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Biological Control of Gray Mold Rot of Perilla Caused by *Botrytis cinerea*
I. Resistance of Perilla Cultivars and Selection of Antagonistic Bacteria

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Resistance of perilla varieties to *Botrytis cinerea* LVF12 was evaluated, while antagonistic bacteria were selected and tested for their efficacy towards biological control of gray mold rot caused by *B. cinerea*. Among 11 perilla varieties tested for disease resistance, Milyang variety showed some degree of resistance, while the rest of varieties showed no resistance. Among 250 bacterial isolates collected from perilla leaves and rhizosphere of perilla plants, six isolates showed high levels of inhibitory effect on mycelial growth and conidial germination of *B. cinerea* in *in vitro* test. Using the pot test in growth chamber, these isolates showed high levels of disease suppression, with N1 isolate showing 95.3% of control value and N4 isolate showing 90.8% of control value. Further test was performed to evaluate the two isolates ability for disease prevention and/or disease therapy, and results showed almost 100% of control value. Isolates N1 and N4 were identified as *Bacillus licheniformis* and *B. megaterium*, respectively, according to Bergey's manual, API 20E and 50CHB test kit, and Transmission electron microscope.

Keywords **:** *Bacillus licheniformis, Bacillus megaterium,* biological control, *Botrytis cinerea,* gray mold rot.

Botrytis cinerea Pers.: Fr. is an economically important pathogen and the most widely distributed among a wide range of cultivated crops in many regions of the world. In recent years, foliar diseases on perilla drew the attention of many researchers because of significant reduction on quality and quantity of the crop.

In 1998, the occurrence and pathogenic ability of *B. cinerea* on perilla as well as the significance of other foliar diseases on perilla leaves have been reported (Moon et al., 1998; Moon et al., 1999). However, with its wide range of hosts and resistance to the arsenal of chemicals used against *B. cinerea*, it became more and more difficult to control gray mold rot. Moreover, the extensive use of chemicals became a public concern due to residual toxicity and environmental contamination (El Ghaouth et al., 1995; Johnson et al., 1994; Kim, 1997; Kim et al., 1996; Kim et al., 1997; Roposo et al., 1995). It has only been in recent years that a more visible trend toward non-chemical strategies has become apparent.

An increasing number of research laboratories worldwide are now focusing their efforts on biological methods of disease control in response to increasing demands. A number of studies have demonstrated the potential to control gray mold rot of bacterial and fungal inoculants such as *Trichoderma harzianum* (Harman et al., 1996; Zimand et al., 1996; Fravel et al., 1998), *Gliocladium roseum* (Yu et al., 1997; Köhl et al., 1998; Sutton et al., 1993), *Bacillus pumilus, B. amyloliquefaciens* (Nari et al., 1996), *B. subtilis* (Ministry of Science and Technology, 1992), and *Pseudomonas syringae* (Fravel et al., 1998).

The objectives of this experiment were to evaluate the resistance of perilla varieties towards gray mold rot caused by *Botrytis cinerea* and to select antagonistic bacteria against *B. cinerea*.

Materials and Methods

Pathogen. *Botrytis cinerea* LVF12 was isolated from leaves and petioles of perilla plants with disease symptoms grown in a greenhouse at Kang-dong, Busan in 1998. The pathogenicity of this fungus has been proven according to Koch's postulate (Moon et al., 1998).

Nutritional properties in conidial suspension on gray mold rot development. *B. cinerea* conidial suspension was prepared in three different media [sterile distilled water, potato dextrose broth (PDB), and 10% tomato juice (Brand name: Kaya)] to test the effect of nutritional properties in the conidial suspension on gray mold rot development on perilla leaves. Conidia of *B. cinerea* LVF12 were collected from 3-week-old PDA culture and mixed

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in the above media. The concentration of each medium was adjusted to a final concentration of 10^6 conidia/ml. These conidial suspensions, prepared in each medium, were used to inoculate the perilla leaves.

Each conidial suspension was sprayed onto 10 perilla plants (three replicates), and inoculated plants were kept in a growth chamber at 20±2°C with greater than 90% relative humidity for 7 days. After incubation, disease development on perilla leaves was recorded (Korean Agricultural Chemical Industrial Association, 2001).

Resistance test among perilla varieties. Eleven perilla varieties, which are commonly used by growers and produce high quality crop, were selected for this experiment. These varieties were Gwangyang, Gurae 3-13, Gosung Jerae, Jinju Jerae, Perilla 1-1, Milyang, Chubu, Okdong, Kyungsin, Hadong, and Gupo.

Conidia of *B. cinerea* LVF12 were collected from 3-week-old PDA culture and suspended at a final concentration of 10^6 conidia/ml in 10% tomato juice.

Conidial suspension was sprayed onto 11 perilla varieties, and inoculated plants were kept in a growth chamber at 20±2°C with greater than 90% relative humidity for 8 days. After incubation period, gray mold rot development on each perilla variety was recorded.

Isolation and selection of antagonistic bacteria. Antagonistic bacteria were isolated from leaves and rhizosphere of perilla plants grown at Kang-dong, Busan. For the initial screening, effects on mycelial growth and conidial germination of *B. cinerea* LVF12 were tested for 250 bacterial isolates. As a final screening procedure, selected isolates were tested against *B. cinerea* LVF12 for the ability of inhibiting gray mold rot on perilla plants. Bacterial isolates that effectively control gray mold rot on perilla were finally selected from perilla leaves and used for further study.

Bioassay of gray mold rot control by antagonists. The efficacy of selected isolates as biological control agents against gray mold rot of perilla was determined by bioassay. In this study, the selected isolates were challenged with LVF12 at different time period to test disease prevention and/or disease curing ability of the selected isolates.

B. cinerea LVF12 were collected from 3-week-old PDA culture and mixed with 10% tomato juice to a final concentration of 10^7 conidia/ml. Selected isolates were raised in nutrient broth for 5 days and adjusted to 10^7 conidia/ml at the time of inoculation. Three, two, and one day prior to spray inoculation of LVF12, five perilla plants were treated with the tested isolates to test disease prevention ability of biological control bacteria for each day. Then, three, two, and one day after spray inoculation of LVF12, five perilla plants were treated with the tested isolates to test disease curing ability of biological control bacteria for each day. After inoculation, plants were incubated in a growth chamber at 20°C with greater than 90% relative humidity for 7 days. After incubation, disease incidence was calculated to disease control value. Disease control value was calculated as follows (Korean Agricultural Chemical Industrial Association, 1997):

$$\text{Disease control value (\%)} = \frac{\text{Di in check plant-DI in treated plants}}{\text{DI in check plants}} \times 100$$

*DI : disease incidence

Identification. The selected isolates N1 and N4, which showed promising results as biological control agents, were subjected for identification. The purity of the isolates was tested by at least three cycles of streaking and re-isolation from single colonies on nutrient agar plates.

The selected isolates were characterized using biochemical, cultural, and physiological property of bacteria based on Bergey's manual, API 20E and 50CHB test kit, and Transmission electron microscope (TEM).

Results

***Botrytis cinerea* LVF12 inoculum preparation.** The occurrences of gray mold rot on perilla leaves varied not only in terms of availability but also types of nutrient. No disease development was observed on perilla leaves inoculated with LVF12 conidial suspensions prepared in sterile distilled water. However, plants inoculated with conidial suspensions prepared in PDB and 10% tomato juice exhibited 76.0% and 85.0% of gray mold rot on perilla leaves, respectively (Table 1).

Disease resistance study of perilla variety. Among 11 perilla varieties tested, not a single variety showed immunity to *B. cinerea* LVF12 infection. However, there were some degrees of difference in disease incidence among the tested varieties. Gwangyang showed the highest disease incidence (83.3%) while Milyang showed the lowest disease incidence (46.7%) to *B. cinerea* LVF12 infection (Table 2). However, not a single variety showed resistance to *B. cinerea* infection in commercially acceptable level.

Selection of antagonistic bacteria and bioassay test. All 250 bacterial isolates from leaves and roots of perilla plants

Table 1. Effects of nutrient supplements in conidial suspension for gray mold rot development caused by *Botrytis cinerea* LVF12 on perilla

Conidial suspension[a]	Disease incidence (%)[b]
SW	0.0x[c]
PDB	76.0y
Tomato	85.0z
Control	0.0x

[a] SW, sterilized water; PDB, potato dextrose broth; Tomato, 10% tomato juice; Control, non-treatment.
[b] Percentage of infected leaf.
[c] Means with the same letter are not significantly different according to the Duncan's multiple test (P=0.05).

Table 2. Resistance of 11 perilla varieties to infection by *Botrytis cinerea* LVF12

Cultivars	Disease incidence (%)
Okdong	61.7x[a]
Gupo	53.3w
Gwangyang	83.3z
Perilla 1-1	76.7y
Milyang	46.7v
Chubu	75.0y
Gurae 3-13	80.0zy
Kyungsin	58.3xw
Gosung jerae	78.3zy
Hadong	56.7xw
Jinju jerae	78.3zy

[a]Means with the same letter are not significantly different according to the Duncan's multiple test (P=0.05).

Table 3. Inhibitory effects of six antagonistic bacteria against the mycelial growth of *Botrytis cinerea* LVF12 on PDA

Antagonistic bacteria	Inhibition zone (mm)[a]
N1	35.0z[b]
N2	24.0y
N3	22.0y
N4	37.0z
N5	25.0y
N6	27.0y

[a]Growth inhibition was determined after 7 days of incubation at 25 °C.

[b]Means with the same letter are not significantly different according to the Duncan's multiple test (P=0.05).

were subjected to mycelial growth inhibition test. Among tested isolates, six isolates showed a wide zone of mycelial growth inhibition. Isolates N1 and N4 were notably superior than the rest of the isolates (Table 3, Fig. 1). All six bacterial isolates, which were capable of inhibiting mycelial growth, were selected and subjected to bioassay study in growth chamber. All isolates tested were capable of reducing disease incidence of gray mold rot on perilla leaves. The control value of the least effective isolate (N2) was 70%, but the control values of the isolates N1 and N4 were 95.3% and 90.8%, respectively (Fig. 2).

Mechanism of inhibition of conidial germination was tested with isolates N1 and N4, which showed strong mycelial growth inhibition ability on PDA media. Conidia of *B. cinerea* LVF12, which were added to the bacterial cultures, rarely germinated on water agar after 48 h incubation under a microscope. Meanwhile, conidia of *B. cinerea* LVF12, which were added to sterile distilled water, readily germinated on water agar after 48 h incubation (Table 4). Even though some conidia germinated, mycelial growth was slow and shape of mycelium was severely distorted (Fig. 3). Based on the results, isolates N1 and N4 were selected as the effective antagonistic bacteria to *B. cinerea* LVF12.

Different timing of application of biological control bacteria. From the above study, isolates N1 and N4 showing the highest control values were further tested for their ability to prevent gray mold rot development on perilla leaves by differentiating inoculation time of the pathogen and biological control agents. No gray mold rot developed on perilla leaves, regardless of time of application of N1

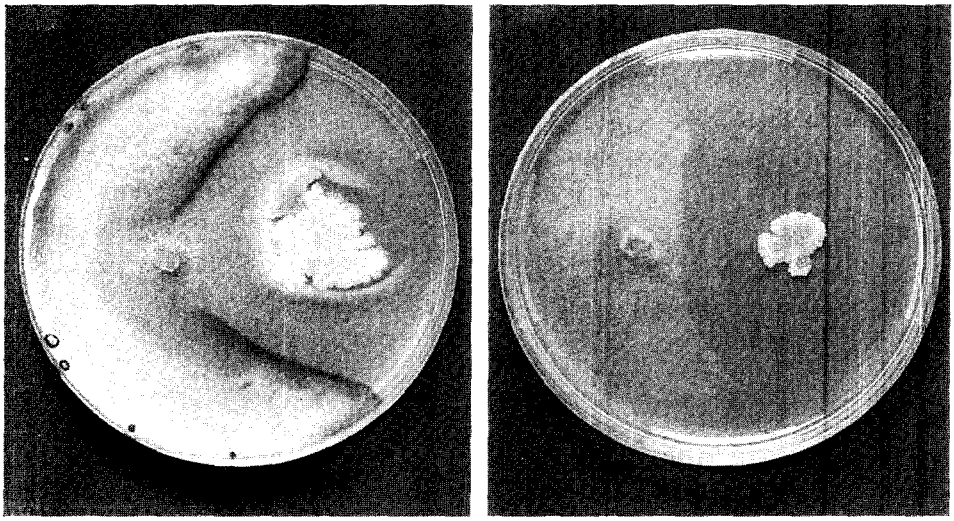

Fig. 1. Growth inhibition of *Botrytis cinerea* LVF12 by two antagonistic bacteria, *Bacillus licheniformis* N1 (**A**) and *B. megaterium* N4 (**B**) on PDA.

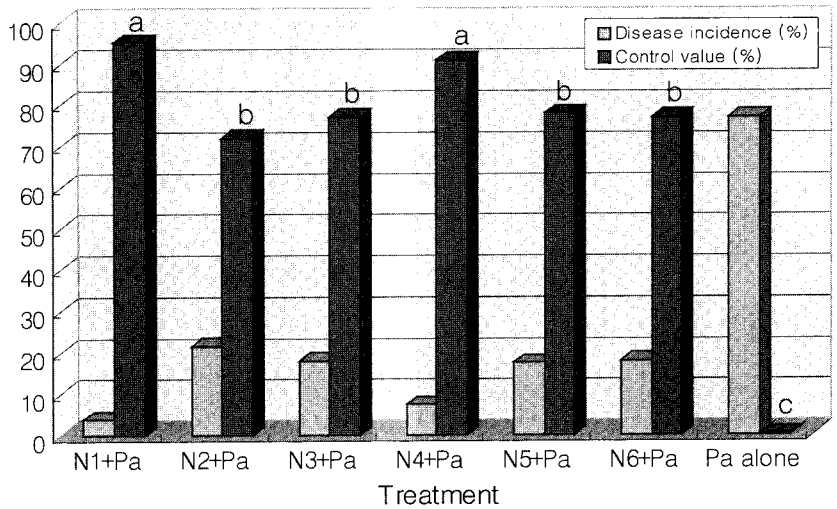

Fig. 2. Suppressive effects of six antagonistic bacteria on disease incidence of gray mold rot on perilla. Perilla seedlings were treated with both antagonistic bacteria and *B. cinerea*. Seedlings inoculated with *B. cinerea* alone served as a check. Means with the same letter are not significantly different according to the Duncan's multiple test (P=0.05).

Table 4. Effects of two antagonistic bacteria on conidial germination of *Botrytis cinerea* LVF12 on water agar

Antagonistic bacteria	Conidial germination (%S.D)[a]	
	12hr	48hr
N1	0	0
N4	0	3±2
SDW[b]	72±4	90±4

[a]Conidial germination was directly counted on water agar under a microscope.
[b]Sterile distilled water.

isolate (Table 5). The same results were found with N4 isolate when applied to plants prior to pathogen application. However, the efficacy of the biological control activity of N4 isolate slightly diminished with co-application or delayed application of N4 from 1 to 3 days after application of pathogen. However, both isolates were capable of preventing disease development on perilla leaves when applied prior to pathogen inoculation (Table 5).

Identification of biological control bacteria. Two iso-

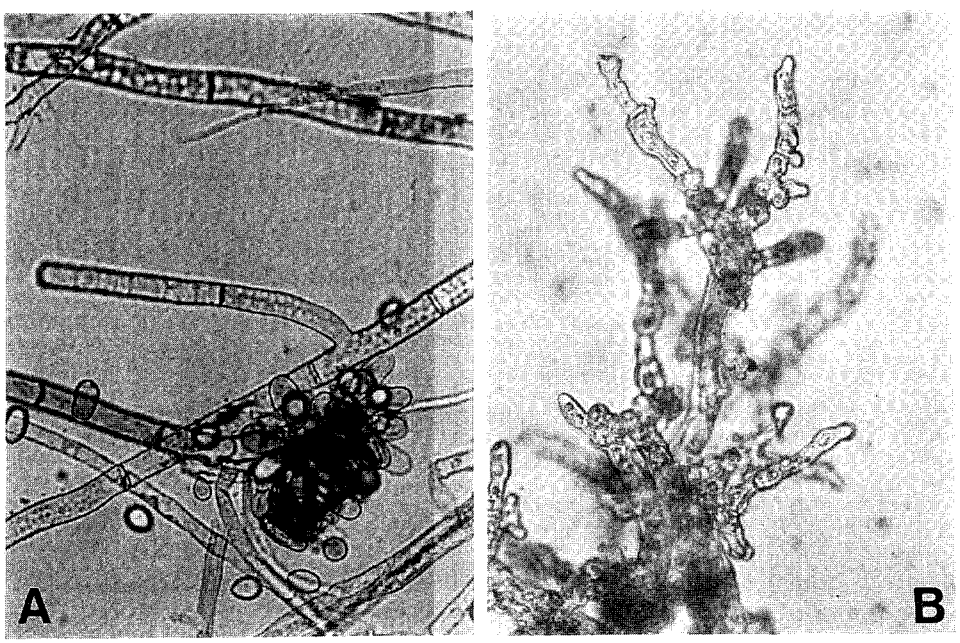

Fig. 3. Abnormal growth of mycelia of *Botrytis cinerea* LVF12 by antagonistic bacteria, *Bacillus licheniformis* N1. (**A**) Normal mycelia (× 400); (**B**) Abnormal mycelia (× 400).

Table 5. Effects of different inoculation time of *Botrytis cinerea* and two antagonistic bacteria on biological control of the gray mold rot of perilla in a growth chamber

Time of bacterial inoculation	Disease incidence (%)[a]			Control value (%)	
	N1[b]+Pa	N4[c]+Pa	Pa[d] alone	N1+Pa	N4+Pa
3 days before	0z[e]	0y		100z	100z
2 days before	0z	0y		100z	100z
1 day before	0z	0y		100z	100z
co-inoculated	0z	8.8z	73.3	100z	88.0y
1 day after	0z	9.0z		100z	87.7y
2 days after	0z	9.3z		100z	87.3y
3 days after	0z	9.3z		100z	87.3y

[a] Percentage of infected leaf.
[b] N1: *Bacillus licheniformis.*
[c] N4: *B. megaterium.*
[d] Pa: *Botrytis cinerea* LVF12.
[e] In a column, means with the same letter are not significantly different according to the Duncan's multiple test (P=0.05).

lates, N1 and N4, showing promising results of biological control of gray mold rot were further tested for their identification according to Bergey's manual by morphological, biochemical, physiological, and cultural properties of bacteria (Fig. 4). Also, these results were confirmed by API kit (bioMerieux, France). Isolates N1 and N4 were identified as *B. licheniformis* (88.8% of similarity by API kit) and *B. megaterium* (83.3% of similarity by API kit), respectively (Table 6).

Table 6. Biochemical, physiological, and cultural characteristics of two antagonistic bacteria compared with *Bacillus* spp.[a]

Test	Isolates		*Bacillus* spp.	
	N1	N4	*B. licheniformis*	*B. megaterium*
Gram stain	+[b]	+	+	+
Endospore	+	+	+	+
Cell diameter > 1.0 μm	-	+	−	+
Anaerobic growth	+	−	+	−
Growth in NaCl 2%	+	+	+	ND
5%	+	+	+	ND
7%	+	+	+	d
Growth at 5 °C	+	−	−	d
10 °C	−	+	−	+
30 °C	+	+	+	+
40 °C	+	+	+	d
50 °C	+	−	+	−
Hydrolysis of Casein	+	+	+	+
Gelatin	+	+	+	+
Starch	+	+	+	+
Nitrate reduction	+	+	+	d
Utilization of Citrate	+	+	+	+
Voges-prosakauer test	+	+	+	−

[a] Data from Bergey's manual of Systematic Bacteriology, 1986. Williams & Wilkins.
[b] Symbol: +, 90% or more of strains are positive; −, 90% or more of strains are negative; ND, no data available; d, 11-89% of strains are positive.

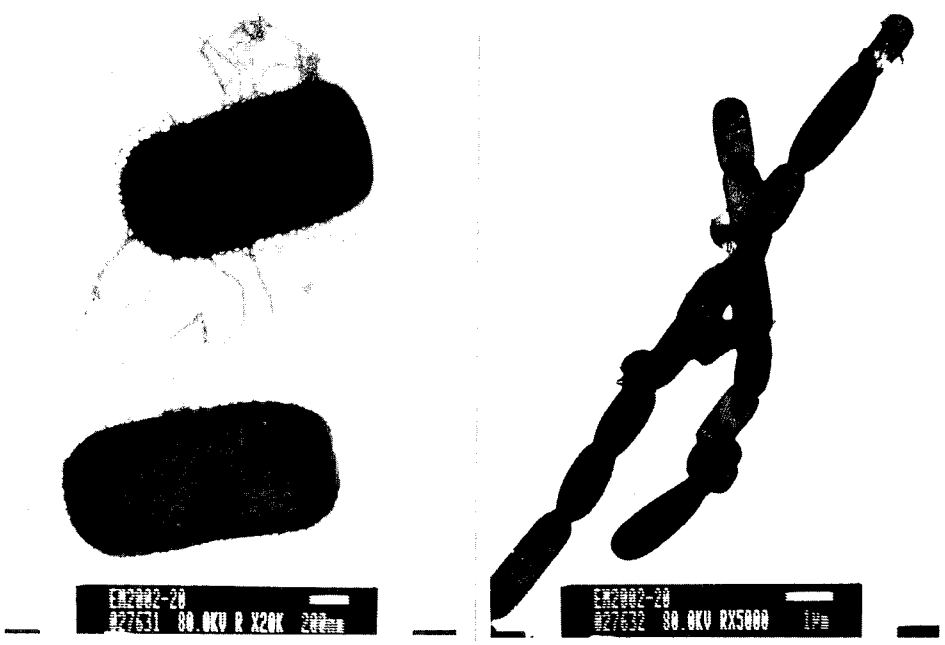

Fig. 4. Transmission electron micrographs of *Bacillus licheniformis* N1 isolated from soil against *B. cinerea.* (Magnification: A, × 20,000; B, × 5,000).

Discussion

Gray mold rot is an important disease of many crops under certain environmental conditions (i.e., period of wetness or high relative humidity). *Botrytis*, its causal pathogen, requires high moisture for reproduction and infection. Conidia germinate rapidly in the presence of water and form primary appressoria at the tip of germ tube. From primary appressoria, multicellular secondary appressoria are formed and penetrate cuticle tissue. Previous investigators have shown that formation of primary appressoria and/or multicellular secondary appressoria of *B. cinerea* was enhanced by different nutrient sources, such as glucose or inosine (Katsumi et al., 1981a; Katsumi et al., 1981b; Katsumi et al., 1987a; Katsumi et al., 1987b; Kim, 1999; Noboru et al., 1985). Based on the results of this inoculation experiment, using conidial suspension prepared in nutrients (PDB or 10% tomato juice) increased the rate of infection compared with conidial suspension prepared in sterile distilled water. Also, the rate of infection increased using 10% tomato juice as germination sap. This suggests that the 10% tomato juice contains more compounds or more concentrated materials responsible for appressoria formation than PDB. This is the first report of the use of 10% tomato juice to make conidial suspension of *B. cinerea* for inoculation test.

Previously reported biological control agents to control gray mold rot on various crops are as follows: post harvest problem of pears with *Bacillus pumillus* and *B. amyloliquefaciens* (Nari et al., 1996); grapes and beans with *Trichoderma harzianum* (Harman et al., 1996; Zimand et al., 1996); cyclamen with *Gliocladium roseum, Ulocladium atrum* (Köhl et al., 1998); apple with yeast (Filonow et al., 1996); petunia with *Pseudomonas fluorescens* (Gould et al., 1996); apple, pear and rose with antibiotic extract from *P. cepacia* (Janisiewicz et al., 1988; Hammer et al., 1993); strawberry with *Gliocladium roseum, Penicillum* spp., and *Trichoderma viride* (Suttin et al., 1993); and onion, lettuce, and apple with *Bacillus* spp. (Choi, 1990; Debao, 1994).

It has been reported that *B. licheniformis* has been shown to be effective against *B. cinerea, Microsporum canis, Mucor mucedo, M. plumbeus*, and *Corynebacterium glutamicum* by producing antifungal and antibacterial peptide (Galvez et al., 1993). Also, *B. megaterium* produces antibiotic peptide which inhibits mycelial growth of *Rhizoctonia solani*, a root rot causal organism on beans (Lebbabi et al., 1994). However, this is the first report showing biological control efficacy of both *B. licheniformis* and *B. megaterium* against gray mold rot caused by *Botrytis cinerea* on perilla.

Results of this study clearly demonstrate the effectiveness of both *B. licheniformis* N1 and *B. megaterium* N4 as potential biological control agents against *B. cinera* on perilla. Commercialization of both *Bacillus* isolates as biofungicide will not only reduce environmentally harmful chemicals but will also help in the control of gray mold rot in greenhouse grown crops like tomato, cucumber, and strawberry.

Acknowledgment

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