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Antagonism of black rot in cabbage by mixtures of plant growth-promoting rhizobacteria (PGPR)

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Abstract Black rot, caused by *Xanthomonas campestris* pv. *campestris* (Xcc) (Xanthomonadales: Xanthomonadaceae), is the most important and potentially destructive disease of cabbage. Twenty-three plant growth-promoting rhizobacteria (PGPR) strains were tested for antibiosis against Xcc in vitro. Seven strains with antibiosis activity significantly reduced disease in the greenhouse. Two mixtures of PGPR strains and four individual strains were then tested three times in the greenhouse and one time in the field. In the greenhouse test, all treatments resulted in significant disease suppression. Mixture-2 and strain AP218 caused the highest and most consistent disease reduction in two of the three trials. In the field test, both mixtures and two individual strains significantly reduced disease incidence and disease severity. In conclusion, mixture-2 exhibited consistent biocontrol of black rot of cabbage.

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Introduction

Cruciferous crops, including broccoli, cabbage, cauliflower, turnip, and collards, are important vegetable crops and widely grown in the United States. The market value of cabbage has an estimated annual value of over $230 million in the United States (NASS/USDA 2015). Black rot, caused by *Xanthomonas campestris* pv. *campestris* (Xcc), is the most important disease of cabbage and occurs in most crucifer growing regions in the world, including North America, Australia, Europe, Asia, and Africa (Alvarez 2000; Vicente and Holub 2013).

Xanthomonas campestris pv. *campestris* can infect most cruciferous crops at any growth stage and cause significant yield loss when warm, humid conditions follow periods of rainy weather during early crop development (Akhtar 1989). Although symptoms vary depending on the host, plant age and environmental conditions, the most characteristic symptoms are yellow to yellow-orange "V"- shaped or "U"- shaped chlorotic and necrotic lesions beginning at the margin of the leaf (Akhtar 1989). Black rot can arise from infected seeds and is spread by splashing rain or sprinkler irrigation. The bacteria enter plants through

hydathodes and wounds (Hugouvieux et al. 1998). As the disease progresses, the midrib of the leaf turns black, and the vein becomes darkened. The disease becomes systemic in the plant when the pathogen enters the stem. Infected plants are stunted, and heads deteriorate rapidly after harvest (Schaad and Alvarez 1993).

A multitude of disease management approaches have been evaluated against Xcc, each with some success, including cultural, physical, chemical, and biological approaches. Cultural control of black rot includes rotating cabbage with plants from other families that are not hosts of black rot, growing plants in fields that have not been in cruciferous crops for at least two years, and draining and drying fields (Mew and Natural 1993). Treating the seed with hot water (50 °C for 25 min) as a physical treatment can significantly reduce bacterial populations on seeds (Nega et al. 2003). Copper-containing fungicides can also inhibit this disease (Krauthausen et al. 2011). However, copper resistance to black rot was first identified at 1972 in a Japanese cabbage cultivar (Early Fuji) (Williams et al. 1972).

Among all the disease management options for black rot, biological control is attractive given the public concern about the environment. Biological control of plant disease by plant growth-promoting rhizobacteria (PGPR) is a promising strategy for plant protection (Saharan and Nehra 2011). Management of black rot on cruciferous crops by antagonistic PGPR strains has been demonstrated in several studies. Mishra and Arora (2012b) reported that *Pseudomonas fluorescens* strain To7 produced 2, 4-diacetylphloroglucinol and managed black rot in cabbage. Wulff et al. (2002) demonstrated that *Bacillus subtilis* strain BB inhibited three strains of Xcc on four *Brassica* crops (cabbage, cauliflower, rape and broccoli) in different types of soil.

Some studies on different plant diseases have shown that mixtures of PGPR strains can more effectively reduce disease than single PGPR strains due to synergistic modes of action (Raupach and Kloepper 1998; Jetiyanon and Kloepper 2002). For example mixtures of several PGPR strains may result in a more stable rhizosphere community, provide several mechanisms of control, and lead to broad-spectrum biocontrol activity on different hosts under diverse field conditions (Jetiyanon et al. 2003; Domenech et al. 2006). However, mixtures of PGPR strains have not been evaluated for biocontrol of black rot of crucifers. The aims of this study were to select individual PGPR strains for suppressing black rot on cabbage through antagonistic activity, and to investigate whether mixtures of PGPR strains could improve the consistency and level of disease reduction and plant growth in the greenhouse and field compared to individual PGPR strains.

Materials and methods

PGPR strains and inoculum preparation

In this research, 23 PGPR strains were obtained from the PGPR lab in the Department of Entomology and Plant Pathology, Auburn University, USA (Table 1). Strain AP218 (1PN-19) inhibited *Phytophthora capasici* in vitro (Zhang et al. 2010), and strains AP218, AP219 and AP295 were active against multiple aquatic pathogens in vitro (Ran et al. 2012). Strains AP136, AP188, AP218, AP219, and AP295 were included in some PGPR blends which increased root and shoot growth of Tifway hybrid Bermudagrass (Murphey Coy et al. 2014). The left strains showed plant growth promotion or biological control (unpublished data). The bacteria were maintained in tryptic soy broth (TSB) (Difco Laboratories, Detroit, MI, USA), supplemented with 30 % glycerol at −80 °C. For in vitro tests, inoculum of PGPR was grown on tryptic soy agar (TSA) at 28 °C for 48 h. A single colony was incubated in 25 ml TSB with continuous shaking (150 rpm) at 28 °C for 48 h. Bacterial cultures were centrifuged at 3500 rpm for 15 min. Pellets were resuspended in sterilized water, and the concentration was adjusted to 10^8 CFU ml^{-1}. For *in planta* tests, these strains were used as spore suspensions at 10^7 CFU ml^{-1} (Zhang et al. 2010).

X. campestris pv. *campestris* inoculum preparation

X. campestris pv. *campestris* strain OHS-001B-92 was provided by J. Olive, Ornamental Horticulture Research Center, Mobile, Alabama, USA, and stored under the conditions described above. For experimental use, Xcc was grown on Yeast Dextrose Calcium Carbonate Agar plate at 28 °C for 72 h (Schaad and Alvarez 1993). Bacterial cultures were prepared as described above, and the concentration was adjusted to 10^8 CFU ml^{-1} for challenge inoculation.

Table 1 In vitro antagonistic activity between individual PGPR and Xcc

Treatment	ID	Isolation source	Inhibition zone (mm)[a]
AP7	*Bacillus safensis*	Cucumber	0.00 ± 0.00^{f}
AP18	*Bacillus safensis*	Cucumber	0.00 ± 0.00^{f}
AP136	*Bacillus velezensis*	Cotton	11.67 ± 0.88^{a}
AP188	*Bacillus velezensis*	Cotton	1.00 ± 0.00^{f}
AP194	*Bacillus velezensis*	Cotton	9.00 ± 0.58^{cd}
AP195	*Bacillus velezensis*	Cotton	11.00 ± 0.58^{ab}
AP197	*Bacillus velezensis*	Cotton	7.33 ± 1.45^{ed}
AP199	*Bacillus velezensis*	Cotton	11.33 ± 0.67^{ab}
AP200	*Bacillus velezensis*	Cotton	11.33 ± 0.33^{ab}
AP201	*Bacillus velezensis*	Cotton	10.67 ± 0.67^{abc}
AP203	*Bacillus velezensis*	Cotton	10.00 ± 1.15^{abc}
AP208	*Bacillus velezensis*	Cotton	10.67 ± 0.88^{abc}
AP209	*Bacillus mojavensis*	Cotton	0.00 ± 0.00^{f}
AP210	*Bacillus velezensis*	Cotton	11.00 ± 0.58^{ab}
AP212	*Bacillus velezensis*	Cotton	10.00 ± 0.00^{abc}
AP213	*Bacillus velezensis*	Cotton	10.67 ± 0.67^{abc}
AP214	*Bacillus velezensis*	Cotton	9.67 ± 0.33^{bc}
AP217	*Fictibacillus solisalsi*	Cucumber	0.00 ± 0.00^{f}
AP218	*Bacillus velezensis*	Cucumber	10.67 ± 1.20^{abc}
AP219	*Bacillus velezensis*	Cucumber	6.00 ± 0.58^{e}
AP282	*Lysinibacillus macroides*	Cucumber	0.00 ± 0.00^{f}
AP295	*Bacillus velezensis*	Red pepper	9.00 ± 0.58^{cd}
AP305	*Bacillus velezensis*	Douglas-fir	10.67 ± 0.88^{abc}
Control			0.00 ± 0.00^{f}

The inhibition zone was measured from the edge of PGPR strain to the pathogen

Values (mean ± SE) in the same column followed by the same letter do not significantly different at $P \leq 0.05$ according to Fisher's protected LSD

Inhibition zone ($F_{23,46} = 52.17$, $P < 0.0001$)

PGPR antagonistic activity to *X. campestris* pv. *campestris*

The antagonism assay was a modified antibiosis technique in which different types of agar were used for the PGPR and the challenged pathogen. Three holes of 13 mm diameter were made into water agar plates, and these were filled with melted TSA. Ten µl PGPR suspension was applied to each TSA disc. One ml of Xcc suspension was mixed with 50 ml soft agar (0.4 % Agar in 50 % TSB) and cooled to 37 °C. After gently swirling, 7 ml of the resulting suspension was added to each plate. Each plate contained two PGPR strains and a water control placed at approximately equal distances from each other at the edge of the plate. Plates were incubated for two days at 28 °C. The inhibition zone was measured from the edge of the PGPR strain to the pathogen. Each treatment was tested three times.

Preliminary screening in the greenhouse

Kaboko hybrid organic Chinese cabbage (*Brassica rapa*) (Park Seed, Hodges, SC 29653) was used. Cabbage seeds were planted in germination trays containing 25 cm^3 holes, grown for two weeks, and then transplanted into 10 cm diameter round pots filled with commercial potting substrate (Sunshine mix, Sun Gro Horticulture, Agawam, MA 01001, UK). Seeds were placed in a temperature-controlled greenhouse at the Plant Science Research Center at Auburn University, Alabama, USA. Ambient air temperature in the greenhouse was maintained at 25 °C day/21 °C night throughout the year. Two weeks after transplanting, plants were sprayed with PGPR suspension (10^7 CFU ml^{-1}). PGPR-inoculated plants were placed into a dark dew chamber (100 % humidity) for two days at 24 °C, and were transferred to the greenhouse. Three days after spraying with PGPR, plants were

challenge-inoculated with Xcc by spraying the whole plant. Pathogen-inoculated plants were placed into the same dew chamber for two days, and then placed in the greenhouse. Pots were watered daily as needed.

Fourteen days after pathogen challenge, total lesion number (TLN) was recorded for each plant. Five leaves from the bottom of each plant were chosen for evaluating the disease index. The disease index for black rot was scored according to a 0–5 rating scale, where 0 = leaves which appeared healthy with no symptoms, 1 = slightly chlorotic at the margins of leaves, 2 = chlorotic at margins—chlorotic blotches at the margin of leaves, 3 = chlorosis progressed toward the midrib of the leaf, 4 = leaves showing extreme chlorotic progression with V-shaped blotches with some one-sided growth and with rapid lower leaf droop, and 5 = brown leaf with some extreme blackening on the vein (Brown et al. 2001). Plants were harvested at the same time and the following plant parameters were measured: plant diameter, shoot fresh weight, shoot dry weight (oven dry at 90 °C), root fresh weight (RFW), and root dry weight.

The experimental design was a randomized complete block with ten treatments and eight replications in each treatment. Treatments included eight PGPR strains (AP136, AP201, AP213, AP214, AP218, AP219, AP295, and AP305), and two controls (a nonbacterized but pathogen-challenged disease control, and a nontreated control).

Advanced tests of selected individual strains and mixtures in the greenhouse and field

Four individual PGPR strains (AP218, AP219, AP295, and AP305) selected in the preliminary greenhouse screening were tested again. These four strains had shown the best antagonistic capacity and were mixed together as mixture-1. Mixture-2 contained the same four strains plus strain AP213 that showed growth promotion. A total of seven treatments were used: four treatments consisting of the single PGPR strains (AP218, AP219, AP295 and AP305), two treatments consisting of strain mixtures (Mixture-1 and Mixture-2), and one control (a nonbacterized but pathogen-challenged disease control). The compatibility of mixed strains was determined by using TSA plates. Bacterial strains were streaked at a 90° angle from each other. The plates were incubated at 28 °C for 48 h and observed for the inhibition zone. Absence of inhibition zone indicated compatibility of the tested strains, and the presence of inhibition zones indicated incompatibility. The mixture of PGPR strains were prepared by combining equal proportions of each strain prior to application to the seed. Methods were the same as previously described, and the biocontrol activity was quantified by the TLN. The experiment was conducted three times. There were six replications in trail 1, seven replications in trail 2, and ten replications in trail 3.

The same treatments were tested once in a field trial conducted at E.V. Smith Research Center, Shorter, AL, USA (32.45 N, 85.88 W). The soil type was sandy loam. The total amount of rainfall during this experiment was 116 mm. The maximum average temperature ranged from 17.2 to 24.4 °C, while the minimum varied from 2.1 to 10.2 °C. Seedlings were transplanted into the field after growing for six weeks in the greenhouse. Two weeks after transplanting, PGPR suspensions (10^7 CFU ml^{-1}) were sprayed on the whole plant. Three days after inoculation with the PGPR, the whole plant was sprayed with the pathogen (10^8 CFU ml^{-1}). The inoculations of PGPR and pathogen were done in the late afternoon to prevent rapid drying. The experimental design was a randomized complete block, and each treatment had six plots. Each plot contained ten plants (two rows of five plants each), and the distance between rows was 60 cm and within rows was 45 cm.

The evaluation was carried out on experimental plots. The biological control effect was quantified by recording disease incidence at three and ten weeks after transplanting, and assessing the external, head surface, and IBR index 71 days after transplanting. For scoring the external black rot index (EBR index), leaves not forming part of the head were examined for black rot symptoms and assessed as follows (Wulff et al. 2002).

$$\text{EBR index} = \frac{0a + 1b + 2c + 3d + 4e}{T} \quad (1)$$

where 0, 1, 2, 3 and 4 indicate respectively none, >0–10, 11–20, 21–30 and >30 % of the surface of a leaf showing black rot symptoms; a–e correspond to the number of leaves in the infection category; T is the total number of external leaves. For recording the head index (HBR index), the surface of the whole head was checked as follows: 0 = no symptom on the head surface; 1 = the symptoms only on the surface; 2 = symptoms on the surface and inside of leaf; 3 = symptoms on the surface and inside of leaf, and form V-shape lesion;

4 = symptoms on the surface and inside of leaf, form V-shape lesion and veil discoloration. For assessing the IBR index, cabbage heads were cut perpendicularly into quarters and the internal symptoms were assessed as follows: 0 = no discoloration, no symptoms on the heart leaves (healthy plants); 1 = vein discoloration extends <1/2 of the stem, no symptoms on the heart leaves; 2 = vein discoloration extends >1/2 of the stem, no symptoms on the heart leaves; 3 = vein discoloration of stem and on 1–3 of the heart leaves and 4 = vein discoloration of stem and on more than three heart leaves (Wulff et al. 2002). For black rot symptoms on the head (HBR index) and IBR index, a–e correspond to the number of plants in the infection category; T is the total number of plants.

Three measurements of yield were conducted. Whole plants were first weighed to assess whole plant yield. After removing the outer leaves that cover the marketable head, weights were recorded as the head yield. The marketable yield was recorded after removing any outer leaves with symptoms.

Statistical analysis

Before statistical analysis, data were evaluated for normality, homoscedasticity, and residual with Kolmogorov–Smirnov test, Levene test, and residual plots respectively. Root fresh weight data in the preliminary screening [log(x + 1)] and disease incidence data at three weeks after transplanting in the field [arcsin ($\sqrt{x}$)] were transformed to satisfy the normality assumption. The left values were analyzed using a normal distribution. Advanced tests in the greenhouse were conducted three times. Since significant differences between the repeated experimental trials were not found, the data were pooled. Subsequently, all the data were analyzed using one-way ANOVA and mean comparisons between treatments were separated through Fisher's protected LSD test at a 0.05 level of significance. Data are presented as mean ± SE.

Results

PGPR antagonistic activity to *X. campestris* pv. *campestris*

In the in vitro test of PGPR strains for antibiosis against Xcc, 18 of the 23 tested stains produced inhibition zones (AP136, AP188, AP194, AP195, AP197, AP199, AP200, AP201, AP203, AP208, AP210, AP212, AP213, AP214, AP218, AP219, AP295, and AP305) (Table 1). Bacterial identification indicated that these 18 PGPR strains belonged to the same group: *B. velezensis*. Twelve of these 18 strains were isolated from cotton, two from cucumber, one from red pepper, and one from douglasfir. Eight strains (AP136, AP201, AP213, AP214, AP218, AP219, AP295, and AP305) to represent strains from a diversity of source plants, including four strains from cotton, two strains from cucumber, one strain from red pepper, and one strain from douglasfir, were selected for preliminary screening in the greenhouse.

Preliminary screening in the greenhouse

In the preliminary screening test in the greenhouse, strains AP201, AP213, AP214, AP218, AP219, AP295, and AP305 caused a significant reduction of TLNs, and strains AP201, AP218, AP219, AP295, and AP305 caused >40 % disease reduction compared with the disease control (Table 2). All the tested strains reduced the disease severity, with strains AP213, AP218, AP219, AP295, and AP305 exhibiting >35 % disease suppression. Among all these effective strains, four PGPR strains (AP218, AP219, AP295, and AP305) showed the best disease reduction of TLN and disease severity.

Two strains, AP213 and AP295, showed better plant growth than other strains or the healthy control (Table 2). Strain AP213 significantly increased the SFW, and strains AP213 and AP295 increased the diameter of the head cabbage.

Advanced tests in the greenhouse and field

PGPR strains that were used in the two mixtures were compatible based on the lack of antagonism among any of the strains (data not shown). In the greenhouse, all the tested PGPR treatments significantly reduced lesion numbers compared to the disease control (Table 3). Individual strain AP218 and mixture-2 showed a higher level of biocontrol activity than other treatments, causing 29.4 and 31.0 % disease reduction respectively. In addition, these two treatments significantly reduced disease in two of three repeated experiments. For the plant growth parameters,

Table 2 Results of the preliminary screening for effects of individual PGPR strains on biocontrol capacity and plant growth parameters

Treatment	Total lesion number	Disease severity	Shoot fresh weight (g)	Shoot dry weight (g)	Root fresh weight (g)	Plant diameter (cm)
Disease control	4.19 ± 0.30^{a}	3.48 ± 0.16^{a}	14.94 ± 1.72^{bc}	0.82 ± 0.09^{abcd}	0.32 ± 0.05^{bcd}	23.50 ± 1.33^{de}
AP136	3.60 ± 0.36^{ab}	2.48 ± 0.14^{bc}	12.63 ± 1.48^{c}	0.59 ± 0.06^{d}	0.33 ± 0.05^{bcd}	21.63 ± 1.13^{e}
AP201	2.25 ± 0.28^{cd}	2.68 ± 0.18^{b}	14.33 ± 2.15^{c}	0.65 ± 0.10^{cd}	0.32 ± 0.03^{bcd}	23.88 ± 0.91^{ed}
AP213	2.94 ± 0.54^{bcd}	2.18 ± 0.30^{bcd}	25.38 ± 2.94^{a}	1.07 ± 0.13^{a}	0.50 ± 0.04^{ab}	29.35 ± 0.68^{a}
AP214	3.00 ± 0.46^{bc}	2.50 ± 0.29^{bc}	12.40 ± 1.00^{c}	0.59 ± 0.05^{d}	0.17 ± 0.04^{d}	23.99 ± 1.14^{cde}
AP218	2.06 ± 0.38^{cd}	2.13 ± 0.10^{bcd}	17.44 ± 2.05^{bc}	0.84 ± 0.07^{abcd}	0.32 ± 0.08^{bcd}	25.89 ± 0.94^{bcd}
AP219	2.31 ± 0.38^{cd}	2.08 ± 0.18^{cd}	14.12 ± 1.50^{c}	0.76 ± 0.09^{bcd}	0.25 ± 0.04^{cd}	24.66 ± 1.18^{bcde}
AP295	2.38 ± 0.21^{cd}	2.25 ± 0.28^{bcd}	20.26 ± 0.43^{ab}	1.03 ± 0.04^{a}	0.50 ± 0.12^{ab}	27.58 ± 0.91^{ab}
AP305	1.94 ± 0.38^{d}	1.80 ± 0.28^{d}	17.37 ± 2.83^{bc}	0.91 ± 0.15^{abc}	0.43 ± 0.13^{abc}	25.46 ± 1.63^{bcd}
Healthy control	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	24.62 ± 2.40^{a}	1.01 ± 0.12^{ab}	0.57 ± 0.08^{a}	27.03 ± 0.74^{abc}

PGPR strains were spayed to the whole plant

Values (mean ± SE) in the same column followed by the same letter do not significantly different at $P \leq 0.05$ according to Fisher's protected LSD

Total lesion number ($F_{9,63} = 9.47$, $P < 0.0001$); disease severity ($F_{9,63} = 18.87$, $P < 0.0001$); shoot fresh weight ($F_{9,63} = 5.13$, $P < 0.0001$); shoot dry weight ($F_{9,63} = 3.50$, $P = 0.0015$); root fresh weight ($F_{9,63} = 2.87$, $P = 0.0066$); plant diameter ($F_{9,63} = 4.09$, $P = 0.0004$)

Table 3 Effects of selected individual PGPR strains and strain mixtures on the lesion number of black rot and plant growth parameters of cabbage in the greenhouse

Treatment	Number of lesions	Shoot fresh weight (g)	Shoot dry weight (g)	Root fresh weight (g)	Root dry weight (g)	Plant diameter (cm)
Disease control	11.03 ± 0.80a	36.49 ± 3.28^{a}	3.00 ± 0.25^{a}	6.62 ± 0.61^{a}	0.47 ± 0.04^{b}	31.33 ± 1.11^{a}
AP218	7.94 ± 0.59c	31.66 ± 2.52^{a}	2.74 ± 0.20^{a}	7.32 ± 0.53^{a}	0.47 ± 0.04^{b}	31.03 ± 1.03^{a}
AP219	8.85 ± 0.46bc	33.05 ± 2.81^{a}	2.78 ± 0.21^{a}	6.84 ± 0.48^{a}	0.43 ± 0.03^{b}	31.68 ± 1.04^{a}
AP295	9.49 ± 0.49b	33.18 ± 2.90^{a}	2.78 ± 0.22^{a}	6.25 ± 0.70^{a}	0.41 ± 0.04^{b}	31.41 ± 1.02
AP305	8.67 ± 0.61bc	34.27 ± 2.59^{a}	2.94 ± 0.22^{a}	7.03 ± 0.58^{a}	0.46 ± 0.03^{b}	32.37 ± 0.90^{a}
Mixture-1	9.09 ± 0.43bc	36.71 ± 3.31^{a}	3.44 ± 0.29^{a}	7.25 ± 0.51^{a}	0.55 ± 0.05^{a}	33.64 ± 1.21^{a}
Mixture-2	7.89 ± 0.54c	35.27 ± 3.43^{a}	2.93 ± 0.28^{a}	6.88 ± 0.49^{a}	0.46 ± 0.05^{b}	32.76 ± 1.23^{a}

Mixture-1: AP218, AP219, AP295 and AP305; Mixture-2: AP213, AP218, AP219, AP295 and AP305

Values (mean ± SE) in the same column followed by the same letter do not significantly different at $P \leq 0.05$ according to Fisher's protected LSD

Data from three trials were pooled together since significant differences between the repeated experimental trials were not found

Number of lesions ($F_{6,120} = 4.40$, $P = 0.0005$) Shoot fresh weight ($F_{6,118} = 0.81$, $P = 0.5652$); shoot dry weight ($F_{6,118} = 2.00$, $P = 0.0710$); root fresh weight ($F_{6,120} = 1.20$, $P = 0.3136$); root dry weight ($F_{6,120} = 2.57$, $P = 0.0224$); plant diameter ($F_{6,120} = 1.37$, $P = 0.2311$)

mixture-1 significantly increased the root dry weight (Table 3).

In the field, all the tested PGPR treatments significantly delayed pathogen infection at three weeks after transplanting (Table 4). Ten weeks after transplanting, treatments AP218 and mixture-1 significantly reduced disease incidence. Although the biocontrol effect of all the treatments was not found to be significant in cabbage leaves (EBR index), all the treatments significantly reduced HBR index compared with the disease control. Internal black rot symptoms (IBR Index) were not observed inside of the cabbage head at harvest time.

Table 4 Effects of individual PGPR strains and strain mixtures on incidence and severity of black rot disease and yield in the field

Treatment	Disease incidence		Disease severity		Yield (kg per plot)		
	Three weeks after transplanting	Ten weeks after transplanting	External black rot index	Head black rot index	Whole yield	Head yield	Marketable yield
Disease control	45.0 ± 4.3^{a}	55.0 ± 6.71^{ab}	1.56 ± 0.09^{a}	2.17 ± 0.30^{a}	9.22 ± 0.41^{a}	6.84 ± 0.30^{a}	5.46 ± 0.30^{a}
AP218	13.3 ± 4.2^{bc}	35.0 ± 5.00^{d}	1.47 ± 0.11^{a}	1.44 ± 0.13^{b}	9.20 ± 0.94^{a}	6.84 ± 0.72^{a}	5.40 ± 0.55^{a}
AP219	13.3 ± 2.1^{b}	46.7 ± 4.94^{bc}	1.57 ± 0.10^{a}	1.42 ± 0.17^{b}	10.08 ± 0.54^{a}	7.55 ± 0.45^{a}	5.77 ± 0.31^{a}
AP295	15.0 ± 3.4^{b}	50.0 ± 9.31^{abc}	1.77 ± 0.09^{a}	1.28 ± 0.13^{b}	9.53 ± 0.16^{a}	7.04 ± 0.18^{a}	5.42 ± 0.22^{a}
AP305	8.3 ± 3.1^{c}	50.0 ± 4.47^{abc}	1.61 ± 0.06^{a}	1.25 ± 0.12^{b}	10.18 ± 0.47^{a}	7.52 ± 0.41^{a}	5.92 ± 0.34^{a}
Mixture-1	16.7 ± 3.3^{b}	41.7 ± 7.92^{cd}	1.53 ± 0.07^{a}	1.36 ± 0.08^{b}	9.69 ± 0.55^{a}	7.23 ± 0.46^{a}	5.72 ± 0.49^{a}
Mixture-2	13.3 ± 3.3^{bc}	60.0 ± 11.25^{a}	1.46 ± 0.10^{a}	1.14 ± 0.11^{b}	10.35 ± 0.52^{a}	7.75 ± 0.48^{a}	6.03 ± 0.37^{a}

Mixture-1: AP136, AP209, AP282 and AP305; Mixture2: AP136, AP209, AP282, AP305, AP7, AP18 and AP218

Whole yield was based on weight of the entire plants. Head yield was the weight after removing outer leaves that were not part of head. Marketable yield was the head after removing any outer head leaves with symptoms

Values (mean ± SE) in the same column followed by the same letter do not significantly different at $P \leq 0.05$ according to Fisher's protected LSD

Disease incidence ($F_{6,\ 30} = 19.47$, $P < 0.0001$ for three weeks after transplanting and $F_{6,\ 30} = 5.04$, $P = 0.0011$ for ten weeks after transplanting); disease severity ($F_{6,\ 30} = 1.70$, $P = 0.1562$ for external black rot index and $F_{6,\ 30} = 4.20$, $P = 0.0035$ for head black rot index); yield ($F_{6,\ 30} = 0.65$, $P = 0.6914$ for whole yield, $F_{6,\ 30} = 0.58$, $P = 0.7398$ for head yield, and $F_{6,\ 30} = 0.42$, $P = 0.8579$ for marketable yield)

Discussion

The results presented here confirmed that selected individual PGPR strains and mixtures significantly reduced the lesion number in the greenhouse and the HBR index in the field. In addition, mixture-2 was more consistent and effective against black rot of cabbage, and the same mixture had the greatest effect on yield (10 % increase) compared with other treatments in the field.

Mixtures of PGPR have been shown to cause more consistent biocontrol and a higher level of protection than individual PGPR strains in some previous studies. For example, Jetiyanon et al. (2003) tested individual PGPR strains and mixtures in Thailand during the rainy season and winter season and reported that mixtures more consistently suppressed disease severity and disease incidence in both seasons than did individual strains. In our studies of three repeated trials in the greenhouse, mixture-2 and individual strain AP218 significantly reduced disease incidence in the first and second trial, while no significant differences were noted among treatments in the third trial (data not shown). Other treatments, i.e., mixture-1, AP219 and AP305, showed a significant reduction of lesion numbers once among three repeated trials. Based on combined data for all three trials in the greenhouse, mixture-2 caused a 31 % disease reduction, while individual PGPR strains AP218, AP219, AP295, and AP305, caused a 29.4, 23.0, 15.0, 23.4 % disease reduction respectively. These results are in agreement with the study by Zhang et al. (2010), in which mixtures of PGPR strains (INR7 + T4 + SE56 and INR7 + IN937a + T4 + SE56) tended to cause higher levels of disease reduction of Phytophthora blight on squash compared to individual PGPR strains. One possible explanation for enhanced biocontrol by mixtures of PGPR strains compared with individual strains is that mixtures could include strains with several plant defense mechanisms, thereby enhancing plant protection.

Formation of mixtures of PGPR can be based on various strategies. Raupach and Kloepper (1998) proposed that strategies for forming mixtures of biocontrol agents include mixtures of organisms with different plant colonization patterns, mixtures of antagonists that control different pathogens, mixtures of antagonists with different mechanisms of disease suppression, mixtures of taxonomically different organisms, or mixtures of antagonists with different

optimum temperature, pH, or moisture conditions for plant colonization. In the current study, mixture-1 included four PGPR strains that showed the best antagonistic capacity in the preliminary screening on plants. In a previous study, Mishra and Arora (2012a) combined *Pseudomonas* sp. KA19 and *Bacillus* sp. SE for biocontrol of Xcc. Both strains were effective against Xcc, while one exhibited better biological control and the other elicited greater plant growth promotion. In the current study, the approach of mixture-2 was to include some strains that exhibited the highest level of biological control and also one strain that elicited greater plant growth promotion in the preliminary screening on plants.

Our results also showed that foliar application of PGPR could protect plants foliar diseases and also cause increased plant growth in the presence of the pathogen (Tables 3, 4). In this study, the capacity to increase plant growth was related to disease protection against black rot. In the greenhouse study, some plant growth parameters were increased by mixtures of PGPR when the lesion number was reduced (Table 3). In the field trial, every time that the head black rot index was reduced, increase of yield was observed (Table 4). These results are in agreement with study by Zhang et al. (2004), in which PGPR strains, applied as drenches, induced disease protection and plant growth promotion. However, treatments that best reduced disease incidence or disease severity were not always the same as those that best enhanced plant growth or yield. In the greenhouse study, mixture-2 exhibited the best biocontrol, while mixture-1 exhibited the best growth promotion. These results are in agreement with the study by Jetiyanon et al. (2003), in which a mixture of IN937a and IN937b caused the highest cucumber fruit weight but not the best protection against *Cucumber mosaic virus*.

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