

Influence of Commercial Antibiotics on Biocontrol of Soft Rot and Plant Growth Promotion in Chinese Cabbages by *Bacillus vallismortis* EXTN-1 and BS07M

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We investigated influence of three commercial antibiotics *viz.*, oxolinic acid, streptomycin, and validamycin A, on biocontrol and plant growth promoting activities of *Bacillus vallismortis* EXTN-1 and BS07M in Chinese cabbage. Plants were pre-drenched with these strains followed by antibiotics application at recommended and ten-fold diluted concentration to test the effect on biocontrol ability against soft rot caused by *Pectobacterium carotovorum* SCC1. The viability of the two biocontrol strains and bacterial pathogen SCC1 was significantly reduced by oxolinic acid and streptomycin *in vitro* assay, but not by validamycin A. In plant trials, strains EXTN-1 and BS07M controlled soft rot in Chinese cabbage, and there was a significant difference in disease severity when the antibiotics were applied to the plants drenched with the two biocontrol agents. Additional foliar applications of oxolinic acid and streptomycin reduced the disease irrespective of pre-drench treatment of the PGPRs. However, when the plants were pre-drenched with EXTN-1 followed by spray of validamycin A at recommended concentration, soft rot significantly reduced compared to untreated control. Similarly, strains EXTN-1 and BS07M significantly enhanced plant growth, but it did not show synergistic effect with additional spray of antibiotics. Populations of the EXTN-1 or BS07M in the rhizosphere of plants sprayed with antibiotics were significantly affected as compared to control. Taken together, our results suggest that the three antibiotics used for soft rot control in Chinese cabbage could affect bacterial mediated biocontrol and plant growth promoting activities. Therefore, combined treatment of the PGPRs and the commercial antibiotics should be carefully applied to sustain environmental friendly disease management.

Keywords : Antibiotics, Biocontrol, Chinese cabbage, Plant growth promotion, Soft rot

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Introduction

Chinese cabbage (*Brassica rapa*) is an important crop belonging to *Brassicaceae* family. However, it is prone to disease such as soft rot caused by *Pectobacterium carotovorum* which lead to serious loss in the plant production (Ren *et al.* 2001). *P. carotovorum* has high pathogenicity, survival ability in plant debris in soils, and broad host range which makes it difficult to control in Chinese cabbage. Therefore, various disease management approaches have been developed to control the disease

(Ren *et al.*, 2001; Vanjildorj *et al.*, 2009). Moreover, cultural practices to suppress the disease are time-consuming and laborious. Recently, genetically modified soft rot resistant crops have been developed but these are yet to attain commercial success. Nowadays, chemicals including commercial antibiotics such as oxolinic acid, streptomycin, and validamycin A are available for control of soft rot (Ishikawa *et al.*, 2004; Kwon *et al.*, 2009; Patricia *et al.*, 2002). Although synthetic chemical application immediately reduce diseases, continuous and excessive use of the chemicals can lead to development of antibiotic resistant pathogens besides having hazardous effect on environment. Streptomycin-resistant strains have emerged in some bacterial pathogens including *Pectobacterium* (Fukusawa *et al.*, 1980), *Pseudomonas* (Huang and Burr, 1999) and *Xanthomonas* (Hyun

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et al., 2012). Therefore, biological control by microorganisms which are also capable of plant growth-promotion, has been studied as one of viable and safe alternatives to reduce disease and increase productivity (Gerhardson, 2002; Whipps, 2001). However, inconsistent result under field condition is a serious concern and therefore, integrated disease management was proposed to compensate the deficiency of PGPR and reduce chemical usage by combined application (Jacobsen et al., 2004; Obradovic et al., 2005).

From our previous study, we selected two biocontrol and plant growth promoting agents, *Bacillus vallismortis* EXTN-1 and BS07M for the present work. EXTN-1 was effective against wilt by *Ralstonia solanacearum* and *Fusarium oxysporum* on tomato and potato, foot rot by *Phytophthora capsici* on black pepper and multiple diseases in other various crops such as rice, tobacco, potato, and cucumber (Park et al., 2006a,b, 2007; Thanh et al., 2009). Mutant strain BS07M was derived from strain BS07, which was capable to protect chili pepper from three pathogens viz., *P. carotovorum* (SCC1), *P. capsici*, and *Colletotrichum acutatum* by induced systemic resistance besides promoting plant growth under field condition (Park et al., 2013).

For integrated biocontrol of soft rot in Chinese cabbage, we examined the possibility of antibiotic application in plants pre-drenched with biocontrol agents (EXTN-1 and BS07M). For this, we studied (i) viability of the two biocontrol agents and the bacterial pathogen, *P. carotovorum* SCC1 grown in commercial antibiotics (oxolinic acid, streptomycin, and validamycin A) (ii) effect of antibiotics on disease suppression and plant growth promoting effects of EXTN-1 and BS07M in pre-drenched plants, and (iii) alteration in rhizosphere population of biocontrol agents by fungicidal spray.

Materials and Methods

Preparation of bacterial strains and antibiotics. Two *B. vallismortis* EXTN-1 and BS07M were used as biocontrol agents against *P. carotovorum* SCC1 in Chinese cabbage (*Brassica rapa* L) cv. Jangmi (Nong Woo Bio Co., Ltd., Korea). Three antibiotics against soft rot in Chinese cabbage viz., oxolinic acid (Ilpum[®], Dongbang Agro, Korea), streptomycin (Agrepto[®], GyungNong, Korea), and validamycin A (Hanwoomul[®], Syngenta Korea, Korea) were studied (Table 1). The antibiotics were prepared at

concentrations recommended by manufacturer (1x); 100 µg a.i./ml for validamycin A, 250 µg a.i./ml for streptomycin, and 0.2 µl a.i./ml for oxolinic acid, and a ten-fold dilution (1/10x) in sterile distilled water. The antibiotics solutions were filtered through 0.2 µm micropore-filter (ADVANTEC, Toyo Roshi Kaisha, Ltd., Japan) for *in vitro* assay.

Effect of three antibiotics on viability of biocontrol agents.

All bacterial strains were cultured on tryptic soy agar (TSA, Difco, USA) and single colony was incubated in 5 ml TSB at 20°C, 150 rpm for 24 h. Bacterial suspensions were centrifuged at 6,000 rpm for 5 min and adjusted to 10⁸ cfu/ml in 10 mM MgSO₄ solution. Antibiotics were prepared at two concentrations as mentioned above. The bacterial suspensions were added to sterile TSB as 10% (v/v), and equal volume (500 µl) of the TSB containing bacterial suspension were mixed with filtered antibiotic solutions in 24 well plates (Costar, Corning, NY, USA). After incubation at 20°C, 100 rpm for 48 h, the mixtures were serially diluted, and plated on TSA. Colony forming unit (cfu) was counted.

Plant trials.

For disease suppression, Chinese cabbage were grown for three weeks in 50-hole tray filled with TKS2 (Floragard, Oldenburg, Germany) containing 10% vermiculite. The bacterial suspension of EXTN-1 and BS07M (10⁸ cfu/ml, 1 ml/1 g of potting mixture), BTH (0.1 mM), and 10 mM MgSO₄ solution (control) were pre-drenched in plants and five days later antibiotics were sprayed at two concentrations (1x and 1/10x) by atomizers. Two days after foliar application, leaf discs (diameter 14 mm) were collected, and wounded by a needle on the centre. The leaf discs were drop-inoculated by SCC1 (20 µl of 10⁸ cfu/ml) on the wounding, and disease severity (%) was assessed (Park et al., 2013). To study the effect on growth promoting ability, Chinese cabbages were grown in pots (diameter 90 mm) for three weeks. Pre-drench treatment and foliar antibiotic application were done as mentioned above. After two weeks of drench treatments, shoots of Chinese cabbage were weight for plant growth evaluation.

Bacterial population of spontaneous rifampicin-resistant mutant strains.

For population test of the EXTN-1 and BS07M, spontaneous rifampicin-resistant mutants were prepared by

Table 1. Commercial antibiotics used in this study

Item name	Formulation type	Active ingredient	Concentrations recommended by manufacturer
Oxolinic acid	Wettable powder	Oxolinic acid 20% surfactant, supplement agent, extender 80%	0.2 µl a.i./ml
Streptomycin	Wettable powder	Streptomycin 20% extender 80%	250 µg a.i./ml
Validamycin A	Water soluble powder	Validamycin A 10% surfactant, coloring, supplement agent, extender 90%	100 µg a.i./ml

Table 2. Effect of three antibiotics on viability of biocontrol agents, *B. vallismortis* EXTN-1 and BS07M and bacterial pathogen *P. carotovorum* SCC1

Antibiotics/ level of concentration	Cell viability [log (CFU/ml+ 1)]		
	EXTN-1	BS07M	<i>P. carotovorum</i> SCC1
TSB	12.14±0.26	13.56±0.05	13.94±0.04
Oxolinic acid			
1/10x	1.14±0.43 a*	3.08±0.35 a*	2.38±0.64 a*
1x	0.00±0.00 b*	1.99±0.75 a*	1.68±0.64 a*
Streptomycin			
1/10x	0.00±0.00a*	2.90±1.10 a*	5.73±0.29 a*
1x	0.00±0.00a*	1.25±0.48 a*	0.00±0.00 b*
Validamycin A			
1/10x	12.86±0.26 a	13.52±0.10 a	13.82±0.07 a
1x	12.73±0.26 a	13.59±0.07 a	13.87±0.05 a

^aMeans ± standard errors followed by different letters are significantly different between concentration in each antibiotic according to the least significant difference (LSD) test at $P < 0.05$. An asterisk indicated difference between each antibiotic treatment and TSB at $P < 0.05$. Each value is the mean of eight replications from repeated experiments.

culturing the strains in TSA amended with rifampicin (100 µg/ml) (TSAR) (Sang and Kim, 2012). Plants and bacterial were prepared and treated as described above; and rhizosphere soils (1g) were sampled at 7 and 14 days after drench treatment, and the CFU were determined on TSAR.

Statistical analysis. Data analyses were conducted using the Statistical Analysis System software (SAS Institute, Cary, NC). Experiments were conducted twice with four replicates each for the assays of bacterial viability, population in rhizosphere, and plant growth promotion, five replicates for disease severity. Pooled data from repeated experiments were used after confirming the homogeneity of the variances using the Levene's test (Levene, 1960). Percentage data for disease severity was statistically analyzed after arcsine-root transformation. Analysis of variance was performed using the general linear model procedure and means were separated by the least significant difference test at $P < 0.05$.

Results and Discussion

In vitro assay for effect of antibiotics on bacterial viability,

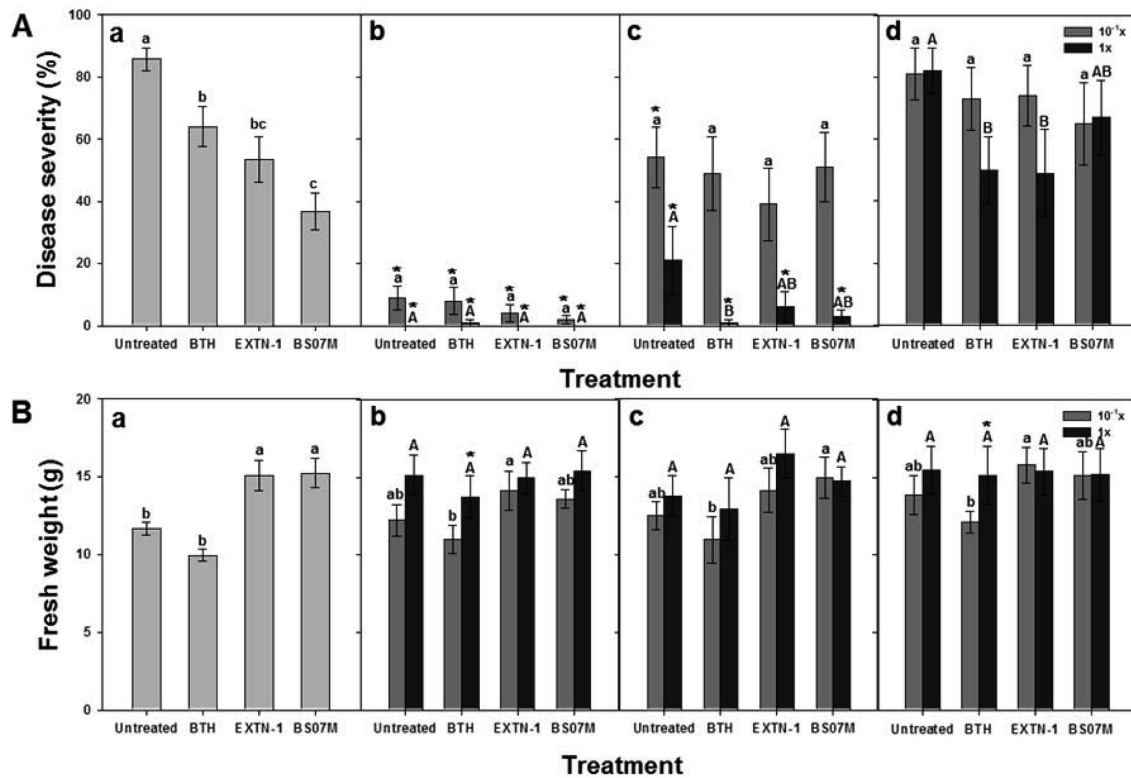


Fig. 1. Disease severity (%) caused by *P. carotovorum* SCC1 (A) and plant growth-promoting (B) on Chinese cabbage pre-drenched with two biocontrol agents, *B. vallismortis* EXTN-1 and BS07M, BTH (0.1 mM), and untreated (10 mM MgSO₄ solution). Five days after drench treatment, distilled water (a), oxolinic acid (b), streptomycin (c), and validamycin A (d) were sprayed at rates of 1/10x (ten-fold diluted) and 1x (recommended by supplier). The bars indicate the mean of 10 replicates for disease severity and eight for plant growth promotion from repeated experiments. The vertical bars show the standard error. Different letters on the bars indicate significant difference among treatments by the LSD test at $P < 0.05$. An asterisk indicates significant difference among antibiotics at a given drench-treatment at $P < 0.05$. Arcsine square root-transformed data for disease severity was used for statistical analysis; however, untransformed data are presented.

Table 3. Bacterial population of spontaneous rifampicin-resistant mutant strains (EXTN-1Rif and BS07MRif) in the potting mixture grown Chinese cabbage sprayed with water and three antibiotics

Antibiotics/ level of concentration	Bacterial population [log (CFU/ g of potting mixture)]			
	EXTN-1Rif		BS07MRif	
	7 DAT ^a	14 DAT	7 DAT	14 DAT
Water	6.73±0.11	6.00±0.12	6.87±0.05	5.97±0.12
Oxolinic acid				
1/10x	6.42±0.14 a ^b	5.86±0.14 a	6.49±0.09 a*	5.84±0.11 a
1x	6.19±0.21 a*	5.70±0.15 a	6.54±0.21 a*	5.37±0.08 b*
Streptomycin				
1/10x	6.46±0.26 a	5.83±0.09 a	6.45±0.18 a*	5.51±0.07 a*
1x	6.65±0.10 a	5.60±0.11 a	6.16±0.08 a*	5.26±0.09 b*
Validamycin A				
1/10x	6.47±0.14 a	5.65±0.10 a	6.63±0.11 a*	5.80±0.07 a
1x	6.10±0.16 a*	5.74±0.21 a	6.64±0.07 a*	5.74±0.09 a

^aDAT=days after treatment.

^bMeans ± standard errors followed by different letters are significantly different between concentration in each antibiotic according to the LSD test at $P < 0.05$. An asterisk indicated difference between each antibiotic treatment and water at $P < 0.05$. Each value is the mean of eight replications from repeated experiments.

oxolinic acid and streptomycin significantly ($P < 0.05$) reduced the number of viable cells of EXTN-1 and BS07M at both recommended (1x) and diluted (1/10x) concentrations compared to control (TSB). However, validamycin A did not inhibit the two bacterial strains (Table 2). Similar results were observed for the bacterial pathogen, *P. carotovorum* SCC1 (Table 2).

Under greenhouse condition, influence of three antibiotics on disease severity and growth in Chinese cabbage pre-drenched with EXTN-1 and BS07M were tested. Plants pre-drenched with BS07M, EXTN-1 and BTH, significantly ($P < 0.05$) reduced soft rot (Fig. 1A-a). However, the disease suppressive effect of the biocontrol strains did not show when oxolinic acid and streptomycin were sprayed at both recommended and diluted concentrations (Fig. 1A-b,c). Pre-drench treatments of BTH and EXTN-1 reduced the disease compared to untreated control when validamycin A was sprayed at recommended concentration, but it did not have a similar effect at diluted concentration (Fig. 1A-d). Similarly, strain EXTN-1 and BS07M significantly ($P < 0.05$) increased fresh weight of shoot in Chinese cabbage (Fig. 1B-a). However, plant growth promoting-effect of EXTN-1 and BS07M was not observed when the antibiotics were sprayed (Fig. 1B-b,c,d).

For changes in the bacterial population in rhizosphere of Chinese cabbage pre-drenched with EXTN-1 and BS07M after the fungicidal spray, spontaneous rifampicin resistant mutants were tested. Population of EXTN-1Rif was significantly ($P < 0.05$) affected when oxolinic acid and validamycin A were sprayed at a rate of recommended concentration at 7 days after treatment (DAT) as compared to water spray; and there were no significant differences between diluted and recommended concentration at 7 and 14 DAT (Table 3). Population of BS07M was also

affected by antibiotic applications at 7 DAT compared to water spray (Table 3). At 14 DAT, the population was lower at recommended spray of oxolinic acid and streptomycin compared to at diluted spray, however, it was not altered by validamycin A treatment at 14 DAT compare to water (Table 3).

Our results showed that oxolinic acid and streptomycin affected biocontrol agents (EXTN-1 and BS07M), and the bacterial pathogen (SCC1) (Table 2). These two antibiotics were developed as bacteriocides which work through inhibition of DNA gyrases; synthesis and cleavage of DNA; and protein synthesis (Smith, 1962; Snyder and Drlica, 1979). These have been used to control various bacterial diseases including soft rot in Chinese cabbages; bacterial spot in red pepper, bacterial grain rot (Kwon *et al.*, 2009). The mode of action of oxolinic acid and streptomycin could directly affect the viability and proliferation of any bacteria whether beneficial or pathogenic. However, validamycin A worked like glucosidase and trehalose inhibitor in plants which led to prevent infection by pathogenic fungus and bacteria (Sisler, 1986; Want *et al.*, 2013).

Our earlier studies indicated EXTN-1 and BS07 as potential biocontrol agents inducing resistance against various fungal and bacterial disease besides enhancing plant growth in various crops (Part *et al.*, 2006a,b, 2007; Park *et al.*, 2013; Thanh *et al.*, 2009). In this study, strains EXTN-1 and BS07M could reduce soft rot on leaves by induced resistance and increase plant fresh weight, however, the effects of the strains were not remarkable compared to untreated control when the tested antibiotics (oxolinic acid, streptomycin, and validamycin A). Foliar applications of oxolinic acid and streptomycin could reduce the disease at a low level to counteract the biocontrol activities of the strains. Similar report was presented by Ahemad and Kahn

(2012) that application of some antibiotics produced concentration-dependent inhibition of plant growth promoting activities of phosphate solubilizing *Pseudomonas putida*. They suggested the application of antibiotic-tolerant PGPR strains as prerequisite so as not to suppress the PGPR activity on treatment with antibiotics.

The population of rifampicin resistant EXTN-1Rif and BS07MR-1 were altered by foliar application of tested antibiotics suggesting that the antibiotic treatments might change certain physiological aspects of the plant including root exudation which might lead to this alteration. The importance of root exudates in plant-plant and plant-microbe interaction in the rhizosphere has been highlighted in earlier works (Bais *et al.*, 2006; Dutta and Podile, 2010). Smith *et al.* (2000) reported potential non-target effects of antibiotic benomyl on changes in soil organisms and nutrient availability. Yang *et al.* (2012) suggested influences of foliar antibiotic application on the composition of rhizobacterial communities.

The results of the present study indicated that disease suppressing and plant growth promoting activities of EXTN-1 and BS07M in pre-drenched Chinese cabbage could be affected by tested antibiotics such as oxolinic acid and streptomycin. Therefore, these foliar antibiotics need to be carefully reconsidered before application to maintain or improve the biological capacities of native or applied biocontrol agents in the rhizosphere.

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