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Biological Control and Plant Growth Promoting Capacity of Rhizobacteria on Pepper under Greenhouse and Field Conditions

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Plant growth promoting rhizobacteria *Ochrobactrum lupini* KUDC1013 and *Novosphingobium pentaromativorans* KUDC1065 isolated from Dokdo Island, S. Korea are capable of eliciting induced systemic resistance (ISR) in pepper against bacterial spot disease. The present study aimed to determine whether plant growth-promoting rhizobacteria (PGPR) strains including strain KUDC1013, strain KUDC1065, and *Paenibacillus polymyxa* E681 either singly or in combinations were evaluated to have the capacity for potential biological control and plant growth promotion effect in the field trials. Under greenhouse conditions, the induced systemic resistance (ISR) effect of treatment with strains KUDC1013 and KUDC1065 differed according to pepper growth stages. Drenching of 3-week-old pepper seedlings with the KUDC-1013 strain significantly reduced the disease symptoms. In contrast, treatment with the KUDC1065 strain significantly protected 5-week-old pepper seedlings. Under field conditions, peppers treated with PGPR mixtures containing E681 and KUDC1013, either in a two-way combination, were showed greater effect on plant growth than those treated with an individual treatment. Collectively, the application of mixtures of PGPR strains on pepper might be considered as a potential biological control under greenhouse and field conditions.

Keywords: Dokdo Island, biological control, induced systemic resistance (ISR), plant growth promoting rhizobacteria (PGPR), pepper (*Capsicum annuum*)

Introduction

In Korea, pepper (*Capsicum annuum*) is one of the most important vegetable crops with a high economic value because pepper and pepper dry powder are used in preparing many traditional Korean foods (Hwang and Kim, 1995; Sang *et al*., 2008). During pepper cultivation in greenhouses and fields, many plant diseases such as bacterial spot disease, phytophthora blight, and damping-off disease cause significant yield loss (Hwang and Kim, 1995; Sang *et al*., 2008). To protect pepper plants from various diseases, many disease control approaches were employed including chemical control methods, cultural control methods and application of biological control agents (Lee *et al*., 2005b; Chung *et al*., 2006). However, continuous chemical applications accumulate residual pesticides in the natural ecosystem and causes human and animal toxicity. The synthetic organic chemical use may also lead to appearance of resistant pathogens and to reduction of microbial diversity and beneficial microorganism populations (Glick and Bashan, 1997). For these reasons, the use of rhizosphere bacteria (rhizobacteria) has been widely studied for eco-friendly biological control as the best alternative to the chemicals. Many researchers have studied biological control agents using rhizobacteria with diverse pathogens and crops such as tomato, cucumber, wheat, sesame and cayenne pepper under greenhouse and field conditions (Mathre and Johnston, 1995; Wei *et al*., 1996; Raupach and Kloepper, 2000; Jetiyanon *et al*., 2003; Ryu *et al*., 2006).

Plant growth-promoting rhizobacteria (PGPR) are free-living or root-associated bacteria in the rhizosphere of many plant species that can reduce plant disease and increase plant growth (Park *et al*., 1988; Arora *et al*., 2001; Ryu *et al*., 2006). Many direct and indirect mechanisms have been presented for growth promotion by PGPR. First, PGPR can directly influence plant growth by producing phytohormones, other plant stimulants and improving uptake of essential nutrients (Lee *et al*., 2005a, 2006; Hameeda *et al*., 2006; Vikaram, 2007). Second, PGPR could promote plant growth indirectly by suppressing plant pathogens. Besides direct suppression of plant pathogen by production of antimicrobial compound from PGPR, PGPR could elicit plant self-defenses referred to as induced systemic resistance (ISR) against a broad range of pathogens including fungi, virus, bacteria, nematodes, and insects (Ramamoorthy *et al*., 2001; Jetiyanon and Kloepper, 2002; Murphy *et al*., 2003; Ryu *et al*., 2003b). These rhizobacteria can decrease plant pathogens or deleterious microorganisms through secretion of antibiotics; further, they can increase the defense response by inducing systemic resistance in plants before pathogens have been introduced (Jetiyanon and Kloepper, 2002; Ryu *et al*., 2003b). Diverse genera of PGPR including *Azospirillum*, *Azotobacter*, *Bacillus*, *Enterobacter*, *Klebsiella*, *Paenibacillus*, *Psuedomonas*, and *Rhizobium* have been shown to promote plant growth

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and elicit induced resistance (Pierson and Weller, 1994; Raupach and Kloepper, 2000; Jetiyanon and Kloepper, 2002; Silva *et al*., 2004; Ryu *et al*., 2006, 2007). However, previous studies have not considered the biological control capacity of genus *Ochrobactrum* and *Novoshingobium* on simultaneous growth promotion and induction of ISR.

The main objective of this research was to determine if *O. lupini* KUDC1013 and *N. pentaromativorans* KUDC1065 isolated from *Solanum nigrum* L. plant roots grown in Dokdo island, S. Korea, and *P. polymyxa* E681 strain which have previously been reported as PGPR strain (Ham *et al*., 2009) could suppress the plant disease as potential of biological control and affect the promotion of plant growth under greenhouse and field conditions.

Materials and Methods

Bacteria and cultures

The 2 PGPR strains KUDC1013 and KUDC1065, which were previously found to demonstrate ISR activity, were used in pepper and tobacco against *Xanthomonas axonopodis* pv. *vesicatoria* and *Erwinia carotovora* subsp. *carotovora*, respectively (Ham *et al*., 2009). For long-term storage, all strains were maintained at -70°C in tryptic soy broth (TSB) or Luria-Bertani (LB) broth containing 15% (v/v) glycerol. For experimental use, KUDC1013 was transferred onto tryptic soy agar (TSA; Difco Laboratories, USA) plates and KUDC1065, onto LB agar plates; the plates were then incubated at 37°C for 24 h. Bacterial cells were suspended in sterilized distilled water (SDW).

Assessment of germination rate

To assess the germination rate of pepper seeds after each treatment, we used the paper towel method of the International Seed Testing Association (ISTA, 1993; Raupach and Kloepper, 2000; Hameeda *et al*., 2006). After bacterization, 100 seeds were transferred on a filter paper (Whatman No. 1, d=125 mm) in a Petri dish (d=14 cm) with 4.5 ml SDW. The Petri dish was incubated at 30°C; after 7 days, the germinated seeds were counted and the weight of all seeds was measured for every treatment. A hundred seeds were used for each treatment with 2 replicates per treatment.

Assessment of the *in vitro* antifungal activity

The antifungal activities of the Dokdo-derived isolates were assessed against the plant pathogenic fungi *Aspergillus niger* and *Rhizoctonia solani*. The isolates were dropped onto a disc (d=8 mm) at the edge of a Petri dish with equal spacing around the perimeter of potato dextrose agar (PDA) medium (Difco); after incubation for 1 day, a fully grown mycelium disk (d=1 cm) was placed at the center of each PDA plate. All fungi were grown on PDA at 25°C for 5 days. Seven days after the incubation of the bacteria, suppression of the fungal growth was evaluated by the appearance of a clear zone between the bacterial disc and the fungus.

Test of ISR activity in peppers under greenhouse conditions

A series of greenhouse experiments was conducted to assess the ability of PGPR to control *X. axonopodis* infection using pepper seeds treated with either of the 3 PGPR strains (KUDC1013, KUDC1015, and KUDC1065). Pepper (*Capsicum annuum* L. cv. Bukang) seeds were surface-sterilized by soaking in 1.2% NaClO for 30 min, rinsing several times in excess tap water, and then air-drying on a sieve for 24 h at room temperature. The seeds were planted in a plug nursery pot (5×10 holes, 5 cm in diameter) filled with commercial soil. After vernalization for 2 days at 25°C in darkness, the pepper seedlings were placed in a greenhouse and were watered daily with tap water. An experiment was conducted to test whether treatment with the bacterial suspensions has different effects on ISR activity in pepper roots at different growth stages. Ten milliliters of a 10^8–10^9 CFU/ml bacterial suspension was applied as a soil drench on the rhizosphere of 3-week-old, 4-week-old, and 5-week-old pepper plants. The same volume of SDW was used as the negative control, while 0.5 mM Benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) and *Bacillus subtilis* GB03 were used as positive controls. Seven days after applying PGPR on the pepper roots, a 10^8 CFU/ml suspension of the phytopathogen *X. axonopodis* was sprayed on the pepper leaves. Immediately after inoculation, peppers were placed in a humidity chamber at 20–24°C and were incubated for 2 days. Ten days after pathogen challenge, disease severity was assessed on a scale from 1 to 5 according to the symptoms on the plant leaves: 1=no symptoms, 2=few lesions to 10% of the leaf area affected, 3=25% of the leaf area affected, 4=50–80% of the leaf area affected, and 5=>80% of the leaf area affected by lesions or dead leaf. The experiment was repeated twice with 20 replications each.

Assessment of plant growth promotion by Dokdo-derived strains in the field

The field experiment was conducted at the Kyungpook National University Farm, Daegu, Korea, from September to November 2009. Each plot was 65 cm wide and 400 cm long and covered with black plastic film; the plants were placed at 40 cm intervals. A randomized complete block (RCB) design was applied using 10 treatments with 3–4 replicates each.

The plants were subjected to the following treatments: non-bacterized control solution; individual PGPR strains E681, KUDC1013, and KUDC1065; 3 mixtures of PGPR strains (E681+KUDC1013, E681+KUDC1065, KUDC1013+KUDC1065); and 2 ISR inducers, namely, acetoin (3-hydroxy-2-butanone; Sigma-Aldrich Co., USA) and 2,3-butanediol (Sigma-Aldrich Co.).

To assess the plant growth promotion ability of the isolates under field conditions, 10^8–10^9 CFU/ml bacterial suspensions were applied on pepper roots by soaking and drenching. Two months after drenching, the growth promotion was evaluated by using 3 parameters: the height, fresh weight, and dry weight of peppers. The dry weight was measured after oven-drying the peppers at 65°C to constant weight.

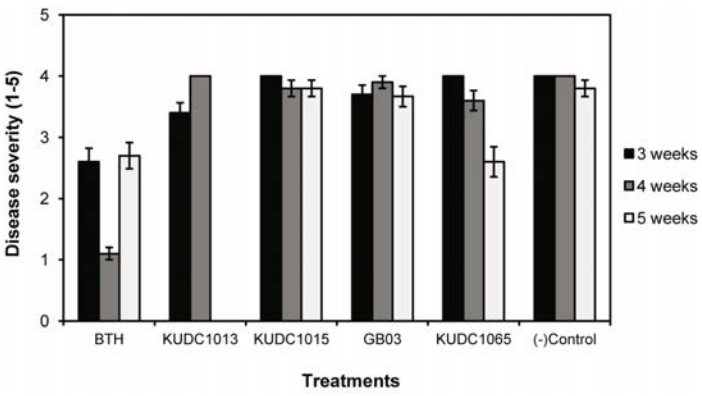

Fig. 1. Induction of systemic resistance to *X. axonopodis* pv. *vesicatoria* by the 2 isolates KUDC1013 and KUDC1065 in pepper plants according to the growth stages under greenhouse conditions. Ten milliliters of bacterial suspensions were used to drench 3-week-old, 4-week-old, and 5-week-old pepper seedlings. The same volumes of SDW and a chemical inducer, 0.5 mM BTH, were used as negative and positive controls, respectively. Seven days after application, the pathogen suspension was sprayed onto pepper leaves. Disease severity was assessed 7 days after pathogen challenge. The experiment was repeated twice with 20 replications for each treatment.

Induction of systemic resistance in pepper plants in the field

In this field trial, we used 2 methods of pathogen application: injection and spraying on the pepper leaves. The *X. axonopodis* suspension was maintained in a vial containing TSB supplemented with 15% glycerol; it was maintained at -70°C for long-term storage. For experimental use, *X. axonopodis* was transferred to TSA plates and incubated at 30°C for 2–3 days. The *X. axonopodis* inoculum was prepared by suspending cells from a 24 h TSA culture in SDW to a final concentration of 10^5–10^6 CFU/ml. The *X. axonopodis* suspension was applied twice by injection and spraying at a weekly interval. Seven days after drenching with PGPR suspension, the pathogen inoculum was injected with a needleless syringe into 3 pepper leaves per plant. One week after injection, the pathogen suspension (10^8 CFU/ml) was sprayed to run-off onto entire plants using a backpack CO_2 sprayer. Six days after injection, the disease severity was assessed by the method that was used at the laboratory level. Disease severity was measured using a disease rating scale from 0 to 5 (0=no lesions, 1=1–20% of the pepper plant covered with lesions, 2=20–40% of the pepper plant covered with lesions, 3=40–60% of the pepper plant covered with lesions, 4=60–80% of the pepper plant covered with lesions, and 5=80–100% of the pepper plant covered with lesions) according to the degree of brown leaf spot infestation caused by spraying the pathogen on peppers (Raupach and Kloepper, 2000). The experiment was repeated twice with 33 replications on an average.

Statistical analysis

Analysis of variance was used for the treatments and the means were separated by the least significant difference test at P=0.05 using the JMP software version 4.0.4 (SAS Institute, USA).

Results

Effect of PGPR strains on seed germination

We assessed the effects of treatment with single PGPR strains or mixtures of strains on the germination rate of pepper seeds. Almost 100% of the pepper seeds treated with single PGPR strains or mixtures of strains germinated; however, the seeds treated with BTH did not germinate (data not shown). Furthermore, the weight of germinated pepper seeds treated with 2,3-butanediol, acetoin, and KUDC1013 was 15.2%, 12.4%, and 7.3% respectively higher compared with the negative control seeds (Table 2).

Suppression of bacterial spot disease by the bacterial isolates under greenhouse conditions

Treatment with PGPR resulted in significant protection from bacterial spot disease as compared to the negative control treatment. Intriguingly, the 2 isolates, KUDC1013 and KUDC1065, yielded different results for different growth stages of the peppers on treatment with the bacterial suspension (Fig. 1). Drenching of 3-week-old pepper seedlings with KUDC1013 significantly reduced the disease symptoms. However, systemic resistance was not induced in 4-week-old and 5-week-old pepper seedlings treated with KUDC1013. Treatment with KUDC1065 resulted in significantly greater protection in 5-week-old pepper seedlings than in seedlings at other growth stages (Fig. 2). The 2 strains might be useful for the suppression of pathogens at various growth stages of peppers.

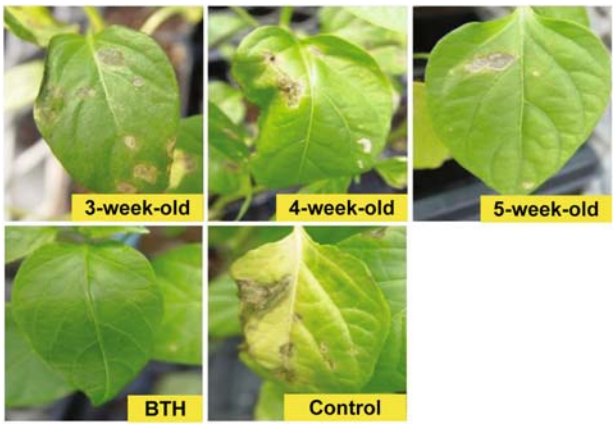

Fig. 2. Induction of protection from X. axonopodis by strain KUDC1065 in 3-week-old, 4-week-old, and 5-week-old pepper seedlings. Drenching of 5-week-old pepper seedlings with KUDC1065 was the most effective in reducing the disease symptoms.

Table 1. Effect of PGPR strains and their mixtures on the height, fresh weight, and dry weight of peppers under field conditions

Treatment[w]	Height (cm)[x]	Fresh weight (g)[y]	Dry weight (g)[z]
Control	27.08±0.31	30.03±3.55	5.02±0.47
BTH	24.06±0.45[b]	24.97±1.66[b]	3.80±0.20[b]
Acetoin	28.77±0.27[a]	36.26±2.52[a]	5.89±0.32[a]
2,3-Butanediol	28.75±0.23[a]	44.52±2.49[a]	7.20±0.34[a]
E681	27.52±0.25[a]	41.45±2.80[a]	6.49±0.43[a]
KUDC1013	29.23±0.23[a]	42.40±2.19[a]	6.50±0.29[a]
KUDC1065	30.11±0.30[a]	40.17±3.86[a]	6.48±0.31[a]
E681/KUDC1013	28.79±0.30[a]	55.73±2.95[a]	8.26±0.42[a]
E681/KUDC1065	27.94±0.27[a]	50.45±2.70 [a]	7.15±0.34[a]
KUDC1013/ KUDC1065	28.68±0.32[a]	46.57±2.44[a]	7.60±0.40[a]

[a,b] Parameters are significantly higher or lower than the control, respectively (P=0.05).
[w] Pepper plants were drenched with strains E681 (*P. polymyxa*), KUDC1013 (*O. lupini*), and KUDC1065 (*N. pentaromativorans*) alone or in combination or pepper roots were soaked with these strains twice at a weekly interval.
[x] Mean height was measured 1 week after drenching of bacterial suspension.
[y] Mean fresh weight in grams was measured 2 months after planting
[z] Mean dry weight in grams was measured 2 months after planting.

Promotion of pepper plant growth by Dokdo-derived strains

To assess the plant growth promotion capability of the isolates under field conditions, bacterial suspensions were applied to pepper roots by soaking and drenching. One week after drenching, growth promotion by PGPR strains was determined using 3 parameters: the height, fresh weight, and dry weight of peppers (Table 1). The peppers subjected to either of the 8 treatments were taller than those subjected to the negative control treatment. Of the 8 treatments, treatment with KUDC1065 resulted in the greatest height of peppers (Table 1). This result indicated that KUDC1065 might be effective in growth promotion with respect to the plant height.

Treatment with PGPR and the chemical inducers of systemic resistance increased the fresh and dry weights of peppers as compared to the control treatment. Plants treated with mixtures of E681 and KUDC1013, either in a two-way combination, were significantly heavier than those treated with E681, KUDC1013, or KUDC1065 alone. Of the 3 mixtures containing PGPR, the mixture containing E681 and KUDC1013 resulted in the highest effect on both fresh and dry weights. Further research is needed to discern the mechanisms underlying the plant growth promotion effects of the PGPR mixtures that were observed in our study.

Table 2. *In vitro* effects of treatment with single PGPR strains, mixtures of PGPR strains, and chemical inducers of systemic resistance on the weight of seeds after 5 days of incubation

Treatment	Weight of 100 germinated seeds (g)
Control	1.91
BTH	1.10
Acetoin	2.14
2,3-Butanediol	2.20
E681	1.90
KUDC1013	2.05
KUDC1065	1.81
E681/KUDC1013	1.71
E681/KUDC1065	1.57
KUDC1013/1065	1.89

Protection from bacterial spot disease in the field

We used 2 different types of application methods: infiltration and spraying on the pepper leaves. With the infiltration

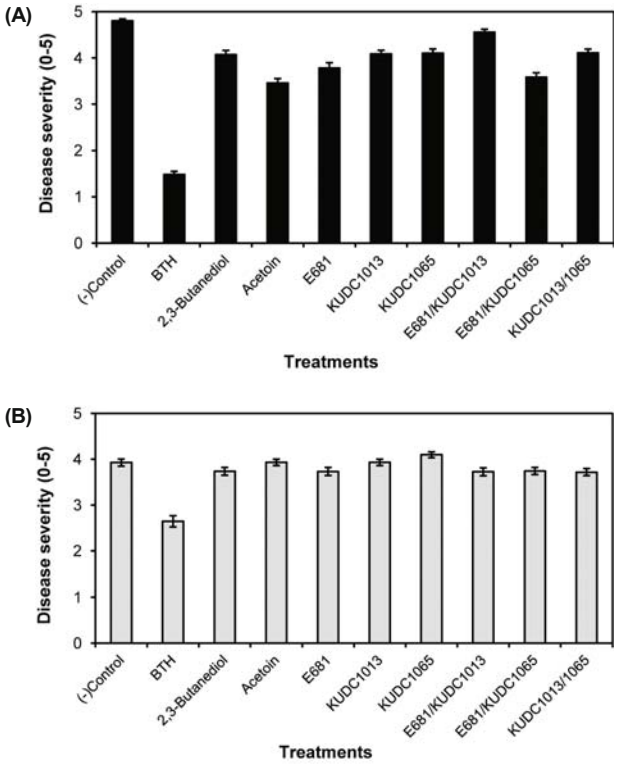

Fig. 3. Induction of disease protection by single PGPR strains and mixtures of strains in pepper plants after challenge with *X. axonopodis* under field conditions. (A) The disease severity was assessed 6 days after pathogen challenge by injection. (B) The disease severity was assessed 20 days after pathogen challenge by spraying. The disease severity was measured using a disease rating scale from 0 to 5 (0, no lesions; 1, 1–20% of the pepper plant covered with lesions; 2, 20–40% of the pepper plant covered with lesions; 3, 40–60% of the pepper plant covered with lesions; 4, 60–80% of the pepper plant covered with lesions, and 5, 80–100% of the pepper plant covered with lesions).

method, all PGPR treatments reduced the area of lesions in peppers. The greatest level of disease suppression in peppers was observed on treatment with the mixture containing E681 and KUDC1065 (Fig. 4). The degree of disease protection by the combinations of PGPR strains did not differ significantly with that of treatment using single strains.

With the spray method, most treatments generally did not have a statistically significant effect on ISR activity as compared to the negative control treatments. However, a trend of greater disease suppression was observed with mixtures of PGPR strains than with single strains. Although the results did not reveal a greater ISR effect on peppers under field conditions, they still indicated the potential of using mixtures of PGPR strains for protection from bacterial spot disease.

Discussion

The Dokdo-derived PGPR strains and their mixtures could induce systemic resistance to bacterial spot disease under field conditions. ISR mediated by strain KUDC1013 or KUDC1065 was associated with increased plant growth for most treatments. However, treatments that showed the highest reduction of disease severity were not the same as those that enhanced the highest plant growth. Protection activity against *X. axonopodis* of the mixtures of E681 and KUDC1065 was the most effective in eliciting systemic resistance in field conditions. PGPR can increase the protection of plants through secretion of antibiotics and through ISR effects (Jetiyanon and Kloepper, 2002; Ryu *et al*., 2003b). The strains KUDC1013 and KUDC1065 have no antifungal activity against the plant pathogenic fungi *A. niger* and *R. solani*. Absence of direct toxic effect by the PGPR against the challenging pathogen is one criterion for ISR activity. The absence of a direct harmful effect on the challenging pathogens suggests that KUDC1013 and KUDC1065 can increase plant defense responses by eliciting ISR.

Previous studies reported that treatment with PGPR strains improved the germination rate of seeds as compared to the negative control treatments (Ashrafuzzaman *et al*., 2009; Gholami *et al*., 2009). Treatments with 2, 3-butanediol, acetoin, and KUDC1013 resulted in 15.2%, 12.4%, and 7.3% higher weights than those of the negative control. In particular, the chemical inducers of systemic resistance, namely, 2,3-butanediol and acetoin, which were previously found to stimulate plant growth and elicit ISR (Ryu *et al*., 2003a, 2004, 2005), could be considered to promote plant growth during seed germination.

A trend of better plant growth was observed with mixtures of PGPR strains than with single strains. This result agrees with those of a previous study (Raupach and Kloepper, 1998). Pierson and Weller (1994) reported that treatment with mixtures of fluorescent pseudomonad strains improved wheat yield as compared to treatment with the same strains used individually. Treatment with mixtures containing the 2 strains IN937a and IN937b increased the yield of tomato, long cayenne pepper, and cucumber as compared to treatment with strain IN937a alone (Jetiyanon *et al*., 2003; Jetiyanon, 2007). All PGPR treatments significantly enhanced the growth of pepper plants in the field as compared to the non-bacterized control treatment. We found that treatment with mixtures containing E681 and KUDC1013 resulted in higher fresh and dry weights of peppers than treatment with the same strains used individually. We suggest that mixtures of strains could be more useful than individual strains for pepper.

PGPR significantly promote plant growth by fixing atmospheric nitrogen, improving essential nutrient uptake in plants, chelating iron from the soil, synthesizing several phytohormones, such as auxins, gibberellins, and cytokinins, that enhance various stages of plant growth, and synthesizing some enzymes modulating plant growth and development (Kloepper *et al*., 1986; Kloepper *et al*., 1989; Arora *et al*., 2001; Frey-Klett *et al*., 2005; Hameeda *et al*., 2006). We found that KUDC1013 and KUDC1065 can fix atmospheric nitrogen (data not shown). Previous studies have revealed that strains KUDC1013 and KUDC1065 can individually produce auxins and siderophores and have phosphate-solubilizing capacity (Ham *et al*., 2009). Further study is needed to determine the mechanisms underlying the plant growth promotion effects of PGPR mixtures that were observed in our study.

This study showed that pepper plants treated with mixtures of PGPR strains have significantly higher fresh and dry weights than those treated with single strains. All PGPR strains used induced systemic resistance against the bacterial spot disease with an increasing trend in disease suppression from single PGPR strains to PGPR mixtures. The disease protection conferred by the mixtures of strains used was not significantly different with that of the single strains. Strain KUDC1013 can elicit systemic protection from bacterial spot disease in young pepper seedlings whereas, the strain KUDC1065 showed ISR effects in old pepper seedlings. This shows that the strains have varied effects on pepper plants which may suggests that these strains have different mechanisms in eliciting resistance. Even if mixtures of PGPR strains do not always result in additive or synergistic effects, a possible advantage of mixtures of strains over single strains is that different strains may have different mechanisms and their combination in mixed applications would provide a greater spectrum of activity useful for plants (Raupach and Kloepper, 2000). We revealed that the treatment of pepper with mixtures of PGPR stains might be considered as potential biological control agents for growth promotion and protection from bacterial spot disease under greenhouse and field conditions.

Acknowledgements

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