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Induction of systemic resistance in Chinese cabbage against black rot by plant growth-promoting rhizobacteria

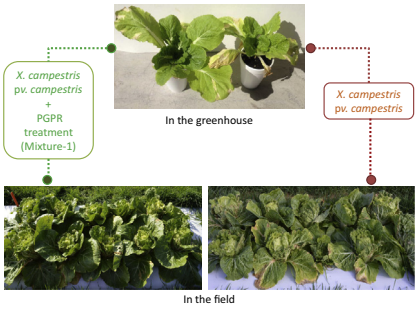
Ke Liu, Carol Garrett, Henry Fadamiro, Joseph W. Kloepper *

Department of Entomology and Plant Pathology, Auburn University, Auburn, AL 36849, USA

HIGHLIGHTS

- Plant growth-promoting rhizobacteria induced systemic resistance to black rot.
- Two mixtures of strains were formed for biocontrol of black rot.
- The marketable yield was increased by individual strains and mixtures of strains.

GRAPHICAL ABSTRACT



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ABSTRACT

Black rot, caused by *Xanthomonas campestris* pv. *campestris*, is the most important and potentially destructive disease of cabbage. The objectives of this study were to select plant growth-promoting rhizobacteria (PGPR) strains and to form strain mixtures with the capacity to elicit induced systemic resistance or to increase plant growth in Chinese cabbage. In preliminary screening, 10 of 12 tested individual PGPR strains (AP136, AP188, AP209, AP213, AP217, AP218, AP219, AP282, AP295, and AP305) reduced the number of foliar lesions, and 2 PGPR strains (AP7 and AP18) increased all tested parameters of plant growth promotion, including shoot fresh weight, shoot dry weight, root fresh weight, root dry weight and plant diameter. In advanced tests, four individual stains (AP136, AP209, AP282 and AP305) were combined into mixture-1. Mixture-2 contained all strains in mixture-1 plus three additional stains (AP7, AP18 and AP218). Both mixtures and three individual strains (AP136, AP209 and AP305) significantly reduced the number of black rot lesions, and mixture-2 increased shoot dry weight and root dry weight in greenhouse tests. In a field test, all the tested treatments significantly reduced disease incidence on whole plants at three weeks after transplanting and reduced head disease severity at harvest time. All treatments also increased marketable yield compared to the nonbacterized control. These results demonstrated that specific individual PGPR strains and strain mixtures induced systemic resistance to black rot in the greenhouse and field.

1. Introduction

Black rot, caused by *Xanthomonas campestris* pv. *campestris* (Xcc), is the most important and potentially destructive disease of cabbage (Williams, 1980; Vicente and Holub, 2013). Black rot may arise from systemic infection (infected seeds) and from secondary spread. Infected seed is a source of secondary infections (Akhtar, 1989) if the bacteria are exuded from the hydathodes, which are natural openings on the leaf edge that connect to the xylem. Splashing rain or sprinkler irrigation can spread the pathogen from the source plant to hydathodes of neighboring plants (Hugouvieux et al., 1998).

* Corresponding author.
E-mail address: kloepjw@auburn.edu (J.W. Kloepper).

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Tactics for management of black rot include using hot water treatment (Nega et al., 2003), certified disease-free transplants and seeds, resistant cultivars, biological control, and chemical control (Mew and Natural, 1993). Biological control can reduce pesticide usage, making it an attractive alternative management option for crop protection (Waard et al., 1993; Chandler et al., 2011). The use of plant growth-promoting rhizobacteria (PGPR) as biopesticides is reported to be an effective way to reduce the use of agrichemicals (Banerjee et al., 2005). PGPR are beneficial bacteria that influence the growth (Khalid et al., 2004), yield (Mia et al., 2010), and nutrient uptake of the plant (Liu et al., 2013). Some PGPR strains also provide biological control of plant disease (Chithrashree et al., 2011; Beneduzi et al., 2012). The two main genera of PGPR strains include asporogenous fluorescent *Pseudomonas* spp. and sporogenous *Bacillus* spp. (Piggot and Hilbert, 2004; Figueiredo et al., 2011). Although the preponderance of PGPR studies have been with fluorescent *Pseudomonas* spp., most commercially available PGPR are bacilli (Sivasakthi et al., 2014). This is because *Bacillus* spp. can form dormant endospores by the process of sporulation, and these spores are tolerant to heat, desiccation, UV irradiation and organic solvents (Nicholson, 2002).

PGPR exhibit two major mechanisms of biological control: including antagonism (Beneduzi et al., 2012), which is a direct mechanism, and induced systemic resistance (Kloepper et al., 2004), which is an indirect mechanism. Biological control of black rot by antagonistic bacteria has been demonstrated experimentally with PGPR on crucifers (Wulff et al., 2002; Massomo et al., 2004; Monteiro et al., 2005; Mishra and Arora, 2012b). Compared to antagonism, with ISR, the physiological (Benhamou et al., 1996) and metabolic response (Ongena et al., 2000) of the host plant is altered, leading to an enhanced synthesis of plant defense chemicals to challenge the pathogen. Some PGPR strains have induced systemic resistance against multiple plant diseases (Kloepper et al., 1997; Ramamoorthy et al., 2001). In addition to having the capacity for biocontrol, PGPR have been reported to enhance plant growth directly by a wide variety of mechanisms, including biological nitrogen fixation (Bhattacharjee et al., 2008), solubilization of mineral phosphate (Yazdani et al., 2009), secretion of plant hormones (Idris et al., 2007), and siderophore production (Sharma and Johri, 2003).

Although the beneficial effects of PGPR on plants usually are separated into two categories: biological control (Beneduzi et al., 2012) and growth promotion (Vessey, 2003), there is a close relationship between them. A single PGPR strain can exhibit both of these effects through multiple mechanisms (Wahyudi and Astuti, 2011). In search of efficient PGPR strains, multiple traits related to plant growth promotion and biocontrol activity were tested together during the screening process, and selected strains showed multiple functions related to crop production (Ahmad et al., 2008; Praveen Kumar et al., 2014).

Currently there is very limited knowledge available regarding the biological suppression of black rot in cabbage by induced systemic resistance. Objectives of this study were to 1) screen individual PGPR strains *in vitro* for multiple traits reported to be related to growth promotion and induction of systemic resistance to black rot *in planta*, and 2) form mixtures of PGPR strains based on results from objective 1 and evaluate them in the greenhouse and field.

2. Materials and methods

2.1. PGPR strains and inoculum preparation

Twelve PGPR strains (*Bacillus velezensis* AP136, AP188, AP213, AP218, AP219, AP295, and AP305; *Bacillus safensis* AP7; *Bacillus altitudinis* AP18; *Bacillus mojavensis* AP209; *Fictibacillus solisalsi* AP217; *Lysinibacillus macrolides* AP282) from the culture collection of Auburn University were used in the study. The bacteria were maintained in tryptic soy broth (TSB) (Difco Laboratories, Detroit, MI, USA), supplemented with 20% glycerol at −80 °C. For *in vitro* tests, inoculum of PGPR was grown on tryptic soy agar (TSA) at 28 °C for 48 h. For *in planta* tests, theses strains were used as spore preparations (Zhang et al., 2010).

2.2. Xanthomonas campestris pv. campestris and inoculum preparation

X. campestris pv. *campestris* strain OHS-001B-92 was provided by J. Olive, Ornamental Horticulture Research Center, Mobile, Alabama, and stored under the conditions described above. For experimental use, Xcc was grown on Yeast Dextrose Calcium Carbonate Agar plate (YDC) at 28 °C for 72 h (Schaad and Alvarez, 1993). 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 for challenge inoculation.

2.3. Preliminary screening

Four traits reported to be related to plant growth promotion were tested *in vitro*: nitrogen fixation, phosphate solubilization, siderophore production, and indole-3-acetic acid (IAA) production. Presumptive nitrogen fixation was qualitatively evaluated by growing the PGPR in the nitrogen-free semisolid medium (JNFb) as described by Olivares et al. (1996). Phosphate solubilizing capacity was qualitatively evaluated by the plate assay using National Botanical Research Institute's phosphate growth medium (NBRIP) which contained calcium phosphate as the inorganic source of phosphate (Nautiyal, 1999). Siderophore production was qualitatively evaluated by Chrome Azurol S medium (Alexander and Zuberer, 1991). IAA production was assayed by the quantitative analysis using ferric chloride-perchloric acid reagent ($FeCl_3$-$HClO_4$) (Gordon and Weber, 1951). Each of these tests was conducted three times.

The induction of systemic resistance to black rot was tested *in planta*. Kaboko hybrid organic Chinese cabbage seeds (Park Seed, Hodges, SC 29653) were planted in germination trays containing 25 cm^2 holes. One ml of PGPR spore suspension (10^7 CFU/ml) was applied to each seed prior to covering with commercial potting substrate (Sunshine mix, Sun Gro Horticulture, Agawam, MA 01001). Seeds were placed in a temperature controlled greenhouse at the Plant Science Research Center at Auburn University. Ambient air temperature in the greenhouse was maintained at 25 °C day/21 °C night throughout the year. Fourteen days after seeding, cabbage seedlings were transplanted into 10 cm diameter round pots. Each pot was drenched with 50 ml of PGPR spore suspension (10^6 CFU/ml) at transplanting time. Freshly prepared suspensions of Xcc were sprayed onto the leaves two weeks after transplanting. Pathogen-challenged plants were placed into a dark dew chamber (100% humidity) for two days at 24 °C, and then moved to the greenhouse. Pots were watered daily as needed. Fourteen days after pathogen challenge, total lesion number was recorded for each plant. Plants were harvested at the same time and the following plant parameters were measured: shoot fresh weight, shoot dry weight (oven dry at 90 °C), root fresh weight, root dry weight, and plant diameter. The experiment included 13 treatments (12 single PGPR strains and a non-bacterized but pathogen-challenged disease control) arranged in a randomized complete block design (RCBD) with 8 replications, with a single cabbage per pot.

2.4. Advanced test of selected individual stains and mixtures of selected stains in the greenhouse and field

Four individual PGPR strains (AP136, AP209, AP282, and AP305) selected in the preliminary screening were tested again. These four strains that showed the induced systemic resistance were mixed together as mixture-1 (AP136, AP209, AP282, and AP305), and three strains (AP7, AP18, and AP218) that showed growth promotion were added to form mixture-2 (AP7, AP18, AP136, AP209, AP218, AP282, and AP305). A total of seven treatments were used: four treatments consisting of single PGPR strains (AP136, AP209, AP282, and AP305), two treatments consisting of strain mixtures (mixture-1 and mixture-2), and one control (a nonbacterized but pathogen-challenged disease). The compatibility of the strains in the mixtures was determined on TSA plates. Bacterial strains were streaked at a 90-degree angle from each other. The plates were incubated at 28 °C for 48 h and observed for presence or absence of an inhibition zone. Absence of inhibition zone indicated compatibility of the tested strains, and the presence of inhibition zones indicated incompatibility. The methods were the same as previously described. The mixture of PGPR strains were prepared by combining equal proportions of each strain prior to application to the seed. The experiment was conducted twice.

The same treatments were tested once at E.V. Smith Research Center, Shorter, Alabama, USA (32.45 N, 85.88 W). The soil type was a sandy loam. The total amount of rainfall during this experiment was 116 mm. The maximum average temperature ranged from 17.17 °C to 24.44 °C, while the minimum varied form 2.11 °C to 10.17 °C. Seedlings were transplanted into the field after growing for six weeks in the greenhouse, and each plant was drenched with 100 ml of PGPR spore suspension (10^6 CFU/ml) at transplanting. Two weeks after inoculation with the PGPR, the whole plant was sprayed with the pathogen (10^8 CFU/ml). 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 10 plants (2 rows of 5 plants each), and the distance between rows was 60 cm and within rows was 45 cm.

The biological control effect was quantified by recording disease incidence at three and ten weeks after transplanting, and assessing the external, head surface, and internal black rot symptoms 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):

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

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 internal black rot index (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 1–3 of the heart leaves and 4 = vein discoloration of stem and on more than 3 heart leaves (Wulff et al., 2002). For HBR index and IBR index, a–e correspond to the number of plants in the infection category; T is the total number of plants.

To determine the yield, the whole plants were first weight to the whole yield. After removing outer leaves covered the head, weights were recorded as the head yield. The marketable yield was the head after removing any outer leaves with symptoms.

2.5. Statistical analysis

All data were analyzed by analysis of variance (ANOVA), and the treatment means were separated by using Fisher's protected least significant difference (LSD) test at $P = 0.05$ using SAS 9.4 software (SAS Institute, Cary, NC, USA).

3. Results

3.1. Preliminary screening

Ten PGPR strains (AP7, AP18, AP136, AP188, AP209, AP213, AP218, AP219, AP295, and AP305) showed presumptive nitrogen fixation capacity (Table 1). Eight PGPR strains (AP136, AP188, AP209, AP213, AP218, AP219, AP295, and AP305) produced siderophores. None of the PGPR strains produced a halo on NPRIP medium. All 12 of the tested PGPR strains produced IAA at levels of 2.11–14.95 μg/ml.

For induced systemic resistance *in planta*, total lesion number was significantly reduced by ten strains (AP136, AP188, AP209, AP213, AP217, AP218, AP219, AP282, AP295, and AP305) compared to the control (Table 2). Treatments AP7 and AP18 increased all tested growth parameters, including shoot fresh weight, shoot dry weight, root fresh weight, root dry weight and plant diameter. Treatments AP136, AP218, and AP295 increased all tested growth parameters except root dry weight. Treatments AP282 and AP305

Table 1
Characterization of PGPR strains for traits reported to be related to plant growth promotion[a].

Strain	ID	Presumptive nitrogen fixation	Phosphate solubilization	Siderophore production	IAA production (μg/ml)
AP7	*Bacillus safensis*	+	–	–	2.28
AP18	*Bacillus altitudinis*	+	–	–	2.66
AP136	*Bacillus velezensis*	+	–	+	6.51
AP188	*Bacillus velezensis*	+	–	+	6.65
AP209	*Bacillus mojavensis*	+	–	+	4.93
AP213	*Bacillus velezensis*	+	–	+	8.19
AP217	*Fictibacillus solisalsi*	–	–	–	3.24
AP218	*Bacillus velezensis*	+	–	+	8.65
AP219	*Bacillus velezensis*	+	–	+	10.16
AP282	*Lysinibacillus macroides*	–	–	–	2.11
AP295	*Bacillus velezensis*	+	–	+	6.51
AP305	*Bacillus velezensis*	+	–	+	14.95

[a] The experiment was conducted three times.

Table 2
Results of preliminary screening of PGPR for induction of systemic resistance (ISR) against black rot.

Treatments[a,b]	Total lesion number	Shoot fresh weight(g)	Shoot dry weight(g)	Root fresh weight(g)	Root dry weight(g)	Plant diameter (cm)
Control	9.44 a	19.93 efg	1.00 ef	0.32 efd	0.016 bc	28.63 ef
AP7	8.93 ab	36.98 ab	1.73 abc	0.75 ab	0.044 a	34.57 abcd
AP18	8.13 abc	45.68 a	2.17 a	0.90 a	0.044 a	35.56 abc
AP136	5.31 d	32.26 bcd	1.62 bcd	0.59 bc	0.023 bc	33.44 bcd
AP188	5.88 cd	13.26 g	0.78 f	0.28 f	0.011 c	23.88 g
AP209	6.25 cd	16.61 fg	0.92 ef	0.31 ef	0.019 bc	24.84 fg
AP213	6.29 cd	27.62 bcde	1.37 bcde	0.54 bcd	0.014 bc	33.21 bcd
AP217	6.56 bcd	22.71 defg	1.18 def	0.46 cdef	0.033 ab	28.81 ef
AP218	6.81 bcd	36.33 ab	1.83 ab	0.65 bc	0.026 abc	38.00 a
AP219	6.00 cd	28.92 bcde	1.43 bcde	0.53 bcde	0.021 bc	32.15 cde
AP282	5.94 cd	28.43 bcde	1.37 bcde	0.60 bc	0.023 bc	30.63 de
AP295	6.50 bcd	34.41 bc	1.65 bcd	0.63 bc	0.021 bc	36.63 ab
AP305	5.69 cd	25.14 cdef	1.24 cdef	0.61 bc	0.015 bc	34.15 abcd
$LSD_{0.05}$	2.5	11.01	0.51	0.22	0.020	4.36

[a] Values followed by the same letter were not significantly different ($P = 0.05$) according to Fisher's protected LSD.
[b] PGPR strains applied as seed treatment and soil drench.

increased the root fresh weight, and treatments AP213 and AP305 increased the plant diameter compared with the control.

3.2. Advanced test of selected individual stains and mixtures of selected stains in the greenhouse and field

Mixed PGPR strains were tested for their compatibility *in vitro*. None of the bacteria were inhibited by each other, so the absence of inhibition zone suggesting that these mixed strains were compatible with each other.

All the tested treatments except AP305 significantly reduced the total lesion number in the greenhouse test (Table 3). Mixture-1 and mixture-2 increased four of the five tested plant growth parameters. Only mixture-2 significantly increased the shoot dry weight, while both mixtures increased the root dry weight. The shoot fresh weight, root fresh weight and plant diameter were increased by treatments AP136 and AP209.

Three weeks after transplanting to the field, all the PGPR treatments significant delayed the pathogen infection (Table 4). At harvest time, treatments of AP136, AP209, AP305 and mixture-1 significantly reduced disease incidence compared to control. The biocontrol effect of all the treatments was not found to be significant in cabbage leaves (EBR index). All the treatments significantly reduced the black rot symptoms on cabbage head (head index) compared with the control. At harvest, no internal black rot symptoms were observed inside of the cabbage head (IBR Index). Strain AP282 significantly increased the whole plant yield, while no statistically significant effects were observed on head yield. However, all the PGPR treatments significantly increased marketable yield.

Table 3
Results of advanced test of selected individual strains and strain mixtures for induction of systemic resistance (ISR) against black rot in the greenhouse.

Treatments[a,b]	Total lesion number	Shoot fresh weight(g)	Shoot dry weight(g)	Root fresh weight(g)	Root dry weight(g)	Plant Diameter (cm)
Control	20.35 a	29.96 b	2.59 b	5.94 d	0.40 c	31.20 c
AP136	15.82 b	37.29 a	2.80 ab	7.00 c	0.46 c	33.44 ab
AP209	17.19 b	35.59 a	2.65 b	7.32 bc	0.51 bc	33.87 ab
AP282	16.03 b	35.66 a	2.87 ab	8.18 b	0.52 bc	32.29 bc
AP305	17.96 ab	34.72 ab	2.86 ab	6.64 cd	0.47 bc	34.58 a
Mixture-1	17.58 b	37.28 a	2.89 ab	8.20 b	0.61 b	33.98 ab
Mixture-2	16.58 b	39.04 a	3.03 a	10.04 a	0.85 a	33.22 abc
$LSD_{0.05}$	2.44	4.92	0.35	1.04	0.14	2.07

[a] The experiment was conducted twice. Values followed by the same letter were not significantly different ($P = 0.05$) according to Fisher's protected LSD.
[b] Mixture-1: AP136, AP209, AP282 and AP305; Mixture-2: AP136, AP209, AP282, AP305, AP7, AP18 and AP218.

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

Treatments[a,b]	Disease incidence		Disease severity			Yield[d] (Lb/plot)		
	3 weeks after transplanting	10 weeks after transplanting	External black rot index	Head black rot index	Internal black rot index	Whole yield	Head Yield	Marketable Yield
Disease	45.0 a	78.3 a	1.88 a	2.65 a	ND[c]	38.13 bc	29.64 ab	21.11 d
AP136	6.7 d	43.3 b	1.84 a	1.03 c	ND	38.29 bc	29.59 ab	25.30 ab
AP209	25.0 bc	41.7 b	1.94 a	1.18 bc	ND	38.47 b	28.75 b	22.51 c
AP282	23.3 bc	61.7 ab	1.96 a	1.45 bc	ND	42.05 a	30.27 a	24.56 b
AP305	20.0 bc	51.7 b	2.16 a	1.23 bc	ND	39.48 b	29.91 ab	25.86 a
Mixture-1	18.3 c	56.7 b	1.96 a	1.18 bc	ND	38.92 b	27.40 c	23.29 c
Mixture-2	30.0 b	58.3 ab	2.09 a	1.22 bc	ND	36.32 c	26.49 c	22.52 c
$LSD_{0.05}$	10.4	21.5	0.32	0.38	–	1.98	1.34	1.21

[a] Values followed by the same letter were not significantly different ($P = 0.05$) according to Fisher's protected LSD.
[b] Mixture-1: AP136, AP209, AP282 and AP305; Mixture2: AP136, AP209, AP282, AP305, AP7, AP18 and AP218.
[c] ND = Not detected.
[d] 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.

4. Discussion

The results presented here confirmed that individual PGPR strains and strain mixtures induced systemic resistance to black rot in the greenhouse and field, and the marketable yield was increased by individual strains and mixtures of strains.

Reduction of total lesion numbers and increase of shoot fresh weight and root fresh weight by PGPR-mediated ISR in the greenhouse, and reduction of disease incidence three weeks after transplanting, head black rot index at harvest and increase the marketable yield were highly correlated, suggesting that plant growth promotion may be partially due to inhibition of infection by the pathogen or reduction of its development in the plant (Tables 3 and 4). ISR elicited by PGPR was previously reported to result in defense against pathogen spread within the plant (Liu et al., 1995; Ahemad and Kibret, 2014). Some *Bacillus* spp. PGPR that elicited ISR also promoted plant growth (Wei et al., 1996; Zhang et al., 2004). However, these previous studies did not test whether plant growth was caused by the direct mechanisms of plant growth promotion. In the current study, traits related to plant growth promotion were tested, including biological nitrogen fixation, solubilization of mineral phosphate, secretion of plant hormones, and siderophore production. Eight of 12 tested strains exhibited three traits related to plant growth promotion. Ten strains showed presumptive nitrogen fixation; however, future work is needed to confirm the presence of nitrogenase activity by gas chromatography using the C_2H_2 reduction technique (Hardy et al., 1973). In the advanced test, the selected individual PGPR strains (AP136, AP209 and AP305) and strain AP218 included in mixture-2 showed positive activities for putative nitrogen fixation, IAA production, and siderophore production that have been directly related to plant growth promotion (Table 1). In addition, strains AP7 and AP18, which are included in mixture-2, were positive for putative nitrogen fixation and IAA production. In previous reports, a single PGPR strain causing biological control and growth promotion through multiple mechanisms was also demonstrated (Ryu et al., 2003, 2004; Wahyudi and Astuti, 2011).

Some previous studies on biological control of black rot focused only on biocontrol effects without evaluating growth parameters in the greenhouse or yield in the field (Wulff et al., 2002; Mishra and Arora, 2012a). However, ideal biocontrol agents should have positive effects on the yield. In one study that recorded yield at harvest, treatments did not significantly increase yield, and some of the tested *Bacillus* strain caused a significant reduction in cabbage head yield by the same PGPR application methods used in the current study (Massomo et al., 2004). In the current study, all the tested PGPR treatments significantly increased marketable yield in the field (Table 4), and, except for strain AP305, promoted shoot and root fresh weights in the greenhouse (Table 3). These results are in agreement with previous reports of increases in several plant growth parameters when induced systemic resistance was demonstrated (Wei et al., 1991).

One criterion for ISR by PGPR is the physical separation of tested bacteria from the target pathogens. In this research, the PGPR were introduced by seed treatment and root drench, and the pathogen was inoculated by the foliar spray. Hence, the PGPR and pathogen were separated spatially and therefore, the reduction of the disease was due to ISR.

Several studies have demonstrated that mixtures of PGPR strains can be more effective due to synergistic modes of action (Raupach and Kloepper, 1998; Jetiyanon and Kloepper, 2002). In this study, we formed two mixtures based on results of the preliminary screening, including mixture-1 that contained 4 PGPR strains (AP136, AP209, AP282, and AP305) that elicited induced systemic resistance and mixture-2 that contained mixture-1 and 3 other PGPR strains (AP7, AP18, and AP218) that exhibited growth promotion. Mixture-1 reduced disease incidence three weeks after transplanting at a greater level than some individual PGPR treatments in the field (Table 4). Mixture-2 elicited the largest growth promotion compared with other treatments, significantly increasing root and shoot dry weights in the greenhouse (Table 3). However, surprisingly, greater biocontrol and yield increase was detected by individual strain AP136 in the field. Although mixtures did not exhibit the greatest growth promotion in the field, they still increased the marketable yield in the field, and only these two mixtures significantly increased the root dry weight in the greenhouse. We tested mixed PGPR strains were compatible with each other *in vitro*. We believe that mixture may be achieving more effective biocontrol effects when increasing the repeat of test in the field. Mixtures of PGPR can have other benefits compared to individual PGPR strains. For example, mixtures have demonstrated increased repeatability of efficacy over multiple field trials (Jetiyanon et al., 2003). In addition, mixtures of several strains may result in a more stable rhizosphere community and provide several mechanisms under field conditions (Domenech et al., 2006). In the future, the PGPR treatments used in the current study should be evaluated in multiple field conditions, to test their consistency.

Strains AP7 (previously SE52) and AP18 (previously INR7) did not reduce black rot of cabbage in this study (Table 2). However, strain INR7 (AP18) showed the capacity to elicit systemic protection on cucumber against to *Erwinia tracheiphila* (Zehnder et al., 2001), on cucumber against to *Pseudomonas syringae* pv. l*achrymans* (Wei et al., 1996), on tomato against to *Ralstonia solanacearum*, on long cayenne pepper against to *Colletotrichum gloeosporioides* (Jetiyanon and Kloepper, 2002), on cucumber against to *C. gloeosporioides* (Raupach and Kloepper, 1998), and strains INR7 (AP18) and SE52 (AP7) elicit systemic protection on loblolly pine against to *Cronartium quercuum* (Enebak et al., 1998). It appears that the broad-spectrum protection resulting from PGPR-ISR can be strain specific. However, the growth promotion activity of INR7 and SE52 in our tests agree with previous reports of enhanced germination rate of loblolly and slash pine by both PGPR strains (Enebak et al., 1998), increased weight of slash pine shoots by INR7 (Enebak et al., 1998), and increases in cucumber main runner length and number of leaves by INR7 (Wei et al., 1996).

In summary, the results reported here showed that PGPR-mediated ISR protected cabbage against black rot and also increased growth parameters in the greenhouse and field condition. In addition to using a single PGPR strain, it may be possible to apply mixtures of PGPR strains. In future, several additional issues should be addressed, including the length of protection and the consistency of mixtures of PGPR. PGPR-mediated ISR should be further evaluated in an integrated pest management approach for controlling black rot of cabbages.

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References

Ahemad, M., Kibret, M., 2014. Mechanisms and applications of plant growth promoting rhizobacteria: current perspective. J. King Saud Univ. Sci. 26, 1–20.

Ahmad, F., Ahmad, I., Khan, M., 2008. Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. Microbiol. Res. 163, 173–181.

Akhtar, M., 1989. *Xanthomonas campestris* pv. *campestris* causing black rot in cabbage. Pak. J. Agric. Res. 10, 311–313.

Alexander, D.B., Zuberer, D.A., 1991. Use of chrome azurol-S reagents to evaluate siderophore production by rhizosphere bacteria. Biol. Fertil. Soils 12, 39–45.

Banerjee, M.R., Yesmin, L., Vessey, J.K., Rai, M., 2005. Plant-growth-promoting rhizobacteria as biofertilizers and biopesticides. In: Rai, M.K. (Ed.), Handbook of Microbial Biofertilizers. Food Products Press, New York, pp. 137–181.

Beneduzi, A., Ambrosini, A., Passaglia, L.M.P., 2012. Plant growth-promoting rhizobacteria (PGPR): their potential as antagonists and biocontrol agents. Genet. Mol. Biol. 35, 1044–1051.

Benhamou, N., Kloepper, J.W., QuadtHallman, A., Tuzun, S., 1996. Induction of defense-related ultrastructural modifications in pea root tissues inoculated with endophytic bacteria. Plant Physiol. 112, 919–929.

Bhattacharjee, R.B., Singh, A., Mukhopadhyay, S., 2008. Use of nitrogen-fixing bacteria as biofertiliser for non-legumes: prospects and challenges. Appl. Microbiol. Biotechnol. 80, 199–209.

Chandler, D., Bailey, A.S., Tatchell, G.M., Davidson, G., Greaves, J., Grant, W.P., 2011. The development, regulation and use of biopesticides for integrated pest management. Philos. Trans. R. Soc. B 366, 1987–1998.

Chithrashree, Udayashankar, A.C., Nayaka, S.C., Reddy, M.S., Srinivas, C., 2011. Plant growth-promoting rhizobacteria mediate induced systemic resistance in rice against bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae*. Biol. Control 59, 114–122.

Domenech, J., Reddy, M., Kloepper, J., Ramos, B., Gutierrez-Manero, J., 2006. Combined application of the biological product LS213 with *Bacillus*, *Pseudomonas* or *Chryseobacterium* for growth promotion and biological control of soil-borne diseases in pepper and tomato. Biocontrol 51, 245–258.

Enebak, S.A., Wei, G., Kloepper, J.W., 1998. Effects of plant growth-promoting rhizobacteria on loblolly and slash pine seedlings. For. Sci. 44, 139–144.

Figueiredo, M., Seldin, L., de Araujo, F., Mariano, R., 2011. Plant growth promoting rhizobacteria: fundamentals and applications. In: Maheshwari, D.K. (Ed.), Plant Growth and Health Promoting Bacteria. Springer Berlin Heidelberg, pp. 21–43.

Gordon, S.A., Weber, R.P., 1951. Colorimetric estimation of indoleacetic acid. Plant Physiol. 26, 192–195.

Hardy, R., Burns, R., Holsten, R.D., 1973. Applications of the acetylene-ethylene assay for measurement of nitrogen fixation. Soil Biol. Biochem. 5, 47–81.

Hugouvieux, V., Barber, C.E., Daniels, M.J., 1998. Entry of *Xanthomonas campestris* pv. *campestris* into hydathodes of *Arabidopsis thaliana* leaves: a system for studying early infection events in bacterial pathogenesis. Mol. Plant-Microbe Interact. 11, 537–543.

Idris, E.E., Iglesias, D.J., Talon, M., Borriss, R., 2007. Tryptophan-dependent production of indole-3-acetic acid (IAA) affects level of plant growth promotion by *Bacillus amyloliquefaciens* FZB42. Mol. Plant-Microbe Interact. 20, 619–626.

Jetiyanon, K., Kloepper, J.W., 2002. Mixtures of plant growth-promoting rhizobacteria for induction of systemic resistance against multiple plant diseases. Biol. Control 24, 285–291.

Jetiyanon, K., Fowler, W.D., Kloepper, J.W., 2003. Broad-spectrum protection against several pathogens by PGPR mixtures under field conditions in Thailand. Plant Dis. 87, 1390–1394.

Khalid, A., Arshad, M., Zahir, Z.A., 2004. Screening plant growth-promoting rhizobacteria for improving growth and yield of wheat. J. Appl. Microbiol. 96, 473–480.

Kloepper, J.W., Tuzun, S., Zehnder, G.W., Wei, G., 1997. Multiple disease protection by rhizobacteria that induce systemic resistance-historical precedence. Phytopathology 87, 136–137.

Kloepper, J.W., Ryu, C.M., Zhang, S.A., 2004. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. Phytopathology 94, 1259–1266.

Liu, F., Xing, S., Ma, H., Du, Z., Ma, B., 2013. Plant growth-promoting rhizobacteria affect the growth and nutrient uptake of *Fraxinus americana* container seedlings. Appl. Microbiol. Biotechnol. 97, 4617–4625.

Liu, L., Kloepper, J., Tuzun, S., 1995. Induction of systemic resistance in cucumber against bacterial angular leaf spot by plant growth-promoting rhizobacteria. Phytopathology 85, 843–847.

Massomo, S.M.S., Mortensen, C.N., Mabagala, R.B., Newman, M.A., Hockenhull, J., 2004. Biological control of black rot (*Xanthomonas campestris* pv. *campestris*) of cabbage in Tanzania with *Bacillus* strains. J. Phytopathol. 152, 98–105.

Mew, T., Natural, M., 1993. Management of *Xanthomonas* diseases. In: Swings, J.G., Civerolo, E.L. (Eds.), Xanthomonas. Chapman & Hall, London, pp. 341–362.

Mia, M.A.B., Shamsuddin, Z.H., Wahab, Z., Marziah, M., 2010. Rhizobacteria as bioenhancer and biofertilizer for growth and yield of banana (*Musa* spp. cv. 'Berangan'). Sci. Hort. 126, 80–87.

Mishra, S., Arora, N.K., 2012a. Management of black rot in cabbage by rhizospheric *Pseudomonas* species and analysis of 2,4-diacetylphloroglucinol by qRT-PCR. Biol. Control 61, 32–39.

Mishra, S., Arora, N.K., 2012b. Evaluation of rhizospheric *Pseudomonas* and *Bacillus* as biocontrol tool for *Xanthomonas campestris* pv *campestris*. World J. Microbiol. Biotechnol. 28, 693–702.

Monteiro, L., Mariano, R.d.L.R., Souto-Maior, A.M., 2005. Antagonism of *Bacillus* spp. against *Xanthomonas campestris* pv. *campestris*. Braz. Arch. Biol. Technol. 48, 23–29.

Nautiyal, C.S., 1999. An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS Microbiol. Lett. 170, 265–270.

Nega, E., Ulrich, R., Werner, S., Jahn, M., 2003. Hot water treatment of vegetable seed–an alternative seed treatment method to control seed borne pathogens in organic farming. J. Plant Dis. Prot. 110, 220–234.

Nicholson, W.L., 2002. Roles of *Bacillus* endospores in the environment. Cell. Mol. Life Sci. 59, 410–416.

Olivares, F.L., Baldani, V.L.D., Reis, V.M., Baldani, J.I., Dobereiner, J., 1996. Occurrence of the endophytic diazotrophs *Herbaspirillum* spp. in roots, stems, and leaves, predominantly of Gramineae. Biol. Fertil. Soils 21, 197–200.

Ongena, M., Daayf, F., Jacques, P., Thonart, P., Benhamou, N., Paulitz, T.C., Bélanger, R. R., 2000. Systemic induction of phytoalexins in cucumber in response to treatments with fluorescent pseudomonads. Plant. Pathol. 49, 523–530.

Piggot, P.J., Hilbert, D.W., 2004. Sporulation of *Bacillus subtilis*. Curr. Opin. Microbiol. 7, 579–586.

Praveen Kumar, G., Mir Hassan Ahmed, S.K., Desai, S., Leo Daniel Amalraj, E., Rasul, A., 2014. In vtro screening for abiotic stress tolerance in potent biocontrol and plant growth promoting strains of *Pseudomonas* and *Bacillus* spp. Int. J. Bacteriol. 2014, 6.

Ramamoorthy, V., Viswanathan, R., Raguchander, T., Prakasam, V., Samiyappan, R., 2001. Induction of systemic resistance by plant growth promoting rhizobacteria in crop plants against pests and diseases. Crop Prot. 20, 1–11.

Raupach, G.S., Kloepper, J.W., 1998. Mixtures of plant growth-promoting rhizobacteria enhance biological control of multiple cucumber pathogens. Phytopathology 88, 1158–1164.

Ryu, C.-M., Farag, M.A., Hu, C.-H., Reddy, M.S., Wei, H.-X., Paré, P.W., Kloepper, J.W., 2003. Bacterial volatiles promote growth in *Arabidopsis*. Proc. Natl. Acad. Sci. 100, 4927–4932.

Ryu, C.-M., Farag, M.A., Hu, C.-H., Reddy, M.S., Kloepper, J.W., Paré, P.W., 2004. Bacterial volatiles induce systemic resistance in *Arabidopsis*. Plant Physiol. 134, 1017–1026.

Schaad, N., Alvarez, A., 1993. *Xanthomonas campestris* pv. *campestris*: cause of black rot of crucifers. In: Swings, J.G., Civerolo, E.L. (Eds.), Xanthomonas. Chapman & Hall, London, pp. 51–55.

Sharma, A., Johri, B.N., 2003. Growth promoting influence of siderophore-producing *Pseudomonas strains* GRP3A and PRS9 in maize (*Zea mays* L.) under iron limiting conditions. Microbiol. Res. 158, 243–248.

Sivasakthi, S., Usharani, G.,P.,S., 2014. Biocontrol potentiality of plant growth promoting bacteria (PGPR) – *Pseudomonas fluorescens* and *Bacillus subtilis*: a review. Afr. J. Agric. Res. 9, 1265–1277.

Vessey, J.K., 2003. Plant growth promoting rhizobacteria as biofertilizers. Plant Soil 255, 571–586.

Vicente, J.G., Holub, E.B., 2013. *Xanthomonas campestris* pv. *campestris* (cause of black rot of crucifers) in the genomic era is still a worldwide threat to brassica crops. Mol. Plant Pathol. 14, 2–18.

Waard, M., Georgopoulos, S., Hollomon, D., Ishii, H., Leroux, P., Ragsdale, N., Schwinn, F., 1993. Chemical control of plant diseases: problems and prospects. Annu. Rev. Phytopathol. 31, 403–421.

Wahyudi, A., Astuti, R., 2011. Screening of *Pseudomonas* sp. isolated from rhizosphere of soybean plant as plant growth promoter and biocontrol agent. Am. J. Agric. Biol. Sci. 6, 134–141.

Wei, G., Kloepper, J.W., Tuzun, S., 1991. Induction of systemic resistance of cucumber to *Colletotrichum orbiculare* by select strains of plant growth-promoting rhizobacteria. Phytopathology 81, 1508–1512.

Wei, G., Kloepper, J., Tuzun, S., 1996. Induced systemic resistance to cucumber diseases and increased plant growth by plant growth-promoting rhizobacteria under field conditions. Phytopathology 86, 221–224.

Williams, P.H., 1980. Black rot: a continuing threat to world crucifers. Plant Dis. 64, 7.

Wulff, E.G., Mguni, C.M., Mortensen, C.N., Keswani, C.L., Hockenhull, J., 2002. Biological control of black rot (*Xanthomonas campestris* pv. *campestris*) of brassicas with an antagonistic strain of *Bacillus subtilis* in Zimbabwe. Eur. J. Plant Pathol. 108, 317–325.

Yazdani, M., Bahmanyar, M.A., Pirdashti, H., Esmaili, M.A., 2009. Effect of Phosphate solubilization microorganisms (PSM) and plant growth promoting rhizobacteria (PGPR) on yield and yield components of Corn (*Zea mays L.*). World Acad. Sci. Eng. Technol. 37, 90–92.

Zehnder, G., Murphy, J., Sikora, E., Kloepper, J., 2001. Application of rhizobacteria for induced resistance. Eur. J. Plant Pathol. 107, 39–50.

Zhang, S., Reddy, M.S., Kloepper, J.W., 2004. Tobacco growth enhancement and blue mold disease protection by rhizobacteria: relationship between plant growth promotion and systemic disease protection by PGPR strain 90–166. Plant Soil 262, 277–288.

Zhang, S., White, T.L., Martinez, M.C., McInroy, J.A., Kloepper, J.W., Klassen, W., 2010. Evaluation of plant growth-promoting rhizobacteria for control of Phytophthora blight on squash under greenhouse conditions. Biol. Control 53, 129–135.