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DISEASE CONTROL

Biological control of Fusarium wilt by *Bacillus amyloliquefaciens* IUMC7 isolated from mushroom compost

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Abstract *Bacillus amyloliquefaciens* IUMC7 isolated from mushroom compost inhibited growth of *Fusarium oxysporum* f. sp. *lycopersici* (FOL) on culture plates, and a culture supernatant of IUMC7 inhibited in vitro germ tube elongation of FOL. When compared with control soils, mushroom compost inoculated with IUMC7 significantly reduced disease severity caused by FOL in tomato plants. PCR tests for expression of PR genes indicated that IUMC7 did not induce resistance in tomato plants. These results suggested that the suppression of disease was mainly caused by antimicrobial compounds produced by IUMC7.

Keywords Compost · Biological control · *Fusarium oxysporum* f. sp. *lycopersici* · *Bacillus amyloliquefaciens*

The soil fungus *Fusarium oxysporum* is a serious plant pathogen throughout the world. The fungus infects a wide range of plant species and causes considerable loss in agricultural output. Since soil-borne pathogens are difficult to control using conventional strategies, such as the use of resistant cultivars and synthetic fungicides, alternative methods of environmentally benign disease control are urgently required (Bailey and Lazarovits 2003; Gamliel et al. 2000).

Biological control using microorganisms such as *Bacillus* spp. (Cao et al. 2011), *Pseudomonas* spp. (Manikandan et al. 2010), *Trichoderma* spp. (Trillas et al. 2006), and some plant growth-promoting rhizobacteria strains (Xue et al. 2009) have been proposed to control diseases caused by soil-borne pathogens. We obtained *Bacillus amyloliquefaciens* IUMC7 from mushroom compost, which had antimicrobial activity against several plant pathogens (Sotoyama et al. 2013). We therefore tested IUMC7 for the control of a tomato disease caused by *Fusarium oxysporum* f. sp. *lycopersici* (FOL) in this study.

First, to investigate *B. amyloliquefaciens* IUMC7 as a possible biocontrol agent, we investigated whether IUMC7 could inhibit growth of FOL on plates. IUMC7 was cultured for 2 days in tryptic soy broth (TSB) (Difco Laboratories, Detroit, MI, USA) at 28 °C on a rotary shaker at 150 rpm. The culture was centrifuged at 3500×*g* for 5 min at 23 °C, the supernatant was discarded, and the bacterial pellet was washed three times with sterilized water. This pellet was resuspended in sterilized water, and the suspension was adjusted to an optical density of 1.0 at 600 nm ($OD_{600} = 1.0$). FOL race 1 was cultured in 100 mL PS medium [extract from 200 g/L potato, 2 % (w/v) sucrose] in a flask with shaking at 130 rpm at 28 °C for 7 days. The cultured medium was filtered through several sheets of sterilized Kimwipes tissues, and then bud cells were collected by centrifuging at 1700×*g* for 5 min. After three washes with sterilized water, the bud cell suspension was adjusted to 1×10^5 cells/mL using a hemocytometer. A 100 μL sample of FOL suspension was mixed with 10 mL melted PSA [extract from 200 g/L potato, 2 % (w/v) sucrose, 1.5 % (w/v) agar] and plated. A 50 μL sample of the IUMC7 suspension was applied to a paper disc (8 mm diameter) on PSA plates containing bud cells of FOL. After 2 days of incubation, the zone of inhibition was measured.

IUMC7 showed antagonistic activity. The average diameter of the inhibition zones was 13.4 ± 1.1 mm.

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Along the border of the inhibition zone, the hyphae of FOL seemed to be damaged; swelling of hyphal tips was often accompanied by exudation compared with the typical, intact hyphae of the control (Fig. 1).

Subsequently, the antimicrobial activity of the culture supernatant of IUMC7 was tested against FOL. IUMC7 was cultured for 2 days in liquid PS at 28 °C. The supernatant after centrifuging the medium at 3500×*g* for 5 min was then filtered through a 0.22 µm membrane (ADVANTEC, Tokyo, Japan). Five-hundred microliters of the FOL bud cell suspension, adjusted to 2×10^5 cells/mL in PS medium, was mixed with an equivalent amount of the supernatant of IUMC7 or PS medium as a control. After 16 h of incubation, the bud cells and germlings were examined with a light microscope (Olympus, Tokyo, Japan).

In the treatment with the IUMC7 supernatant, all germ tubes were abnormally and irregularly swollen (Fig. 2).

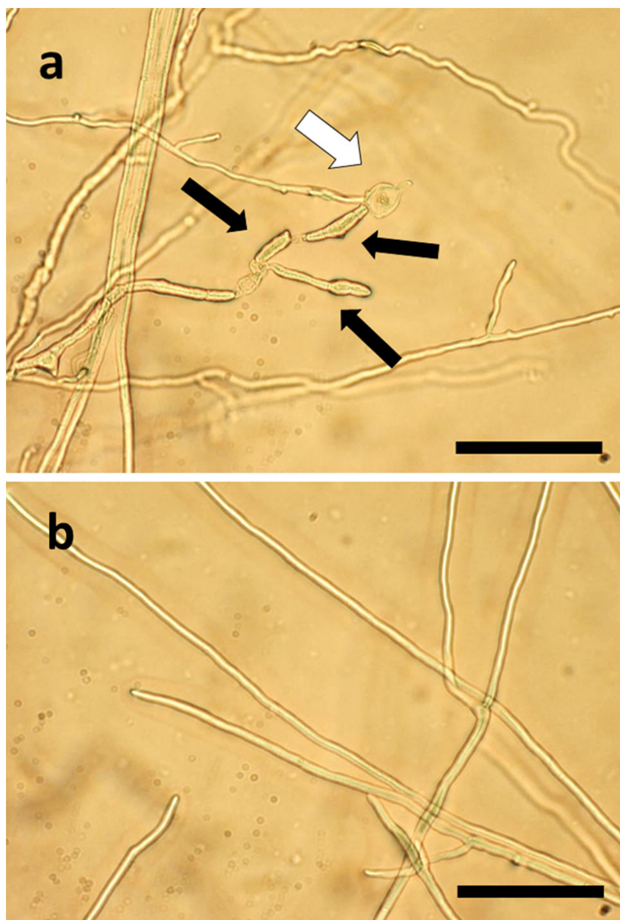

Fig. 1 Light micrographs of *Fusarium oxysporum* f. sp. *lycopersici* (FOL) in co-cultivation test with strain IUMC7 of *Bacillus amyloliquefaciens*. **a** Lysed FOL hyphae along the border of the inhibition zone. *Black arrows* Swollen hyphal tips; *white arrow* exudation of hyphal protoplasm. **b** Apparently healthy FOL hyphae outside of inhibition zone. *Scale bar* is 100 µm

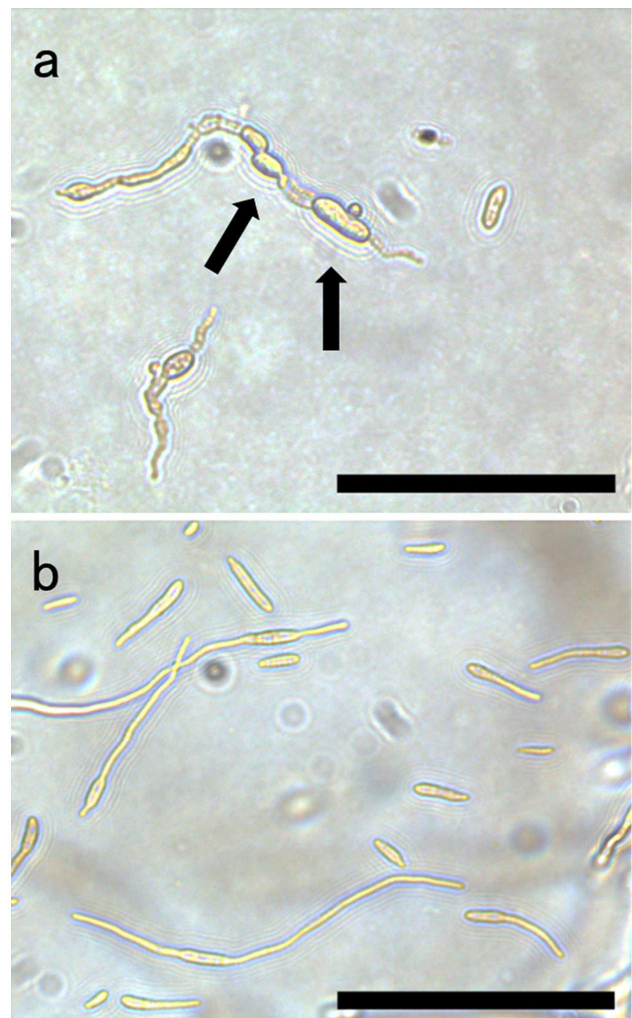

Fig. 2 Effect of 2-day treatment with culture supernatant from strain IUMC7 of *Bacillus amyloliquefaciens* on *Fusarium oxysporum* f. sp. *lycopersici* (FOL) bud cells. **a** FOL bud cell suspension (500 µL of 2×10^5 cells/mL in PS medium) was mixed with 500 µL of the supernatant. *Arrows* Swollen area on germ tube. **b** Bud cells (500 µL) mixed only with PS medium (500 µL) as a control. *Scale bar* is 100 µm

These results showed that IUMC7 produces an extracellular antimicrobial substance(s).

We then examined the biocontrol efficiency of IUMC7 against Fusarium wilt of tomato. A suspension of IUMC7 was prepared as described, adjusted to $OD_{600} = 0.7$, then mixed with sterilized culture soil (Sakata Super Mix, Sakata Seed Co., Tokyo, Japan) or sterilized mushroom compost (Mushsoil, JA Ibaraki Kasumi Compost Center, Ibaraki, Japan) at a ratio of 1:4 (w/w), which gave an IUMC7 concentration of approximately 10^8 cfu/g soil. As inoculum, a bud cell suspension of FOL was prepared using the same method described above and adjusted to 4×10^5 -cells/mL using a hemocytometer. This suspension was used to inoculate four types of soil prepared as follows: (1)

IUMC7 + sterilized mushroom compost (sterilized mushroom compost inoculated with IUMC7 and mixed 1:1 v/v with sterilized culture soil); (2) IUMC7 + sterilized culture soil (sterilized culture soil inoculated with IUMC7 and mixed 1:1 v/v with sterilized culture soil; (3) sterilized mushroom compost (uninoculated sterilized mushroom compost mixed 1:1 v/v with sterilized culture soil); (4) sterilized culture soil alone. Samples (200 g each) of each soil type were inoculated with 50 mL of FOL suspension and placed in a plastic tray (about 17 × 12 × 6 cm), then incubated in a growth chamber for 1 week at 31 °C. Tomato seedlings (cv. Ponderosa), which had been grown in culture soil for 3 weeks in a growth chamber (12 h light/12 h dark, 31 °C), were transplanted into the four soils (15 plants in each type) that had been inoculated with the pathogen. Disease severity was recorded at weekly intervals for 3 weeks using a disease index based on a scale of 0 to 2, where 0 = no wilting, 1 = chlorosis and yellowing of leaves or wilting, 2 = dead plants. Disease severity was calculated as follows: Disease severity = [∑(Number of diseased plants at each index value × Disease index value)/(Total number of plants investigated × 2)] × 100 %. The population density of FOL in each treatment was also estimated at weekly intervals using Komada selective medium (Komada 1975). Three independent experiments were run.

Compared with the disease severity in the two control treatments without IUMC7, disease severity on plants grown in both soils containing IUMC7 was lower for the first 2 weeks. However, the severity of disease in all treatments gradually increased, and after 3 weeks, there were no significant differences among the IUMC7 treatments and the controls (Fig. 3). The FOL population in the two control treatments increased each week, whereas it remained constant in two IUMC7 treatments (Fig. 4). These findings suggested that the initial low disease severity in the IUMC7 treatment was due to suppression of FOL proliferation by IUMC7. These results indicated that IUMC7 may have potential as a biocontrol agent and that the mushroom compost may contain nutrients for the growth of IUMC7.

To investigate the suitability of the mushroom compost for IUMC7 growth, we compared the population dynamics of IUMC7 in field soil and in compost-amended field soil. A suspension of IUMC7 prepared as described was mixed with sterilized field soil or field soil containing sterilized compost (1:1 v/v) at a ratio of 1:4 (w/w). One gram of each soil was sampled weekly and suspended in 9 mL of sterilized water. Dilutions of the suspension were spread on tryptic soy agar (TSA; Difco) plates. After 1 day of incubation at 37 °C, the colonies were counted.

The density of IUMC7 in the soil containing mushroom compost gradually increased until 2 weeks, whereas it decreased in control soil until 3 weeks (Fig. 5). From 3 weeks on, the IUMC7 density of both treatments both decreased, then increased, but the density differed significantly between the treatments. This result showed that mushroom compost contained nutrients for growth of IUMC7.

The mode(s) of action by biological agents can involve a complex syndrome of factors, including nutrient competition, site exclusion, attachment of the antagonist to the

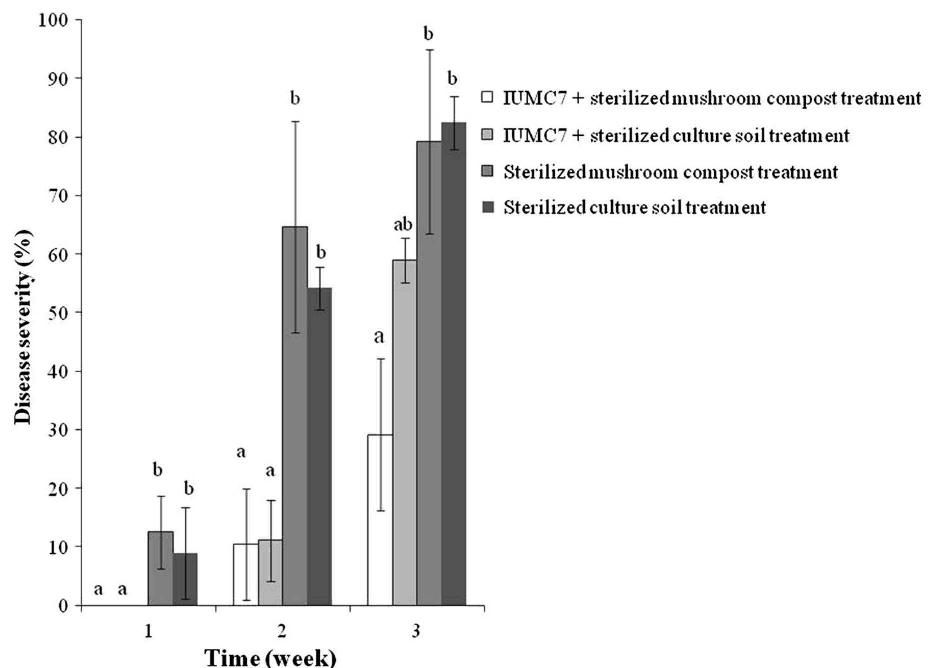

Fig. 3 Effect of four soil treatments (IUMC7 + sterilized mushroom compost treatment, IUMC7 + sterilized culture soil treatment, sterilized mushroom compost treatment, sterilized culture soil treatment) on severity of Fusarium wilt disease in tomatoes over time. Each treatment consisted of 15 plants; error bars indicate 95 % confidence intervals of the means from three independent experiments. *Different letters* indicate significant differences ($P < 0.05$) according to a Tukey's test after arcsine transformation

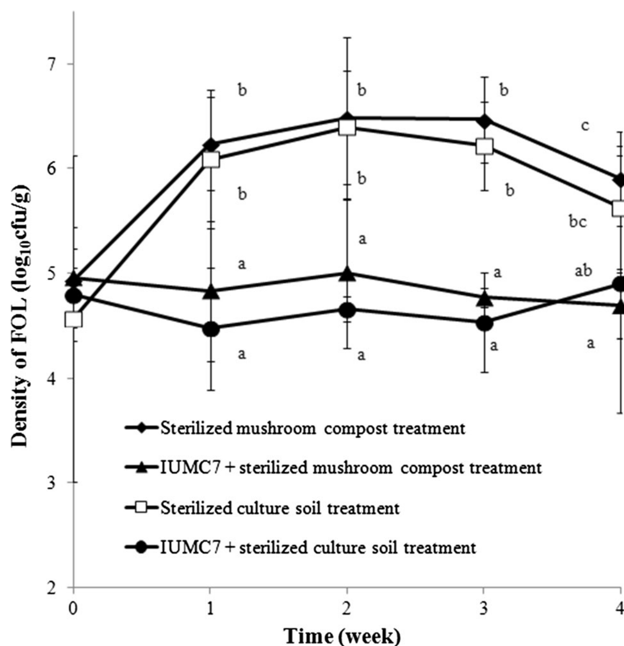

Fig. 4 Density of *Fusarium oxysporum* f. sp. *lycopersici* (FOL) in four soil treatments (IUMC7 + sterilized mushroom compost treatment, IUMC7 + sterilized culture soil treatment, sterilized mushroom compost treatment, sterilized culture soil treatment) measured weekly for 4 weeks after inoculation. Suspensions of four soil types inoculated with FOL were diluted and spread on Komada selective medium. Colonies were counted after 5 days of incubation. Values are means and the 95 % confidence interval of three independent experiments. *Different letters* indicate significant differences ($P < 0.05$) according to Tukey's test

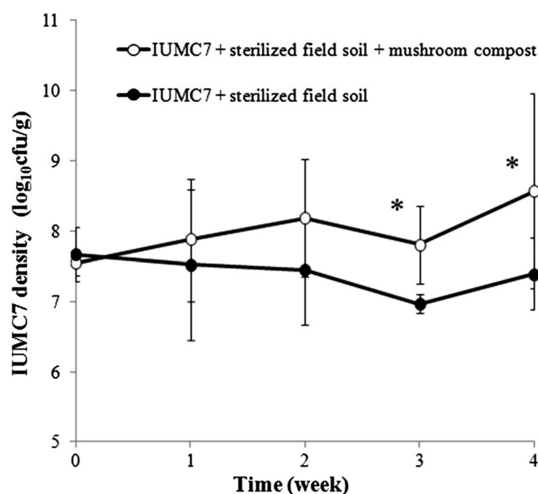

Fig. 5 Density of *Bacillus amyloliquefaciens* IUMC7 in two inoculated soil types (field soil alone, field soil + mushroom compost) after 4 weeks. Suspensions of IUMC7 at various dilutions from the two soil types inoculated with IUMC7 were spread on TSA medium, and the colonies were counted after 24 h of incubation. Values are means and the 95 % confidence interval of three independent experiments. *Significantly different at $P < 0.05$ according to Student's *t* test

pathogen, induced resistance, and direct parasitism (Handelsman and Stabb 1996). Some *Bacillus* spp. can activate plant defense, thereby suppressing diseases caused by various pathogens (Choudhary and Johri 2009). For example, Tan et al. (2013) showed that *B. amyloliquefaciens* suppresses bacterial wilt of tomato by inducing systemic resistance. In addition, Ongena et al. (2007) demonstrated that fengycin and surfactin, lipoproteins produced by *B. subtilis*, act as plant resistance elicitors. With these precedents in mind, we investigated whether systemic resistance was induced in plants by IUMC7. Tomato plants 3 weeks of age were transplanted into IUMC7 + sterilized mushroom compost treatment, sterilized mushroom compost treatment, or sterilized culture soil, then placed in a growth chamber (12 h light/12 h dark, 31 °C). One week later, total RNA was extracted from roots using the RNeasy Plant Mini Kit (Qiagen, Maryland, USA), and samples were immediately treated with DNase I. cDNA was synthesized using the PrimeScript 1st strand cDNA synthesis kit (Takara Bio, Shiga, Japan). All methods were done according to the manufacturer's protocol. The expression of several pathogenesis-related (PR) protein genes were analyzed using specific primer sets (Table 1). The primer set for the glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) gene was used as the internal control. All primer sets were designed using the Primer3-Plus program. Amplification was performed using a Bio-Rad iCycler iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and iQ SYBR Green Supermix (Bio-Rad). Quantitative RT-PCR was performed in a volume of 25 µL containing 12.5 µL iQ SYBR Green Supermix, 1 µL each primer set, 8.5 µL sterilized water and 2 µL template, which was diluted 100-fold. Each program consisted of an initial step at 95 °C for 3 min and 45 cycles of 95 °C for 5 s, 55 °C for 30 s, and 72 °C for 30 s. Settings to measure fluorescence were programmed in advance. As shown in Fig. 6, the expression of PR genes was not significantly induced by the bacterium.

Of the various biocontrol agents tested for the control of soil-borne pathogens in recent years, *B. amyloliquefaciens* has been used extensively as a biocontrol agent (Mari et al. 1996; Yu et al. 2002). Some *Bacillus* spp. synthesize numerous antimicrobial or bioactive compounds (Arguelles-Arias et al. 2009; Stein 2005), which provide effective protection (Chen et al. 2009). In this study, IUMC7 inhibited the growth of FOL in vitro, and an antimicrobial substance(s) in the supernatant of IUMC7 physically damaged the fungus. Now, this antibacterial substance(s) produced by IUMC7 needs to be isolated and characterized.

In summary, we showed that mushroom compost mixed with *B. amyloliquefaciens* IUMC7 can suppress Fusarium

Table 1 Primers used by real time RT-PCR

Gene	Supplement	Forward primer	Reverse primer
PR1b	Basic PR1	CTTGCGGTTCATAACGATGC	TAGTTTTGTGCTCGGGATGC
PR2a	Acidic glucanase	TCCCTTTTACTTGTTGGGCTTC	GGGCATTAAAGACATTTGTTTCTGG
PR2b	Basic glucanase	AGGATTACTTGTCGCCACCAAC	ATGGCAAGTTGTTCCCCATC
PR3a	Acidic chitinase	GCTGCCTTTTTCGGTCAAAC	TGCCATAGTATCTGTCTGACTGCTC
PR3b	Basic chitinase	GCCCAAACTTCCCATGAAAC	CAAGGCCATTGACTACTTGGTG
PR5	Osmotin-like PR5	TACGCCTTGGACCAGTTTAGC	GTTGGGGCAAAAGTCATTGG
gapdh	Glyceraldehyde-3-phosphate dehydrogenase	CTGTTGCTGGCTTACAAACCTC	CATGCCAGCACCTCAAGAAG

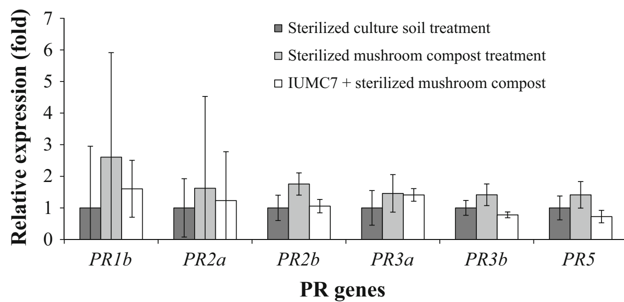

Fig. 6 Relative expression of several PR proteins from tomato roots grown in three soils (IUMC7 treatment, sterilized mushroom compost treatment, sterilized culture soil treatment) 1 week after transplanting. Values are means and 95 % confidence intervals of three independent experiments

wilt. IUMC7 has potential as a biocontrol agent, and applying it with mushroom compost will contribute to the success of biocontrol. Because low disease severity index might be mainly due to the antimicrobial compounds produced by IUMC7, we are currently investigating which substance(s) produced by *B. amyloliquefaciens* is responsible for disease suppression to further elucidate the mechanism of action.

References

Arguelles-Arias A, Ongena M, Halimi B, Lara Y, Brans A, Joris B, Fickers P (2009) *Bacillus amyloliquefaciens* GA1 as a source of potent antibiotics and other secondary metabolites for biocontrol of plant pathogens. Microb Cell Fact 8:63

Bailey KL, Lazarovits G (2003) Suppressing soil-borne diseases with residue management and organic amendments. Soil Till Res 72:169–180

Cao Y, Zhang Z, Ling N, Yuan Y, Zheng X, Shen B, Shen Q (2011) *Bacillus subtilis* SQR9 can control *Fusarium* wilt in cucumber by colonizing plant roots. Biol Fertil Soils 47:495–506

Chen XH, Koumoutsi A, Scholz R, Schneider K, Vater J, Süssmuth R, Piel J, Borriss R (2009) Genome analysis of *Bacillus amyloliquefaciens* FZB42 reveals its potential for biocontrol of plant pathogens. J Biotechnol 140:27–37

Choudhary DK, Johri BN (2009) Interactions of *Bacillus* spp. and plants—with special reference to induced systemic resistance (ISR). Microbiol Res 164:493–513

Gamliel A, Austerweil M, Kritzman G (2000) Non-chemical approach to soilborne pest management—organic amendments. Crop Prot 19:847–853

Handelsman J, Stabb EV (1996) Biocontrol of soilborne plant pathogens. Plant Cell 8:1855–1869

Komada H (1975) Development of a selective medium for quantitative isolation of *Fusarium oxysporum* from natural soil. Rev Plant Prot Res 8:114–125

Manikandan R, Saravanakumar D, Rajendran L, Raguchander T, Samiyappan R (2010) Standardization of liquid formulation of *Pseudomonas fluorescens* Pf1 for its efficacy against *Fusarium* wilt of tomato. Biol Control 54:83–89

Mari M, Guizzardi M, Brunelli M, Folchi A (1996) Postharvest biological control of grey mould (*Botrytis cinerea* Pers.: Fr.) on fresh-market tomatoes with *Bacillus amyloliquefaciens*. Crop Prot 15:699–705

Ongena M, Jourdan E, Adam A, Paquot M, Brans A, Joris B, Arpigny JL, Thonart P (2007) Surfactin and fengycin lipopeptides of *Bacillus subtilis* as elicitors of induced systemic resistance in plants. Environ Microbiol 9:1084–1090

Sotoyama K, Kawamura I, Nakajima M, Akutsu K (2013) Antimicrobial substances produced by *Bacillus* sp. isolated from mushroom compost (abstract in Japanese). Jpn J Phytopathol 79:30

Stein T (2005) *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. Mol Microbiol 56:845–857

Tan S, Dong Y, Liao H, Huang J, Song S, Xu Y, Shen Q (2013) Antagonistic bacterium *Bacillus amyloliquefaciens* induces resistance and controls the bacterial wilt of tomato. Pest Manag Sci 69:1245–1252

Trillas MI, Casanova E, Cotxarrera L, Ordovás J, Borrero C, Avilés M (2006) Composts from agricultural waste and the *Trichoderma asperellum* strain T-34 suppress *Rhizoctonia solani* in cucumber seedlings. Biol Control 39:32–38

Xue QY, Chen Y, Li SM, Chen LF, Ding GC, Guo DW, Guo JH (2009) Evaluation of the strains of *Acinetobacter* and *Enterobacter* as potential biocontrol agents against Ralstonia wilt of tomato. Biol Control 48:252–258

Yu GY, Sinclair JB, Hartman GL, Bertagnolli BL (2002) Production of iturin A by *Bacillus amyloliquefaciens* suppressing *Rhizoctonia solani*. Soil Biol Biochem 34:955–963