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Antifungal Activities of *Bacillus thuringiensis* Isolates on Barley and Cucumber Powdery Mildews

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Abstract Fourteen *Bacillus thuringiensis* isolates having both insecticidal activity and *in vitro* antifungal activity were selected and tested for *in vivo* antifungal activity against tomato late blight, wheat leaf rust, tomato gray mold, and barley powdery mildew in growth chambers. All the isolates represented more than 70% disease control efficacy against at least one of four plant diseases. Specifically, 12 isolates exhibited strong control activity against barley powdery mildew. Under glasshouse conditions, four (50–02, 52–08, 52–16, and 52–18) of the isolates also displayed potent control efficacy against cucumber powdery mildew. To our knowledge, this is the first report of *B. thuringiensis* isolates that have disease control efficacy against powdery mildew of barley and cucumber as well as insecticidal activity.

Keywords: *Bacillus thuringiensis*, antifungal activity, insecticidal activity, powdery mildew, disease control

Management of plant disease and harmful insect is essential to agricultural productivity and efficiency. In particular, powdery mildew is one of the most serious plant diseases, causing large yield losses in crops such as cucumber, pea, rose, strawberry, and apple [24]. Biological control using microorganisms to suppress powdery mildew fungi offers an attractive alternative to currently used pest control practices, especially as chemical pesticides are deemphasized because of the development of resistance in pest populations and concerns about human health and environmental quality [2]. Many studies have focused on integrated control of powdery mildew fungi, with the aim of reducing the input of fungicides [5, 16, 19, 20].

The genus *Bacillus* includes a variety of industrially important species that are "generally recognized as safe" (GRAS) in food, industry, and agriculture [3, 8, 14]. Among them, *B. thuringiensis* is used worldwide as a biological control agent acting on susceptible insects by producing sporulation parasporal crystals containing delta-endotoxins [6, 7, 15]. Moreover, it is able to produce several biologically active molecules such as bacteriocins, insecticidal proteins, and hydrolytic enzymes among which are chitinases [1, 3, 9, 17].

Recently, a few studies on the use of *B. thuringiensis* against plant pathogenic fungi have been conducted [3, 4, 11, 23]. Stabb *et al.* [23] found that zwittermicin A-producing strains of *B. thuringiensis* represented control efficacy against alfalfa damping-off, which is caused by *Phytophthora medicaginis*. Kim *et al.* [11] reported that a fengycin-like lipopeptide produced by *B. thuringiensis* CMB26 showed *in vitro* antifungal activity against *Colletotrichum gloeosporioides*, pathogenic fungi of plant anthracnose. In addition, a chitinase, belonging to the chitobiosidase class, from *B. thuringiensis* subsp. *kurstaki* inhibited the mycelial growth of *Aspergillus niger* that causes black mold of onion and peanut [4]. As far as is known, little work has been done on the strains of *B. thuringiensis* that have not only *in vivo* antifungal activity against powdery mildew fungi but also insecticidal activity. In the present study, we obtained *B. thuringiensis* isolates from fecal samples and selected 14 isolates showing not only insecticidal activity against diamondback moth (*Plutella xylostella*) and/or yellow fever mosquito (*Aedes aegypti*) but also *in vitro* antifungal activity against *Botrytis cinerea*, a pathogen of gray mold disease, in dual culture tests. To develop environmental-friendly biopesticides, their *in vivo* antifungal activities were examined against four plant pathogenic fungi in growth chambers and against cucumber powdery mildew fungus under glasshouse condition.

For isolation of *B. thuringiensis*, fecal samples were collected from mammalian species at the Fukuoka Municipal Zoo, Fukuoka, Japan. The sample of 1 g (wet mass) was suspended in 9 ml of sterile phosphate-buffered saline and heated at 65°C for 30 min to kill heat-sensitive microorganisms. Tenfold serial dilutions of the suspension were prepared in phosphate-buffered saline and plated on nutrient agar (pH 7.6) consisting of meat extract (10 g), polypeptone (10 g), NaCl (2 g), agar (25 g), and distilled water (1 l). The plates

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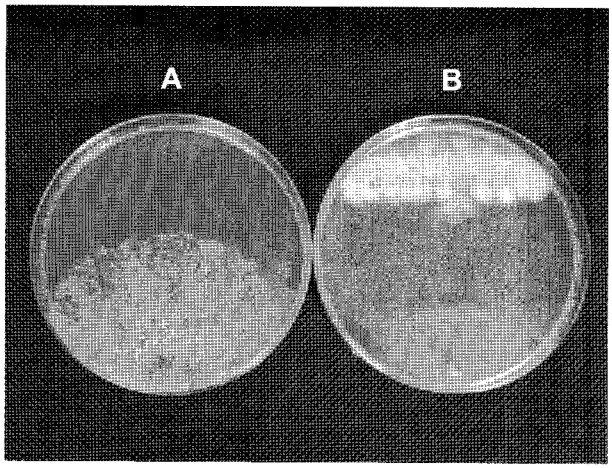

Fig. 1. *In vitro* antifungal activity of 17-29 against the mycelial growth of *Botrytis cinerea* (A, only *B. cinerea*; B, 17-29 and *B. cinerea*).

were incubated at 28°C for 3–4 days. Of the colonies formed, those with morphological features characteristic of the *Bacillus cereus* group were chosen for phase-contrast microscopy of the sporulating cells [13, 18, 22]. Finally, parasporal-inclusion-forming colonies were collected as *B. thuringiensis*. We examined their insecticidal activity against yellow fever mosquito (*A. aegypti*) and diamondback moth (*P. xylostella*). *B. thuringiensis* isolates showing insecticidal activity (data not shown) against yellow fever mosquito and/or diamondback moth were tested further for *in vitro* antifungal activity against *B. cinerea* in dual culture tests (Fig. 1). In consequence, 14 *B. thuringiensis* isolates showing *in vitro* antifungal activity (data not shown) as well as insecticidal activity were selected.

For identification of H antigen serotypes, reference H antisera against *B. thuringiensis* H serotypes 1–55 were raised in rabbits [12]. The reference strains of these H serotypes were obtained from the Institute Pasteur, Paris, France. Antisera against H antigenic subfactors, existing in several H serotypes, were prepared by a cross-saturation technique. H serotyping was done with a slide agglutination test [18]. In brief, one drop of 3- to 4-h-old flagellated broth culture was mixed on a glass slide with one drop of H antiserum. Specific agglutination was determined after 1–2 min. Table 1 shows the serological identification of H antigens associated with 14 *B. thuringiensis* isolates. Fourteen strains of *B. thuringiensis* were divided into 5 subspecies.

In order to examine *in vivo* antifungal activity of the 14 isolates against tomato late blight (*Phytophthora infestans*), wheat leaf rust (*Puccinia recondita*), tomato gray mold (*B. cinerea*), and barley powdery mildew (*Blumeria graminis* f. sp. *hordei*), each isolate was inoculated in a flask containing tryptic soy broth (TSB; Becton and Dickinson Co.) or nutrient broth (NB; Becton and Dickinson Co.) medium. The flask was incubated on a rotary shaker at

Table 1. *Bacillus thuringiensis* isolates used in this study.

Serotype	Isolate name
B. thuringiensis subsp. *aizawai*	15-11, 52-18
B. thuringiensis subsp. *galleriae*	45-27
B. thuringiensis subsp. *kurstaki*	17-29, 19-17, 19-22, 20-48, 42-15, 42-20, 52-08, 52-12, 52-16
B. thuringiensis subsp. *morrisoni*	50-02
B. thuringiensis subsp. *sotto*	46-12

150 rev/min for 72 h at 30°C. The culture broth was harvested and then Tween 20 was added at 250 μg/ml. In order to select bacterial isolates with potent *in vivo* antifungal activity, 28 culture broths of 14 isolates incubated in TSB and NB media were tested *in vivo* for antifungal activity against the four plant diseases. Tomato (*Lycopersicon esculentum* Mill., cv. Seokwang), barley (*Hordeum sativum* Jessen, cv. Dongbori), and wheat (*Triticum aestivum* L., cv. Chokwang) plants were grown in the plastic pots (4.5-cm diameter) in a greenhouse at 25±5°C for 1 to 3 weeks. The plant seedlings were sprayed with the suspension until run-off. Control plants were sprayed with Tween 20 (250 μg/ml) solution. After 24 h, the treated plant seedlings were inoculated with spore suspensions of one of four plant pathogenic fungi, followed by rating disease symptoms at 3–7 days after inoculation [10]. Pots were arranged as a randomized complete block with three replicates per treatment. Dimethomorph (10 μg/ml) for tomato late blight, fludioxonil (50 μg/ml) for tomato gray mold, flusilazole (10 μg/ml) for wheat leaf rust and barley powdery mildew were applied as positive controls. Experiments were conducted twice in a growth chamber, and the mean value of six estimates for each treatment was converted into percentage disease control. The percentage of disease control was determined using the following equation: % control= 100[(A–B)/A], where A=the area of infection (%) on leaves sprayed with Tween 20 solution alone and B=the area of infection (%) on treated leaves.

All *B. thuringiensis* isolates displayed disease control activity of more than 70% against at least one of the four plant diseases (Table 2). Of the isolates incubated in TSB, twelve exhibited control values of more than 80% against barley powdery mildew and one (*B. thuringiensis* subsp. *kurstaki* 19-17) displayed potent control activity against tomato late blight. Among the bacterial isolates incubated in NB medium, *B. thuringiensis* subsp. *kurstaki* 17-29 and subsp. *morrisoni* 50-02 were active against tomato gray mold and barley powdery mildew, respectively. On the other hand, the bacterial isolates hardly controlled the development of wheat leaf rust caused by *P. recondita*. The above results indicate that most of the *B. thuringiensis* isolates tested have potent control activity against the barley powdery mildew caused by *B. graminis* f. sp. *hordei*. *B. thuringiensis* is

Table 2. *In vivo* antifungal activity of *Bacillus thuringiensis* isolates against four plant pathogens.[a]

Isolate	Medium	Disease control (%)			
		TGM	TLB	WLR	BPM
15-11	TSB	0[b]	13	0	83
	NB	8	33	0	0
17-29	TSB	0	19	20	0
	NB	75	8	20	8
19-17	TSB	0	81	0	75
	NB	0	8	0	17
19-22	TSB	0	13	20	83
	NB	25	0	3	0
20-48	TSB	0	0	3	95
	NB	25	0	0	0
42-15	TSB	0	6	33	92
	NB	8	0	13	0
42-20	TSB	0	0	20	85
	NB	0	8	20	17
45-27	TSB	0	31	13	90
	NB	0	0	13	8
46-12	TSB	0	31	20	88
	NB	8	8	0	0
50-02	TSB	0	0	43	87
	NB	8	17	3	72
52-08	TSB	0	6	3	83
	NB	0	25	20	0
52-12	TSB	0	6	33	90
	NB	0	8	0	0
52-16	TSB	0	19	33	80
	NB	8	33	33	67
52-18	TSB	0	13	3	93
	NB	17	42	33	8
Chemical	Concentration (μg/ml)				
Fludioxonil	50	100	–[c]	–	–
Dimethomorph	10	–	98	–	–
Flusilazole	10	–	–	100	100

[a]*Bacillus thuringiensis* isolates were inoculated in tryptic soy broth and nutrient broth and then incubated at 30°C for 3 days on a rotary shaker. Tween 20 (250 μg/ml) was added in the culture broth, and then sprayed to run-off the following seedlings; tomato (3-leaf stage), wheat (1-leaf stage), and barley (1-leaf stage). After 24 h, the treated plant seedlings were inoculated with spores of the fungi. TGM, tomato gray mold; TLB, tomato late blight; WLR, wheat leaf rust; BPM, barley powdery mildew.
[b]Each value represents the mean of disease controls (%) of two runs with three replicates.
[c]Not tested.

widely used in agriculture as a bioinsecticide for the control of many insect pests. It produces a characteristic proteinaceous crystalline toxin with a specific activity against certain insect species, causing paralysis of the larval gut [21]. Recently, a few studies on antifungal activity have been reported. Kim *et al.* [11] reported that *B. thuringiensis* CMB26 isolated from soil inhibited the mycelial growth of

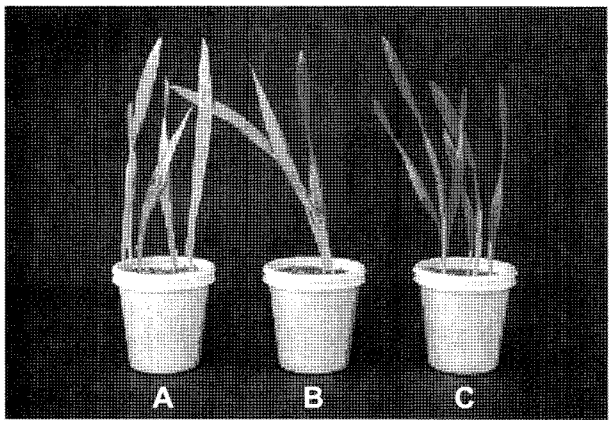

Fig. 2. *In vivo* antifungal activity of 19-22 against barley powdery mildew (A, Control; B, 19-22; C, Flusilazole 10 μg/ml).

C. gloeosporioides and produced a lipopeptide as toxic principal. The molecular mass was estimated as 1,447 Da. Zwittermicin A (an aminopolyol antibiotic) produced by *B. thuringiensis* strains also displayed control efficacy against alfalfa damping-off caused by *P. medicaginis* [23]. Cherif *et al.* [3] reported that the novel bacteriocin entomocin 9 produced by *B. thuringiensis* subsp. *entomocidus* HD9 showed antifungal activity against *Aspergillus nidulans* and *Fusarium graminis* in dual culture tests. A chitinase Chi255 from *B. thuringiensis* subsp. *kurstaki* showed *in vitro* antifungal activity against *A. niger* that causes black blight of onion and peanut [4]. To our knowledge, *B. thuringiensis* isolates having *in vivo* antifungal activity on powdery mildew fungi as well as insecticidal activity have not been reported yet.

To examine the disease control efficacy of the 12 active isolates against cucumber powdery mildew under glasshouse conditions, each isolate was inoculated in a flask containing TSB medium and incubated in the dark on a rotary shaker (150 rpm; 30°C; 3 days). Each culture broth was harvested and serially diluted to 2 concentrations (1/10 and 1/30 dilution) and then Tween 20 was added at 250 μg/ml. The suspensions were sprayed, using hand sprayers to run-off, on potted cucumber plants at the six-leaf stage. Treatments were made twice, with an interval of a week, starting immediately after the appearance of symptoms of powdery mildew on the first and second leaf. Tween 20 (250 μg/ml)-treated plants were used as controls. In addition, fenarimol (30 μg/ml) was applied as positive controls. Pots were arranged as a randomized complete block with four replicates per treatment. The experiments were repeated twice, for a total of eight plants tested in each variant. Disease severity was rated on ten leaves (leaves 3–12) per plant, 7 days after the second treatment. The eight estimates for each treatment were converted into percentage fungal control (±standard deviation) as compared with the control plants. The twelve isolates, evaluated at 1/10 dilution of

Table 3. Control efficacy of *Bacillus thuringiensis* isolates against cucumber powdery mildew under glasshouse conditions.[a]

Bacterial isolate	Concentration (dilution)	
	1/10	1/30
15-11	38±11[b]	28±13
19-22	44±11	27±16
20-48	40±9.5	16±14
42-15	18±8.0	14±6.0
42-20	38±3.0	30±11
45-27	49±5.5	28±2.3
46-12	48±9.0	18±8.5
50-02	68±3.5	22±4.6
52-08	51±2.4	38±16
52-12	49±14	25±11
52-16	54±3.1	14±12
52-18	51±3.6	23±5.5
Chemical	Concentration (μg/ml)	
	30	
Fenarimol	96±2.1	

[a]Cucumber plants at the six-leaf stage were sprayed with the diluted culture broth of *Bacillus thuringiensis* twice, with an interval of a week, starting immediately after the appearance of the first symptoms of powdery mildew on foliage. Disease severity was rated at 7 days after the second treatment.
[b]Each value represents the mean of disease controls (%)±standard deviation of two runs with four replicates.

the culture broth for control efficacy against cucumber powdery mildew, represented various control values of 18% to 68% on the disease. Among them, the 4 isolates 50-02, 52-08, 52-16, and 52-18 controlled development of powdery mildew more than 50% in glasshouse application (Table 3). In particular, *B. thuringiensis* 50-02 at 1/10 a dilution showed disease control value of 68%.

The above results indicate that the *B. thuringiensis* isolates simultaneously are able to control powdery mildews as well as insects infesting various crops in fields. Their high efficacy against powdery mildew fungi in a glasshouse led us to test them under field conditions. Further studies on the characterization of the antifungal principal from the active isolates of *B. thuringiensis* are in progress.

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References

1. Ahern, M., S. Verschueren, and D. van Sinderen. 2003. Isolation and characterization of a novel bacteriocin produced by *Bacillus thuringiensis* strain B439. *FEMS Microbiol. Lett.* **220:** 127–131.
2. Carlton, B. C. 1993. Development of improved bioinsecticides based on *Bacillus thuringiensis*, pp. 258–266. *In* S. O. Duke, J. J. Menn, and J. R. Plimmer (eds.), *Pest Control with Enhanced Environmental Safety.* American Chemical Society, Washington, D.C.
3. Cherif, A., S. Chehimi, F. Limem, B. M. Hansen, N. B. Hendriksen, D. Daffonchio, and A. Boudabous. 2003. Detection and characterization of the novel bacteriocin entomocin 9, and safety evaluation of its producer, *Bacillus thuringiensis* ssp. *entomocidus* HD9. *J. Appl. Microbiol.* **95:** 990–1000.
4. Driss, F., M. Kallassy-Awad, N. Zouari, and S. Jaoua. 2005. Molecular characterization of a novel chitinase from *Bacillus thuringiensis* subsp. *kurstaki*. *J. Appl. Microbiol.* **93:** 374–379.
5. Falk, S. P., D. M. Gadoutry, P. Cortesi, R. C. Pearson, and R. C. Steem. 1995. Parasitism of *Uncinula necator* cleistothecia by the mycoparasite *Ampelomyces quisqualis*. *Phytopathology* **85:** 794–800.
6. Glare, T. R. and M. O'Callaghan. 2000. *Bacillus thuringiensis: Biology, Ecology and Safety.* John Wiley and Sons Ltd., Chichester, U.K.
7. Höfte, H. and H. R. Whiteley. 1989. Insecticidal proteins of *Bacillus thuringiensis*. *Microbiol. Rev.* **53:** 242–255.
8. Jung, H. K. and S.-D. Kim. 2005. An antifungal antibiotic purified from *Bacillus megaterium* KL39, a biocontrol agent of red-pepper *Phytophthora*-blight disease. *J. Microbiol. Biotechnol.* **15:** 1001–1010.
9. Kim, J. S., J. Y. Choi, J. H. Chang, H. J. Shim, J. Y. Roh, B. R. Jin, and Y. H. Je. 2005. Characterization of an improved recombinant baculovirus producing polyhedra that contain *Bacillus thuringiensis* Cry1Ac crystal protein. *J. Microbiol. Biotechnol.* **15:** 710–715.
10. Kim, J.-C., G. J. Choi, J.-H. Park, H. T. Kim, and K. Y. Cho. 2001. Activity against plant pathogenic fungi of phomalactone isolated from *Nigrospora sphaerica*. *Pest Manag. Sci.* **57:** 554–559.
11. Kim, P. I., H. Bai, H. Chae, S. Chung, Y. Kim, R. Park, and Y.-T. Chi. 2004. Purification and characterization of a lipopeptide produced by *Bacillus thuringiensis* CMB26. *J. Appl. Microbiol.* **97:** 942–949.
12. Lecadet, M.-M., E. Frachon, V. Cosmao Dumanoir, H. Ripouteau, S. Hamon, P. Laurent, and I. Thiery. 1999. Updating of the H-antigen classification of *Bacillus thuringiensis*. *J. Appl. Microbiol.* **86:** 660–672.
13. Lee, D.-H., I. H. Cha, D. S. Woo, and M. Ohba. 2003. Microbial ecology of *Bacillus thuringiensis*: Fecal populations recovered from wildlife in Korea. *Can. J. Microbiol.* **49:** 465–471.
14. Lee, H.-J., K.-H. Park, J.-H. Shim, R.-D. Park, Y.-W. Kim, J.-Y. Cho, H. HwangBo, Y.-C. Kim, G.-S. Cha, H. B. Krishnan, and K.-Y. Kim. 2005. Quantitative changes of plant defense enzymes in biocontrol of pepper (*Capsicium annuum* L.) late blight by antagonistic *Bacillus subtilis* HJ927. *J. Microbiol. Biotechnol.* **15:** 1073–1079.

15. Lereclus, D., A. Delecluse, and M. Lecadet. 1993. Diversity of *Bacillus thuringiensis* toxins and genes, pp. 37–69. *In* P. F. Entwistle, J. S. Cory, M. J. Bailey, and S. Higgs (eds.), *Bacillus thuringiensis, a Environmental Biopesticide: Theory and Practice*. John Wiley & Sons Ltd., U.K.
16. Nofal, M. A. and W. M. Haggag. 2006. Integrated management of powdery mildew of mango in Egypt. *Crop Prot.* **25:** 480–486.
17. Oh, S.-T., J.-K. Kim, S.-Y. Yang, and M.-D. Song. 2004. Characterization of *Bacillus thuringiensis* having insecticidal effects against larvae of *Musca domestica*. *J. Microbiol. Biotechnol.* **14:** 1057–1062.
18. Ohba, M. and K. Aizawa. 1986. Distribution of *Bacillus thuringiensis* in soils of Japan. *J. Invertebr. Pathol.* **47:** 277–282.
19. Paulitz, T. C. and R. R. Belanger. 2001. Biological control in greenhouse systems. *Annu. Rev. Phytopathol.* **39:** 103–133.
20. Romero, D., A. Perez-Garcia, M. E. Rivera, F. M. Cazorla, and A. de Vicente. 2004. Isolation and evaluation of antagonistic bacteria towards the cucurbit powdery mildew fungus *Podosphaera fusca*. *Appl. Microbiol. Biotechnol.* **64:** 263–269.
21. Schnepf, E., N. Crickmore, J. Van Rie, D. Lereclus, J. Baum, J. Feitelson, D. R. Zeigler, and D. H. Dean. 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev.* **62:** 775–806.
22. Shisa, N., N. Wasano, A. Ohgushi, D.-H. Lee, and M. Ohba. 2002. Extremely high frequency of common flagellar antigens between *Bacillus thuringiensis* and *Bacillus cereus*. *FEMS Microbiol. Lett.* **213:** 93–96.
23. Stabb, E. V., L. M. Jacobson, and J. Handelsman. 1994. Zwittermicin A-producing strains of *Bacillus cereus* from diverse soils. *Appl. Environ. Microbiol.* **60:** 4404–4412.
24. Yarwood, G. E. 1957. Powdery mildews. *Bot. Rev.* **23:** 235–300.