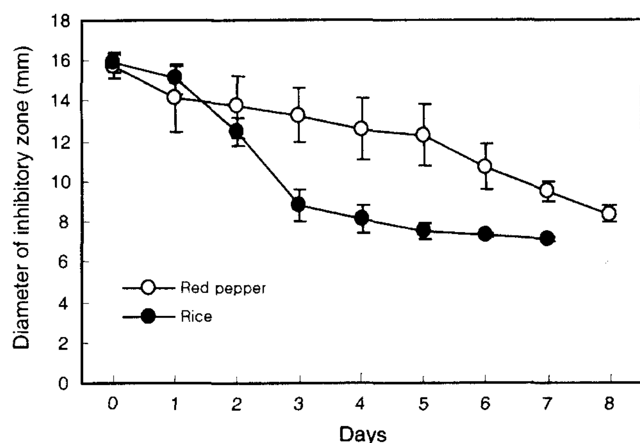

Fig. 5. Changes of glycinecinA activity on pepper and rice leaves with time after treatment. After the cell extract, including the glycinecinA, was sprayed onto leaves of red pepper and rice, bacteriocin activity was determined for 1 week by measuring the diameter of inhibition zones.

after treatment on rice leaves. However, the bacteriocin activity on leaves reduced gradually with time after treatment.

Population changes of pathogen on red pepper and rice. The pathogen population densities were significantly reduced on red pepper and rice leaves sprayed with cell extract containing glycinecinA (12,800 AU/ml), compared with that of the untreated controls (Fig. 6). Bacterial populations on leaves were reduced drastically in the first day after treatment, and maintained at the reduced population densities for 7 days.

Control effect of the bacteriocin in the greenhouse. In the greenhouse experiments, disease severities were significantly reduced by both the bacteriocin and chemical treatments (Fig. 7). In red pepper, disease severity index was 3.1 in the untreated control, 0.17-0.47 in the bacteriocin treatments, and 0.75 in the chemical treatment. There were no significant differences in disease severity among the spraying times of the bacteriocin. In rice, diseased areas in the bacteriocin treatments were 9.3-11.4%, while the diseased area was 27.7% in the plots inoculated with the bacterial pathogen only. The chemical treatment reduced the diseased area to 16.1%.

Control effect of the bacteriocin in the field. In red pepper, disease severity was significantly reduced by the bacteriocin treatments in the field experiment (Fig. 8). In 1998, the untreated control had disease index of 2.89, but the bacteriocin treatments had disease indices of 1.64, 1.40, and 1.38 for 1pr, 1pr-1po, and 1pr-2po, respectively, 3 weeks after inoculation. The chemical treatment reduced the disease severity to 1.75. Four weeks after inoculation, the disease severity increased in the control. However, the control efficacy of the bacteriocin treatments was not changed significantly. Also in 1999, disease severity was lower in the bacteriocin (0.72-1.76) and chemical (1.44) treatments than that in the control (3.22). In the rice field, disease severity of rice bacterial blight was also lower in the bacteriocin treatments than the untreated control and the chemical treatment (Fig. 9).

Discussion

Biological control studies using living microorganisms usually begin with laboratory plate assays to examine antibi-

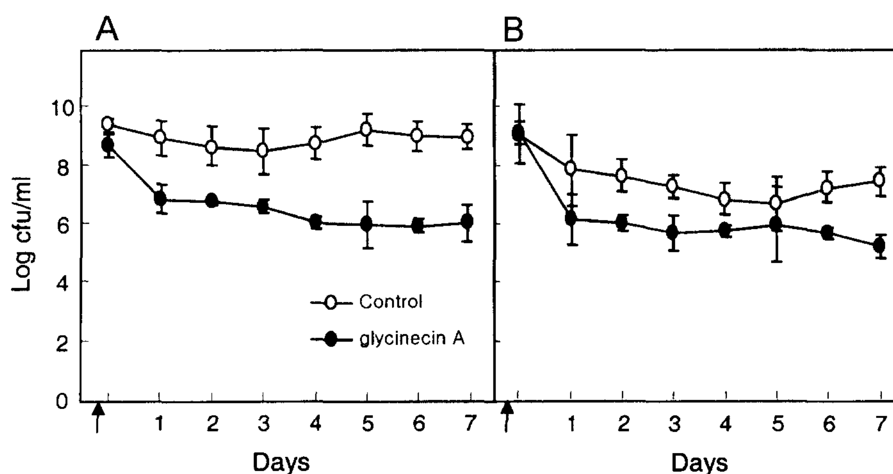

Fig. 6. Population changes of *Xanthomonas vesicatoria* on red pepper leaves and *X. oryzae* pv. *oryzae* on rice leaves. Bacterial cell extract adjusted to 12,800 AU/ml of glycinecinA was prepared and sprayed onto leaves of red pepper and rice 1 day before pathogen inoculation. Numbers of colonies were counted daily as described in the Materials and Methods. **A**: Red pepper (cv. Nogkwang), **B**: Rice (cv. Milyang 23).

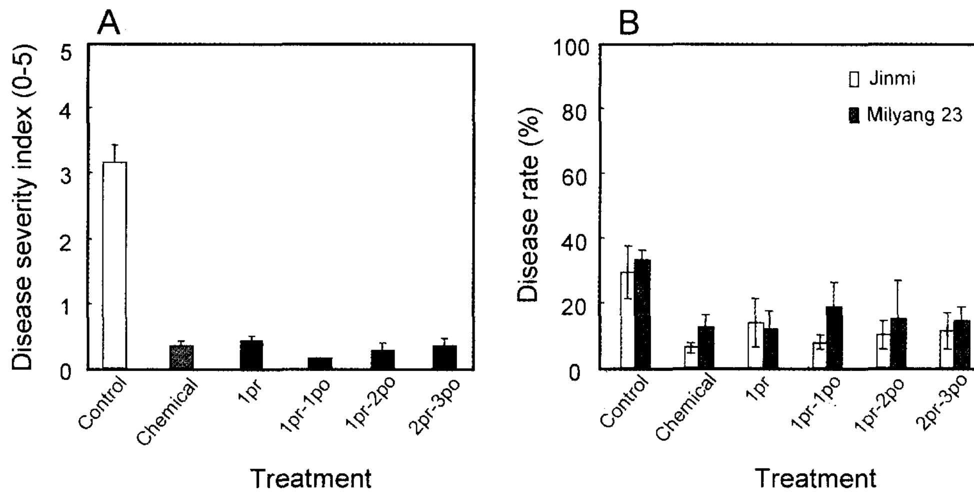

Fig. 7. Effect of cell extract including glycinecinA against bacterial leaf spot and bacterial leaf blight in the greenhouse. Cell extract (12,800 AU/ml glycinecinA) treatments (1 day before inoculation only (1pr); 1 day before and 1 week after inoculation (1pr-1po), 1 day before and two times at intervals of 1 week after inoculation (1pr-2po); and two times at 1 and 7 days before and three times at intervals of 1 week after inoculation (2pr-3po)), control (pathogen inoculation only) and chemical treatment (copper hydroxide application (2 mg/ml, 1 day before inoculation and at 1 week intervals)). NI indicates no inoculation. **A**: Red pepper (cv. Nogkwang), **B**: Rice.

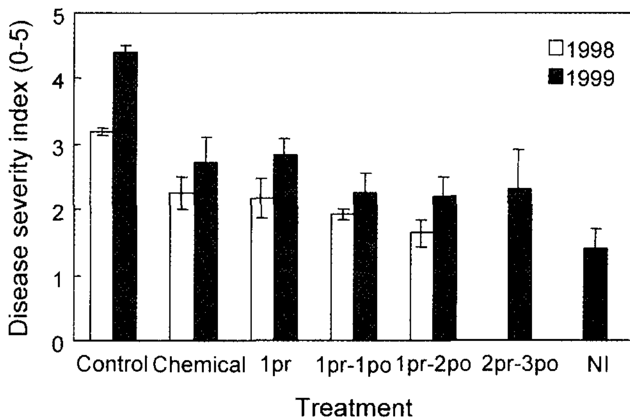

Fig. 8. Effect of cell extract (glycinecinA) treatment on the disease severity of bacterial leaf spot of pepper in the field for 2 years (1998-1999). Cell extract (12,800 AU/ml glycinecinA) treatments (1 day before inoculation only (1pr); 1 day before and 1 week after inoculation (1pr-1po), 1 day before and two times at intervals of 1 week after inoculation (1pr-2po); and two times at 1 and 7 days before and three times at intervals of 1 week after inoculation (2pr-3po)), control (pathogen inoculation only) and chemical treatment (copper hydroxide application (2 mg/ml, 1 day before inoculation and at 1 week intervals)). NI indicates no inoculation.

otics or microparasitism on a specific medium. However, the *in vitro* results have not matched well with the ability to reduce or prevent diseases in greenhouse or field conditions. This is probably because survival and activity of the living microbial antagonists on host plants are importantly involved in the efficacy of biological control besides antibiosis and microparasitism (Handelsman and Stabb, 1996).

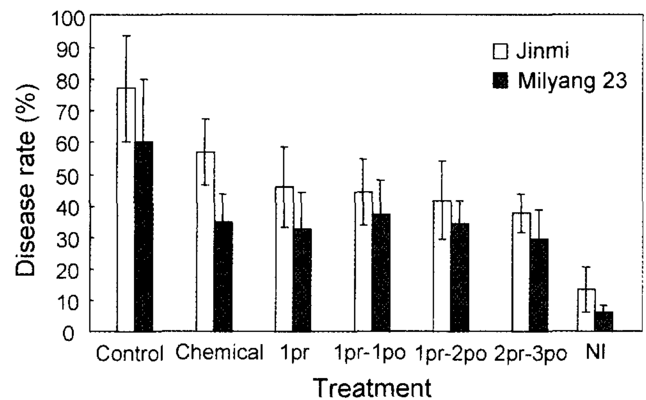

Fig. 9. Effect of cell extract (glycinecinA) treatment on the disease rate of bacterial leaf blight of rice in the field in 1999. Cell extract (12,800 AU/ml glycinecinA) treatments (1 day before inoculation only (1pr); 1 day before and 1 week after inoculation (1pr-1po), 1 day before and two times at intervals of 1 week after inoculation (1pr-2po); and two times at 1 and 7 days before and three times at intervals of 1 week after inoculation (2pr-3po)), control (pathogen inoculation only) and chemical treatment (copper hydroxide application (2 mg/ml, 1 day before inoculation and at 1 week intervals)). NI indicates no inoculation.

The introduced biological agents in fields or greenhouses must sustain an active population to give high levels of antibiosis against target microorganism or to compete with other microorganisms, especially on the phylloplane.

In case of bacteriocins, which are active at the extremely low level, there are some advantages over microbial biocontrol agents for foliar diseases in that they do not require reproduction on the active sites. In this respect, bacteriocins have been known as effective antimicrobial agents for foliar

diseases (Harding and Shaw, 1990). Bacteriocins are safe for users and the environments, and can inhibit only target organisms without giving any harm to other beneficial organisms.

High level of stability of glycinecinA suggests that the cell extract (including glycinecinA) may be an effective biocontrol agent that can be commercialized. Mass production of the bacteriocin can be achieved by mass culture of a good bacteriocin producer, *E. coli* DH5α (pBL5), instead of *X. axonopodis* pv. *glycines* 8ra.

In the biological control of plant diseases, antibiotic activity against pathogens is a key factor for success. The experiments showed that glycinecinA was the more effective biological control against *X. vesicatoria* and *X. oryzae* pv. *oryzae*. However, the control efficiency did not increase at concentrations above 12,800 AU/ml of glycinecinA. Therefore, in an economical point of view, the cell extract solution with 12,800 AU/ml activity may be appropriate for practical use.

The treatment of red pepper and rice leaves with cell extract having glycinecinA significantly reduced the populations of subsequently inoculated pathogenic bacteria. Also, the bacterial diseases of red pepper and rice were effectively controlled by the bacteriocin treatments both in the greenhouse and in the field. The control efficacies were as much, as or even greater than, the chemical treatment. As there was no significant difference in the control efficacy among the application times, it was believed that 1 or 2 sprays will give a satisfactory result to control the diseases.

Variation of the concentration of glycinecinA in the cell extract depends on the conditions of incubation, and these problems need to be solved or considered in the application of glycinecinA as control agent for bacterial diseases.

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