**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

*Corresponding author at: Entomology and Plant Pathology, Auburn University, Auburn, AL
36849, USA.

E-mail address: kloepjw@auburn.edu (J. Kloepper).

Fax: +1 334-844-1950

Phone: +1 334-844-5067

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 strains (AP136, AP209, AP282 and AP305) were combined into mixture-1. Mixture-2 contained all strains in mixture one plus three additional strains (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.

Keywords: Induction of systemic resistance; plant growth-promoting Rhizobacteria; black rot; Chinese cabbage.

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).

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, b). 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; *B. safensis* AP7; *B. altitudinis* AP18; *B. 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, these strains were used as spore preparations (Zhang et al., 2010).

2.2. X. 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 10⁸ 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 ($\text{FeCl}_3\text{-HClO}_4$) (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 cm² 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 strains and mixtures of selected strains 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 from 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):

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

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 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 antagonistic 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.

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. *lachrymans* (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.

315 **Acknowledgments**

316 We acknowledge and thank John McInroy and Dr. Chia-Hui Hu for their advice and technical
317 help and John Olive for the source of *Xanthomonas campestris* pv. *campestris*. Funding was
318 provided in part by the USDA National Institute of Food and Agriculture of Organic
319 Agriculture Research and Extension Initiative (Grant No. 2011 – 51300 – 30634).

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. Fertility 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.

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

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

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

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

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

356 Gordon, S.A., Weber, R.P., 1951. Colorimetric estimation of indoleacetic acid. Plant Physiol. 26,
 357 192-195.

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

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

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

366 Jetiyanon, K., Kloepper, J.W., 2002. Mixtures of plant growth-promoting rhizobacteria for
 367 induction of systemic resistance against multiple plant diseases. Biol. Control 24, 285-
 368 291.

369 Jetiyanon, K., Fowler, W.D., Kloepper, J.W., 2003. Broad-spectrum protection against several
 370 pathogens by PGPR mixtures under field conditions in Thailand. Plant Dis. 87, 1390-
 371 1394.

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

374 Kloepper, J.W., Tuzun, S., Zehnder, G.W., Wei, G., 1997. Multiple disease protection by
 375 rhizobacteria that induce systemic resistance-historical precedence. Phytopathology 87,
 376 136-137.

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

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

382 Liu, L., Kloepper, J., Tuzun, S., 1995. Induction of systemic resistance in cucumber against
 383 bacterial angular leaf spot by plant growth-promoting rhizobacteria. Phytopathology 85,
 384 843-847.

385 Massomo, S.M.S., Mortensen, C.N., Mabagala, R.B., Newman, M.A., Hockenhull, J., 2004.
 386 Biological control of black rot (*Xanthomonas campestris* pv. *campestris*) of cabbage in
 387 Tanzania with *Bacillus* strains. J. Phytopathol. 152, 98-105.
 388 Mew, T., Natural, M., 1993. Management of *Xanthomonas* diseases. In: Swings, J.G., Civerolo,
 389 E.L. (Eds.), *Xanthomonas*. Chapman & Hall, London, pp. 341-362.
 390 Mia, M.A.B., Shamsuddin, Z.H., Wahab, Z., Marziah, M., 2010. Rhizobacteria as bioenhancer
 391 and biofertilizer for growth and yield of banana (*Musa* spp. cv. 'Berangan'). Sci. Hort.
 392 126, 80-87.
 393 Mishra, S., Arora, N.K., 2012a. Management of black rot in cabbage by rhizospheric
 394 *Pseudomonas* species and analysis of 2,4-diacetylphloroglucinol by qRT-PCR. Biol.
 395 Control 61, 32-39.
 396 Mishra, S., Arora, N.K., 2012b. Evaluation of rhizospheric *Pseudomonas* and *Bacillus* as
 397 biocontrol tool for *Xanthomonas campestris* pv *campestris*. World J. Microbiol.
 398 Biotechnol. 28, 693-702.
 399 Monteiro, L., Mariano, R.d.L.R., Souto-Maior, A.M., 2005. Antagonism of *Bacillus* spp. against
 400 *Xanthomonas campestris* pv. *campestris*. Braz. Arch. Biol. Technol. 48, 23-29.
 401 Nautiyal, C.S., 1999. An efficient microbiological growth medium for screening phosphate
 402 solubilizing microorganisms. FEMS Microbiol. Lett. 170, 265-270.
 403 Nega, E., Ulrich, R., Werner, S., Jahn, M., 2003. Hot water treatment of vegetable seed—an
 404 alternative seed treatment method to control seed borne pathogens in organic farming. J.
 405 Plant Dis. Protect. 110, 220-234.
 406 Nicholson, W.L., 2002. Roles of *Bacillus* endospores in the environment. Cell. Mol. Life Sci. 59,
 407 410-416.

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

411 Ongena, M., Daayf, F., Jacques, P., Thonart, P., Benhamou, N., Paulitz, T.C., Bélanger, R.R.,
 412 2000. Systemic induction of phytoalexins in cucumber in response to treatments with
 413 fluorescent pseudomonads. Plant Pathol. 49, 523-530.

414 Piggot, P.J., Hilbert, D.W., 2004. Sporulation of *Bacillus subtilis*. Curr. Opin. Microbiol. 7, 579-
 415 586.

416 Praveen Kumar, G., Mir Hassan Ahmed, S.K., Desai, S., Leo Daniel Amalraj, E., Rasul, A.,
 417 2014. *In vitro* screening for abiotic stress tolerance in potent biocontrol and plant growth
 418 promoting strains of *Pseudomonas* and *Bacillus* spp. Int. J. Bacteriol. 2014, 6.

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

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

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

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

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

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

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

437 Vessey, J.K., 2003. Plant growth promoting rhizobacteria as biofertilizers. Plant Soil 255, 571-
438 586.

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

442 Waard, M., Georgopoulos, S., Hollomon, D., Ishii, H., Leroux, P., Ragsdale, N., Schwinn, F.,
443 1993. Chemical control of plant diseases: problems and prospects. Annu. Rev.
444 Phytopathol. 31, 403-421.

445 Wahyudi, A., Astuti, R., 2011. Screening of *Pseudomonas* sp. isolated from rhizosphere of
446 soybean plant as plant growth promoter and biocontrol agent. Ameri. J. Agri. Biol. Sci 6,
447 134-141.

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

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

454 Williams, P.H., 1980. Black rot: a continuing threat to world crucifers. *Plant Dis.* 64, 7.
 455 Wulff, E.G., Mguni, C.M., Mortensen, C.N., Keswani, C.L., Hockenhull, J., 2002. Biological
 456 control of black rot (*Xanthomonas campestris* pv. *campestris*) of brassicas with an
 457 antagonistic strain of *Bacillus subtilis* in Zimbabwe. *Eur. J. Plant Pathol.* 108, 317-325.
 458 Yazdani, M., Bahmanyar, M.A., Pirdashti, H., Esmaili, M.A., 2009. Effect of Phosphate
 459 solubilization microorganisms (PSM) and plant growth promoting rhizobacteria (PGPR)
 460 on yield and yield components of Corn (*Zea mays* L.). *World Acad. Sci. Engineer.*
 461 *Technol.* 37, 90-92.
 462 Zehnder, G., Murphy, J., Sikora, E., Kloepper, J., 2001. Application of rhizobacteria for induced
 463 resistance. *Eur. J. Plant Pathol.* 107, 39-50.
 464 Zhang, S., Reddy, M.S., Kloepper, J.W., 2004. Tobacco growth enhancement and blue mold
 465 disease protection by rhizobacteria: relationship between plant growth promotion and
 466 systemic disease protection by PGPR strain 90-166. *Plant Soil* 262, 277-288.
 467 Zhang, S., White, T.L., Martinez, M.C., McInroy, J.A., Kloepper, J.W., Klassen, W., 2010.
 468 Evaluation of plant growth-promoting rhizobacteria for control of *Phytophthora* blight on
 469 squash under greenhouse conditions. *Biol. Control* 53, 129-135.
 470

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	<i>Bacillus safensis</i>	+	–	–	2.28
AP18	<i>Bacillus altitudinis</i>	+	–	–	2.66
AP136	<i>Bacillus velezensis</i>	+	–	+	6.51
AP188	<i>Bacillus velezensis</i>	+	–	+	6.65
AP209	<i>Bacillus mojavensis</i>	+	–	+	4.93
AP213	<i>Bacillus velezensis</i>	+	–	+	8.19
AP217	<i>Fictibacillus solisalsi</i>	–	–	–	3.24
AP218	<i>Bacillus velezensis</i>	+	–	+	8.65
AP219	<i>Bacillus velezensis</i>	+	–	+	10.16
AP282	<i>Lysinibacillus macroides</i>	–	–	–	2.11
AP295	<i>Bacillus velezensis</i>	+	–	+	6.51
AP305	<i>Bacillus velezensis</i>	+	–	+	14.95

^aThe experiment was conducted three times.

Table 2

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

Treatments ^{ab}	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

^aValues followed by the same letter were not significantly different ($P=0.05$) according to Fisher's protected LSD.

^bPGPR strains applied as seed treatment and soil drench.

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 ^{ab}	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

^aThe experiment was conducted twice. Values followed by the same letter were not significantly different ($P=0.05$) according to Fisher's protected LSD.

^bMixture-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 ^{ab}	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.

X. campestris
pv. campestris
+
PGPR
treatment
(Mixture-1)

In the greenhouse

X. campestris
pv. campestris

In the field