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Biological Control of Strawberry Gray Mold Caused by *Botrytis cinerea* Using *Bacillus licheniformis* N1 Formulation

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Abstract *Bacillus licheniformis* N1 is a biological control agent to control gray mold diseases caused by *Botrytis cinerea.* Various formulations of *B. licheniformis* N1 were generated and evaluated for the activity to control strawberry gray mold. The wettable powder type formulation N1E was selected in pot experiments with remarkable disease control activity on both strawberry leaves and flowers. The N1E formulation contained 400 g of corn starch, 50 ml of olive oil, and 50 g of sucrose per a liter of bacterial fermentation culture. Optimum dilution of N1E to appropriately control the strawberry gray mold appeared to be 100-fold dilution in plastic house artificial infection experiments. The significant reduction of symptom development in the senescent leaves was apparent by the treatment of N1E at 100-fold dilution when N1E was applied before *Bo. cinerea* inoculation, but not after the inoculation. Both artificial infection experiments in a plastic house and natural infection experiments in the farm plastic house under production conditions revealed that the disease severity of gray mold on strawberry leaves and flowers was significantly reduced by N1E treatment. The disease control value of N1E on strawberry leaves was 81% under production conditions, as compared with the 61.5% conferred by a chemical fungicide, iprodione. This study suggests that our previously generated formulation of *B. licheniformis* N1 will be effective to control strawberry gray mold by its preventive activity.

Keywords: *Bacillus licheniformis*, biological control, *Botrytis cinerea*, strawberry gray mold

Strawberry gray mold, caused by *Botrytis cinerea* Pers.:fr., is one of the most important airborne diseases of the strawberry resulting in poor fruit quality and serious yield loss. The fungal pathogen infects the leaves and flowers of strawberry plants and subsequently resulting in fruit rot when the fruit begins to ripen [5]. The open flower and senescent flower stages are most susceptible to infection by the fungal pathogen *Bo. cinerea.* The fungal pathogen infects young leaves, does not produce symptom until the leaves senesce, and grows and sporulates rapidly when they die [15]. Conidia from the infected flowers and leaves are the primary inoculums for disease spread and infection of fruits [3, 4]. The fruit rot by the fungal pathogen is the most critical factor causing the severe yield loss of strawberry by gray mold.

Since no cultivar is highly resistant to fruit rot by *Bo. cinerea,* control of strawberry gray mold mainly depends on fungicide application by protecting flowers from infection during early fruiting periods [13]. Leaf sanitation (removal of senescent and necrotic leaves) and fruit sanitation was also effective to reduce the incidence of Botrytis fruit rot, although the sanitation did not increase the strawberry yield [16]. Therefore, fungicide application on flowers and infected leaves could be the major disease management strategy for strawberry gray mold. However, alternative control measure such as biological control has been tried [1, 9, 14, 20, 24]. This is mainly because of the increasing environmental concern on pesticide use and frequent appearance of fungicide resistance strains of *Bo. cinerea* [6, 11, 25].

One of the bacteria biocontrol agents that has received much attention is the genus *Bacillus* because of their advantages over other Gram-negative bacteria. *Bacillus* species produce broad-spectrum antibiotics and maintain viability for a long time as a result of endospores production [7]. Interestingly, some of the *Bacillus* species are also involved in plant growth promotion [10, 17]. Kodiak (Gustafson, Inc., Dallas, U.S.A.) and Serenade

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(AgraQuest, Inc., Davis, U.S.A.) are the successful examples of biofungicides developed from antagonistic *B. subtilis* strains [2]. Currently, the critical steps toward the biofungicide development with promising antagonists are the scale-up production of the organism and effective formulation [21]. Since endospores-forming *Bacillus* species could be readily formulated because of heat- and desiccation-tolerant spores [7], we focused mostly on the *Bacillus* species for biofungicide development to control gray mold on various vegetable crops. In fact, the many commercialized biofungicides of *Bacillus* species are spore formulations.

We were previously successful in generating effective formulations of biofungicide with antagonistic *B. licheniformis* N1 using *Biji* medium for mass production of bacterial cells and corn-starch-based formulations [12, 23]. One of the laboratory-made formulations, N1E, was effective to control tomato gray mold. One-hundred-fold dilution of the N1E showed the equivalent disease control activity of chemical fungicide [12]. In this study, we report that the N1E was also effective to reduce the gray mold infection on strawberry leaves and flowers. Therefore, it is likely that biological control using *B. licheniformis*-based N1E could be applied to decrease the strawberry fruit rot and severe yield loss.

MATERIALS AND METHODS

Microorganisms and Cultivation

A biocontrol bacterium *B. licheniformis* N1 was previously used for the biological control of tomato gray mold disease [12]. *B. licheniformis* N1 was routinely grown in nutrient agar medium at 30°C and cultured in *Biji* medium (5% dried soybean curd residue in distilled water) for mass production. Soybean curd residue ("*Biji*" in Korean) was obtained from a Korean traditional tofu factory. Dried *Biji* was produced to make *Biji* medium as previously described [12]. A fungal pathogen, *Botrytis cinerea* LVF12, which causes strawberry gray mold, was routinely grown on potato dextrose agar (PDA) for 15 days at 25°C in a 12-h light period to trigger conidia formation. The conidia were suspended in a 30% tomato juice solution supplemented with 0.1 M KH_2PO_4 to a concentration of 1.6×10^6 conidia/ml [22].

Formulations of Microbial Fungicides

Previously, we have generated various formulations of *B. licheniformis* N1 using *Biji* broth culture [12]. Briefly, the 400-ml preculture of bacterial cells was added directly to 4 l of *Biji* broth in a 7-l jar fermenter with 10 ml of 10-fold diluted antifoam emulsion (DB-110A, Dowcorning) and grown for three days at 300 rpm, pH 7.0 (automatic control with 1 M HCl and 1 M NaOH), and 35°C. The grown to approximately 4×10^9 CFU/ml bacterial cells were thoroughly mixed with various materials, as previously described [12]. The previously generated formulations of *B. licheniformis* N1 included a wettable powder (WP) formulation, soluble concentrate (SL) formulation, and the emulsifiable concentrate (EC) formulations. The various formulations were stored at 4°C until use. A control formulation lacking bacterial cells was prepared with *Biji* broth mixed with corn starch for the WP formulation. Fungal pathogen inoculation and the control formulation lacking bacterial cells were used as controls for pot experiments and plastic house experiments.

Plant Material, Screening Process, and Disease Control Values

The strawberry plants used in this study, the cultivar Reiko, were grown in a plastic house using routine cultivation practices. Pathogen inoculation was performed as previously described [22]. Briefly, conidia suspensions of *Bo. cinerea* LVF12 in 30% tomato juice with 0.1 M of KH_2PO_4 were sprayed on strawberry plants maintained in pots or in a plastic house. The disease severity on strawberry leaves and flowers was rated on various days after pathogen inoculation. The fungicide used in this study for the control treatment was iprodione (Rovral) purchased from Bayer CropScience Co., Korea, and was used after 1,000-fold dilution with tap water. Strawberry plants of the 3–4 leaves stages were treated with the formulated microbial fungicides to evaluate biocontrol activity on leaves in pot experiments. The activity on strawberry flowers was conducted by treating the microbial fungicides on 5–6 flowers per strawberry plant in plastic house experiments.

The disease severity index of gray mold on the strawberry plants was defined as the percentage of diseased leaf area, where 0=no disease symptoms, 1=0.1–5%, 2=5.1–20%, 3=20.1–40%, and 4=40.1–100%. The disease severity value was calculated using the following formula: Disease severity (%)=((Σ(the number of diseased leaves×disease severity index))/(4×the number of leaves rated))×100. The disease control value was calculated using the following formula: Disease control value (%)=((A–B)/A)×100, where A is the disease severity caused by pathogen inoculation alone, and B is the disease severity after various treatments. Under field conditions, the natural disease severity was rated without pathogen inoculation.

Pot Experiments

Selection of the formulation of *B. licheniformis* N1 that most efficiently controlled strawberry gray mold was performed in pot experiments. Strawberry plants were grown in 15-cm-diameter pots in a plastic house until the four-leaf stage. The various biofungicide formulations were diluted 100-fold with tap water before being sprayed on the strawberry plants. The 100-fold dilutions contained 1 g of WP formulation, 1 ml of SL formulation, or 1 ml of

EC formulation suspended in 100 ml of tap water. The plants were inoculated by spraying the fungal conidia suspension on strawberry leaves until runoff one day after chemical or microbial fungicide treatment, and the plants were maintained in a controlled growth room for one day (20±2°C, 90% RH). The inoculated plants were then transferred into a plastic house (25±5°C). Each treatment included 10 pots per treatment with three replications.

Evaluation of Biofungicides in a Plastic House

Disease control activity on leaves and flowers and dilution effect of the selected microbial fungicide were examined in a plastic house. Strawberry plants were first grown in a pot for 30 days and then transplanted into cropping soil in a plastic house. The strawberry plants were maintained in the plastic house, after which they were treated with chemical fungicide or the N1E biofungicide dilutions as described above.

The two formulations were chosen based on disease control activity against gray mold on strawberry leaves and flowers. The formulations N1E and N1W3 and a chemical fungicide were applied to strawberry leaves and flowers, and the fungal pathogens were inoculated by spraying conidial solutions one day after the treatment of biofungicide or chemical fungicide. Each experiment included five plants per treatment with three replications. The disease severity was rated at five and two days after pathogen inoculation on strawberry leaves and strawberry flowers, respectively. The number of diseased flowers per plant was determined in the flower disease control experiment.

Plastic house experiments were also conducted with the N1E formulation of *B. licheniformis* N1 to investigate the optimum dilution for the effective control of strawberry gray mold. N1E dilutions of 25-, 50-, 100-, 200-, 400-, and 800-fold were tested for disease control activity. To determine whether the gray mold control effect of the N1E treatment was curative or preventive, the N1E formulation was applied to strawberry plants before or after fungal pathogen inoculation. Each experiment included five plants per treatment with three replications. The disease severity was rated at seven days after pathogen inoculation.

Trials Under Field Conditions

A field experiment under strawberry production condition was carried out from March to April 2002 in a farmer's plastic house at Hanlim, Kimhae, Korea, utilizing naturally occurring gray mold on strawberry plants. The strawberry cultivar Reiko was transplanted into a plastic house on September 5, 2001. The strawberry plants were grown in the plastic house using routine cultivation practices. The three treatments comprised the N1E formulation of *B. licheniformis* N1, the iprodione fungicide, and a control. The N1E biofungicide and the iprodione were diluted 100-fold and 1,000-fold before treatment, respectively. From among several candidate plastic houses, we chose an experimental plastic house containing strawberry plants showing an initial appearance of gray mold symptoms, on which a heavy disease occurrence was expected. The first sprayings of the chemical fungicide and N1E were performed on March 25, 2002. The treatment was repeated three times at seven-day intervals, and the disease severity was rated at seven days after the final treatment. The disease severities were converted into disease control values as described above. In the experimental plastic house, each plot consisted of thirty strawberry plants planted in an area of 1 m×2 m. The three treatments were arranged in a completely randomized design, with five replicates per treatment. Natural disease occurrence without any treatment was a control in this experiment.

Statistical Analysis

Data from the pot experiments, the plastic house experiments, and the field trials under production conditions were analyzed using analysis of variance (ANOVA) in a complete randomized design. Duncan's multiple range test was used to compare the means of the treatments in each experiment. All statistical analyses were conducted using SAS/STAT software (SAS Institute, 1989).

RESULTS

Selection of N1E to Control Strawberry Gray Mold

Our previous study for *B. licheniformis* N1 mass cultivation selected *Biji* medium, which supports bacterial growth up to 10^9 cell/ml and a corn-starch-based formulation of the N1 strain proven to be effective to maintain the biological control activity against tomato gray mold [12]. Since our *in vitro* analysis of antifungal activity of *B. licheniformis* N1 indicated the broad-spectrum antifungal activity against several plant pathogen fungi, we expected a wide range of applications of the N1 strain for plant disease biological control. To investigate if the previously generated formulations of *B. licheniformis* N1 are effective to control strawberry gray mold, we tested three different forms of formulations (WP, SL, EC forms) for strawberry gray mold control after 100-fold dilution on strawberry plants grown in pots. At 100-fold dilution, the N1W3 SL formulation exhibited the highest disease control activity at 95.6%, and the activity was significantly different from that of N1E WP formulation with 84.2% (Fig. 1). The disease control activity of N1W3 and N1E was significantly higher than that of chemical fungicide iprodione with 64.9%. The rest of the tested formulations did not show a significantly higher activity than chemical fungicide. Therefore, N1E and N1W3 were selected to evaluate the control effect of strawberry gray mold in plastic house experiments by artificial infection.

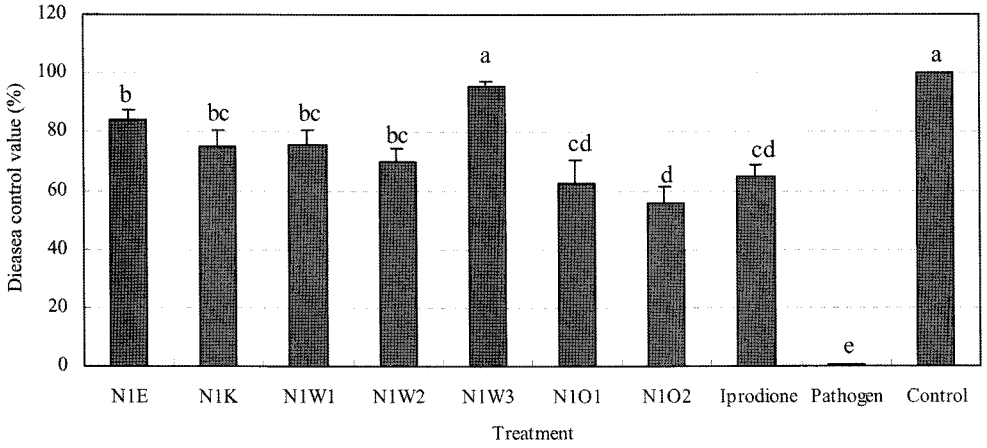

Fig. 1. Effect of various formulations of *Bacillus licheniformis* N1 on the control of the gray mold *Botrytis cinerea* on strawberry plants in pots in a plastic house.
Iprodione represents a chemical fungicide. Pathogen and control indicate artificial inoculation of the fungal pathogen and no treatment, respectively. Error bars represent the standard deviation of three replications.

Evaluation of N1E and N1W3 in Plastic House Experiments

The disease control activity of N1E and N1W3 on strawberry leaves and flowers in plastic house experiments was examined. The N1E exhibited a remarkably higher disease control activity on flower infection control, whereas disease control activity on strawberry leaves was not significantly different between N1E and N1W3 treatments (Fig. 2). Compared with pot experiments, the disease control values of N1E and N1W3 on strawberry leaves in plastic house experiments were slightly low at 82.6% and 88.2%, respectively. Yet, those were still significantly higher than that of iprodione treatment at 65.1% (Fig. 2). However, the disease control activity on strawberry flower infection by N1E (73.7%) was much higher than that of N1W3 (9.8%) (Fig. 3). The disease control activity on flowers by N1W3 was significantly lower than that of chemical fungicide at 42.5%, whereas that of N1E was significantly higher than that of chemical fungicide. The pathogen inoculation without any prior treatment resulted in heavy infection of strawberry flowers with 94.7% of disease severity. The heavy infection of strawberry flowers resulted in dramatic decrease of the number of fertilized fruits. The number of fertilized fruits was different between treatments. Since N1W3 treatment yielded less flower disease control activity, the number of fertilized fruits in N1W3 treatment was much less than chemical fungicide treatment (data not shown). Thus, the N1E was finally

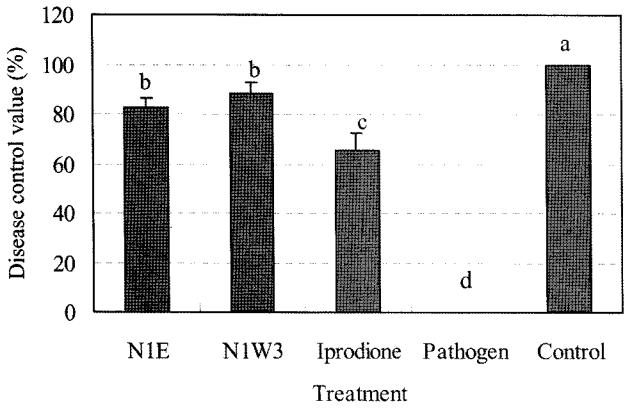

Fig. 2. Effect of N1E and N1W3 of *Bacillus licheniformis* N1 on the control of the gray mold *Botrytis cinerea* on strawberry plants grown in a plastic house.
Iprodione represents a chemical fungicide. Pathogen and control indicate artificial inoculation of the fungal pathogen and no treatment, respectively. Error bars represent the standard deviation of three replications.

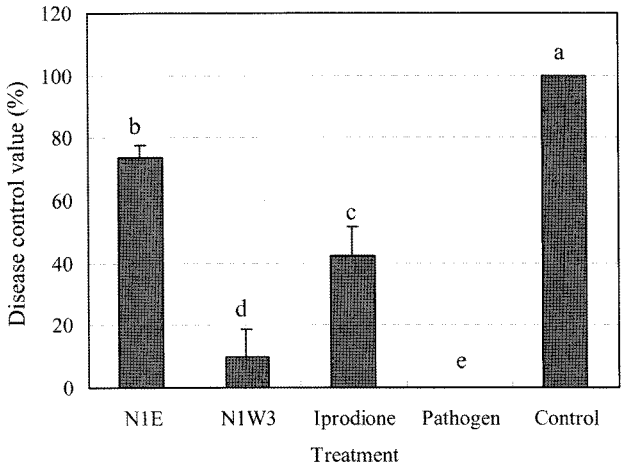

Fig. 3. Effect of N1E and N1W3 of *Bacillus licheniformis* N1 on the control of the gray mold *Botrytis cinerea* on strawberry flowers grown in a plastic house.
Iprodione represents a chemical fungicide. Pathogen and control indicate artificial inoculation of the fungal pathogen and no treatment, respectively. Error bars represent the standard deviation of three replications.

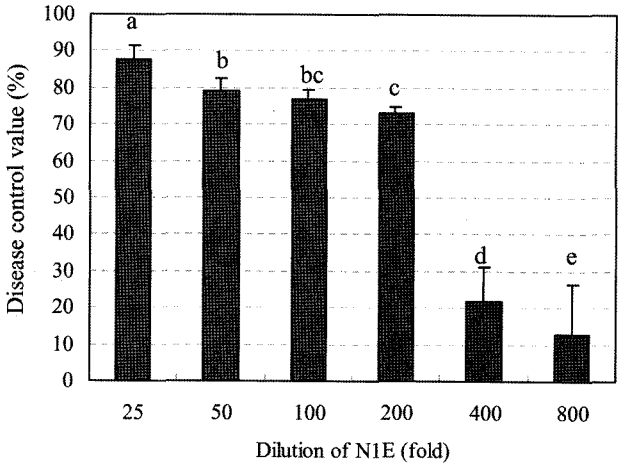

Fig. 4. Effect of dilutions of the N1E formulation on the control of the gray mold *Botrytis cinerea* on strawberry plants in pots in a plastic house.
Error bars represent the standard deviation of three replications.

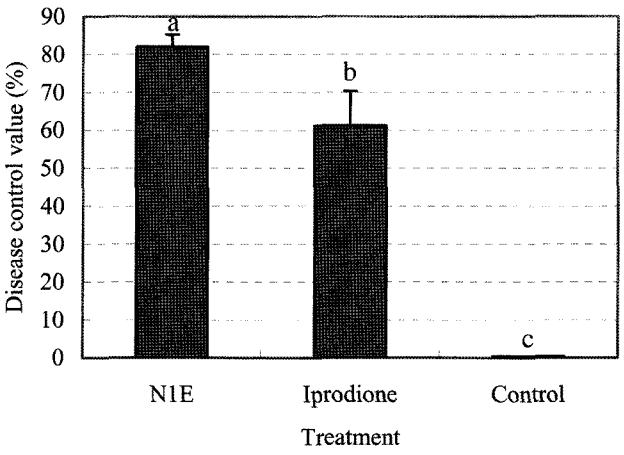

Fig. 6. Control of gray mold on strawberry plants by the N1E formulation under production conditions.
Iprodione represents a chemical fungicide. Error bars represent the standard deviation of five replications.

chosen for further experiments and field trials under strawberry production condition.

Effects of N1E Dilution and its Preventive Activity

Stepwise dilutions of N1E revealed a decreased disease control activity in plastic house experiments. Dilution of N1E from 25- to 200-fold exhibited over 70% disease control activities, whereas 400-fold and 800-fold dilution showed the dramatic decrease of disease control activity of 21.6% and 12.8%, respectively (Fig. 4). Disease control activity by 100-fold dilution was not significantly different from 200-fold dilution treatment, but that of 200-fold dilution was different from 50-fold dilution, whereas that of 100-fold dilution was not. For field trials, we used 100-fold dilution treatment, which is comparable to the case to control the tomato gray mold.

N1E treatment before, after, or simultaneously with *Bo. cinerea* inoculation on strawberry plants revealed the preventive effect of the biofungicide against gray mold on strawberry plant. N1E treatment after pathogen inoculation did not show an acceptable level of disease control activity, indicating the lack of disease curative effect of N1E. However, N1E treatment 1 or 2 days before pathogen inoculation and simultaneous treatment yielded 69.1%, 59.8%, and 73.1% of disease control activity, respectively (Fig. 5). This result indicated that the N1E treatment had a preventive control effect against gray mold on strawberry but not a curative effect.

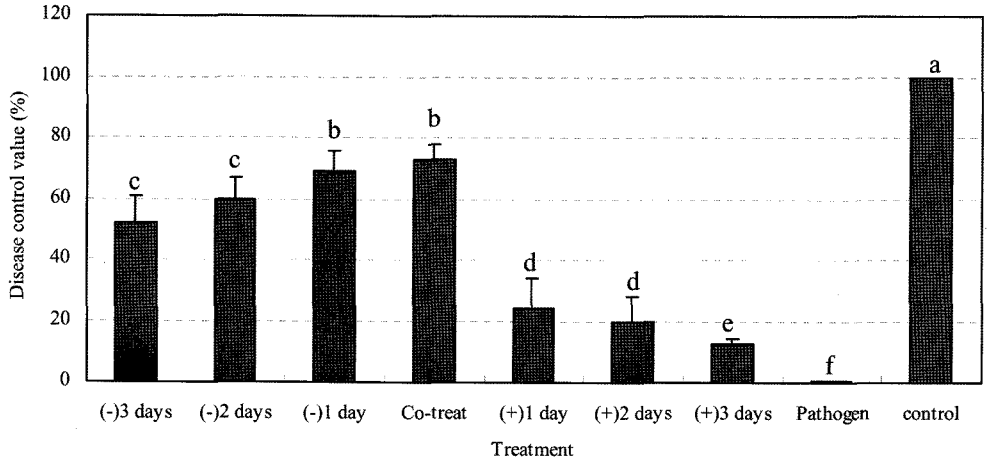

Fig. 5. Preventive and curative effects on the control of gray mold on strawberry plants grown in a plastic house, evaluated by varying the time of spraying of N1E formulations.
(-) indicates N1E treatment before pathogen inoculation and (+) indicates treatment after pathogen inoculation. Pathogen and control indicate artificial inoculation of the fungal pathogen and no treatment, respectively. Error bars represent the standard deviation of five replications.

Biological Control of Strawberry Gray Mold by N1E Under Production Condition

Our field experiments in the Kimhae farmhouse was performed by spraying N1E and iprodione three times with a one-week interval under production conditions. The natural disease occurrence during the experimentation period was 18.7% (first week) and 67.2% (third week) of disease severity, indicating a heavy infection of gray mold disease on the strawberry without any control practices in the farmer's plastic house. The disease control value of the N1E treatment was 82%, much more effective than that of the chemical fungicide, which gave a disease control value of 61.2% (Fig. 6). This result was similar to that obtained in the plastic house and pot experiments that utilized artificial infection of the fungal pathogen.

DISCUSSION

Mass production of antagonistic bacteria and its formulation with an appropriate carrier are the critical steps to develop biofungicides with extended shelf life and prolonged activity [8, 21]. We have previously generated a corn-starch-based wettable powder-type formulation from the fermentation culture of antagonistic *B. licheniformis* N1. One of the products, N1E, was effective to suppress tomato gray mold caused by *Bo. cinerea* [12]. In this study, we investigated the activity of the various types of formulations to control strawberry gray mold caused by the same fungal pathogen. Since our previously produced formulations were derived from fermentation culture of N1 strain in *Biji* medium and formulated using corn starch, the overall cost to produce the formulations was highly effective. *Biji* (soybean curd residue) is rich in nutrients and often used for solid fermentation in some *B. subtilis* strains [18, 19]. However, our formulation, N1E was based on a liquid fermentation method using dried soybean curd residue [12]. In this study, N1E was shown to be the most effective to control strawberry gray mold. It exhibited a great disease control activity from the experiments both in plastic house by artificial inoculation of pathogen and in the field under natural infection.

The biological control activity by N1E on strawberry gray mold is mainly due to its preventive effect (Fig. 4). The absence of a curative effect by N1E suggests that *B. licheniformis* N1 may not enter inside the plant to attack the fungal pathogen. Therefore, the absence of a curative effect by N1E also indicated that any components in the N1E had no direct effect on *B. cinerea* inside the plant or on the plant surface. Since the N1E formulation is a mixture of bacterial cells and culture fluids, the disease control activity by N1E treatment was probably due to the strong antagonistic activity of *B. licheniformis* N1 cells and antifungal compounds against the gray mold pathogen. In fact, a formulation with culture supernatant only was more effective than that with bacterial cells only (data not shown), suggesting the contribution of antifungal compounds in the biological control activity. A preliminary experiment with unformulated bacterial culture supernatant and crude extract of bacterial culture broth exhibited strong antifungal activity and biological control activity (data not shown).

One of the most critical steps to remove potential inoculum of strawberry gray mold on fruit is to reduce the infection of leaves and flowers by fungal pathogen. In particular, young leaves infected with the pathogen remained without any symptom until they senesce, and many infections in flowers are latent [2, 3]. Therefore, an initial reduction of infection on leaves and flowers is necessary for strawberry gray mold control. Leaf and fruit sanitation was effective to decrease fruit infection in strawberry [16]. Since our N1E application on strawberry leaves and flowers significantly reduced the fungal infection, biofungicide application on strawberry plants could be the effective way to remove the initial inoculum of strawberry gray mold.

Cultivation of strawberry plants in a plastic house may also be a factor for the enhanced biological control activity by preventing the removal of the biofungicide from the plant surface by rainfall. Our overall experimental results suggest that the spray schedule of the biofungicide N1E should commence before fungal infection of the strawberry leaves and flowers, with at least three sprayings at one-week intervals.

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