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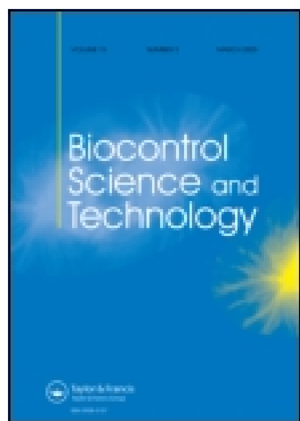

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Biological control of bacterial spot disease and plant growth-promoting effects of lactic acid bacteria on pepper

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RESEARCH ARTICLE

Biological control of bacterial spot disease and plant growth-promoting effects of lactic acid bacteria on pepper

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In our previous studies, we observed the biological control effect of lactic acid bacteria strains (LABs) KLF01, KLC02 and KPD03 against different plant pathogenic bacteria *in vitro* against *Ralstonia solanacearum*, and strains KLF01 and KLC02 against *Pectobacterium carotovorum* under greenhouse and field experiments, respectively. In this study, we observed the efficacy of these bacteria against bacterial spot pathogen (*Xanthomonas campestris* pv. *vesicatoria*) and their plant growth-promoting activities in pepper (*Capsicum annuum* L. var. *annuum*), under greenhouse and field conditions. LABs significantly ($P < 0.05$) reduced bacterial spot on pepper plants in comparison to untreated plants in both the greenhouse and the field experiments. The plant growth-promoting effect of LABs on pepper varied; some strains had a significant effect on growth promotion ($P < 0.05$) compared with untreated plants, while some showed no significant effect in the greenhouse and field experiments. Additionally, LABs were able to colonise roots, produce indole-3-acetic acid (IAA), siderophores and solubilise phosphate. These findings indicate that application of LABs could provide a promising alternative for the management of bacterial spot disease in pepper plants and could therefore be used as a healthy plant growth-promoting agent.

Keywords: bacterial spot; lactic acid bacteria; plant growth promotion; *Xanthomonas campestris* pv. *vesicatoria*

Introduction

Bacterial spot of pepper (*Capsicum annuum* L. var. *annuum*) caused by *Xanthomonas campestris* pv. *vesicatoria* (Jones, Lacy, Bouzar, Stall, & Schaad, 2004) is distributed worldwide and is destructive for pepper production. It is characterised by irregular yellow necrotic areas on pepper leaves and ultimately affects almost all parts of the plants such as stems, leaves and fruits (Sherf & MacNab, 1986). Various practices have been implemented to control this disease including sanitation, use of pathogen-free seed and other cultural strategies (Goode & Sasser, 1980; Jones, Jones, Stall, & Zitter, 1991; Mew & Natural, 1993; Sherf & MacNab, 1986). Various tactics have been used for biological control of *X. campestris* pv. *vesicatoria*, including an *hrp* mutant of this pathogen (Moss et al., 2007), antagonist microbes such as *Rahnella aquatilis* (El-Hendawy, Osmaon, & Sorour, 2005) and *Bacillus* strains (Mirik, Aysan,

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& Cinar, 2008). Integrated use of rhizobacteria or biological control strains with harpin or acibenzolar-*S*-methyl (Abo-Elyousr & El-Hendawy, 2008; Fayette, Roberts, Pernezny, & Jones, 2012; Obradovic et al., 2005) and fertilizers (e.g. ammonium lignosulfonate, potassium phosphate; Abbasi, Soltani, Cuppels, & Lazarovits, 2002) is another approach. Phosphorus acid salts (Wen, Balogh, Momol, Olson, & Jones, 2009) and plant essential oils are also used as biocontrol agents against *X. campestris* pv. *vesicatoria* (Lucas, Alves, Pereira, Perina, & de Souza, 2012).

Lactic acid bacteria strains (LABs) are known as probiotic organisms and are generally recognised as safe (Stiles & Holzapfel, 1997). LABs are reported to produce various antibacterial compounds, such as acetic acid, lactic acid (Ariyapitipun, Mustapha, & Clarke, 1999), hydrogen peroxide (Chang, Kim, & Shin, 1997), several bacteriocins (Klaenhammer, 1988) and even antifungal compounds (Axel et al., 2012; Hamed, Moustafa, & Abdel-Aziz, 2011; Laitila, Alakomi, Raaska, Mattila-Sandholm, & Haikara, 2002; Visser, Holzapfel, Bezuidenhout, & Kotze, 1986). Interestingly, LABs cultures or their supernatants have been used as biological control agents on plant diseases in chilli, tomato and cucumber caused by the fungi *Colletotrichum capsici* (El-Mabrok, Hassan, Mokhtar, Hussain, & Kahar, 2012), *Fusarium oxysporum* and *Pythium ultimum*, respectively (Hamed et al., 2011; Lutz, Michel, Martinez, & Camps, 2012). Several species of LABs have been recognised as producers of bioactive metabolites that act against a broad spectrum of undesirable microorganisms such as fungi, oomycetes and other bacteria (Axel et al., 2012). Moreover, significant effects of LABs (KLF01 and KLC02) against soil-borne diseases (e.g. bacterial wilt and bacterial soft rot) were shown in our previous studies (Shrestha, Choi, Lim, Hur, & Cho, 2009a; Shrestha et al., 2009b). Therefore, we investigated biological control effects of LABs against *X. campestris* pv. *vesicatoria* to observe whether these bacteria are equally effective against air-borne disease.

Plant growth-promoting bacteria are reported to produce siderophores (Bullen, Rogers, & Griffiths, 1978; Crosa, 1984; Matzanke, Müller, & Raymond, 1984; Neilands, 1981), volatile compounds (Ryu, Hu, Reddy, & Kloepper, 2003) and phytohormones (Idris, Iglesias, Talon, & Borriss, 2007; Patten & Glick, 1996; Swain, Naskar, & Ray, 2007). These beneficial bacteria can solubilise phosphate (Angerer, Klupp, & Braun, 1992; Leong, 1986; Milagres, Machuca, & Napoleão, 1999) and colonise plant roots (Kloepper, Ryu, & Zhang, 2004; Timmusk & Wagner, 1999; Yao et al., 2006). Recently, LABs isolated from organic agricultural soils have been reported to show biological control activity and plant growth-promoting activity in cabbage and tomato seedlings (Lutz et al., 2012; Somers, Amke, Croonenborghs, Overbeek, & Vanderleyden, 2007).

There is limited information available on characterisation of the potential plant growth-promoting and antibacterial activity of LABs against *X. campestris* pv. *vesicatoria*. Therefore, in this study we evaluated (1) the biological control effects of LABs against bacterial spot disease and (2) the growth-promoting activity of LABs in pepper plants under greenhouse and field conditions.

Materials and methods

Bacterial strain, culture and preservation

LABs (KLF01, KLC02 and KPD03) were routinely grown in de Man-Rogosa-Sharpe (MRS) agar plates and stored in nutrient broth containing 20% glycerol at

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−70°C. Phytopathogenic bacterium *X. campestris pv. vesicatoria* KACC (Korean Agricultural Culture Collection) 1157 was routinely cultured in Yeast extract Dextrose Calcium carbonate (YDC) medium.

Antagonistic effect of LABs

Initial screening of antagonistic effects of LABs against *X. campestris* pv. *vesicatoria* was performed using the agar well diffusion method as described by Benkerroum and Sandine (1988) with some modifications. Briefly, MRS agar plates were overlaid with 7 mL soft mannitol glutamate yeast extract agar (containing 0.75% agar) inoculated with 100 μL of the overnight growth culture of the pathogenic bacterium and incubated for 3 h. After incubation, wells were punched out of the agar and 10 μL of streptomycin (200 ppm as a positive control), water (negative control) and 10 μL LABs were poured separately into each well. Antibacterial activity was assayed by observing inhibitory zones in the background of the LABs after 12 and 24 h of incubation. Each assay was performed in triplicate. The degree of antagonism shown was determined by measuring the average diameter of the clear zone of inhibition: −, no inhibition (<1 mm); +, weak inhibition (<5 mm); ++, mild inhibition (=5 mm); and +++, strong inhibition (>10 mm).

Greenhouse test

Pepper (cultivar 'Buja') seedlings were grown in a greenhouse in 3 × 3 × 5 cm plug trays filled with commercial potting mixture. After 3 weeks, they were transplanted into 10 cm pots and watered daily before reaching the four-leaf stage. Six- to 7-week-old pepper plants were incubated in the greenhouse at high relative humidity for 24 h prior to LABs treatment. The experiment was carried out in a completely randomised block design with six plants as replicates in each of the following treatments against *X. campestris* pv. *vesicatoria*: (1) water control, (2) copper hydroxide, 200 ppm (3) KLF01, 4.28×10^3 CFU mL^{-1}; (4) KLC02, 3.15×10^4 CFU mL^{-1}, and (5) KPD03, 2.5×10^3 CFU mL^{-1}. Treatments were administered to pepper plants for 3 consecutive weeks by spray inoculation onto the abaxial and adaxial leaf surfaces and by 10 mL drench inoculation. After the last treatment, 10 mL of *X. campestris* pv. *vesicatoria* (1×10^8 CFU mL^{-1}) along with 0.01% Silwet was applied (foliar treatment) to all treated and control plants. Ten pepper leaflets per replicate were randomly sampled 10 days after inoculation with the pathogen. Lesions on individual leaflets were counted and quantified, based on the method presented in previous studies (Byrne et al., 2005; Moss et al., 2007). Reduction in disease severity was evaluated in comparison to the untreated control using the following formula:

$$\text{Disease reduction}\,(\%) = \frac{\text{Disease severity}_{\text{control}} - \text{Disease severity}_{\text{treatment}}}{\text{Disease severity}_{\text{control}}} \times 100$$

Field test

The field test was carried out from early July to late October 2011 in the experimental fields of Chuncheon city (Site A) and Hongcheon county (Site B), Republic of Korea. The LABs inocula (biological control treatment) were sprayed onto the leaves, and 10 mL drench inoculation was applied to pepper roots for 3 consecutive weeks. Pepper plants were artificially inoculated with *X. campestris* pv. *vesicatoria* 1×10^8 CFU mL^{-1} after the last treatment, and foliar disease severity

was determined by counting lesions on each of 50 randomly sampled leaflets per replicate at 20 days after inoculation.

Plant growth promotion under greenhouse conditions

On the basis of antagonistic activity results from *in vitro* experiments and preliminary shoot and root elongation assay, the three LABs were further investigated in a greenhouse pot trial for their plant growth-promoting effects. Pepper seeds were sown on trays containing commercially available potting mixture. The seeds were watered regularly until seedling emergence was complete. Seedlings were then transferred to 12 × 10 cm diameter pots containing a manure and soil mixture. One week after transplantation, 100 mL of the bacterial inocula was applied as spray and soil drench treatment for 4 consecutive weeks. Control plants were inoculated with distilled water (DW) only. The pots were watered twice daily with a sprayer. The experiment was arranged in a randomised block design with three replicates. The following plant growth-promoting parameters were analyzed after 6 weeks: shoot height, shoot fresh weight, shoot dry weight, root fresh weight, root dry weight and leaf chlorophyll. Chlorophyll content was measured with a SPAD 502 Chlorophyll Meter (Konica Minolta Sensing Americas, Inc., made in Japan) and expressed in SPAD units.

Plant growth promotion under field conditions

Thirty seedlings chosen randomly from each flat of pepper plants were transplanted into field plots on 15 May 2011 at the experimental farm of Kangwon National University, Republic of Korea. The field plots consisted of three raised beds (20 cm high × 60 cm wide, spaced 1 m apart on centre), covered with plastic mulch. Seepage irrigation practices were used, and all plots were sprayed weekly with the LABs. Each field plot was 15 m long × 2 m wide, and 30 seedlings per treatment were planted in each plot: three replicates were sampled for analysis of growth parameters. Field treatments were initiated 6 weeks after transplanting. Pepper plants were subjected to LABs via foliar application and 10 mL drench application; the first application of LABs was on 1 July followed by application on 15, 21 and 28 July. Increase in shoot length and chlorophyll content was measured similar as in the greenhouse experiments. The first observation was performed 2 weeks after inoculation with the first treatment; the second observation was made 1 week after inoculation with third treatment. During the observation, plant height, chlorophyll content and marketable fruit were recorded.

Detection of plant growth-promoting parameters

Pepper seedlings were grown in sterile soil and treated with LABs for 4 consecutive weeks. The roots were then washed carefully with sterile water. Root tips were stored in 4% glutaraldehyde for 1–2 h, after which simple staining was performed using 0.1% safranin dye. The stained root segments were examined microscopically (40×) using phase contrast microscopy with an Olympus BX50F-3™ (Olympus Optical Co. Ltd., Japan).

Siderophore production was detected by the universal chemical assay using chrome azurol S (CAS) agar (Schwyn & Neilands, 1987). Cultures of selected LABs were grown in MRS medium at 37 ± 2°C for 24 h at 200 rpm on a rotary shaker. Three equidistant wells were made on the CAS agar plate using a cork borer, after

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which 20 μL of bacterial culture filtrate was deposited into the wells. Control plates received sterile broth media without bacteria. The plates were incubated at 37°C for 24, 48 and 72 h, and changes in the medium were recorded.

For indole-3-acetic acid (IAA) production, strains were grown in MRS broth for 24 h, and 20 μL aliquots were transferred into 50 mL flasks containing 10 mL of MRS broth supplemented to reach 50, 200 and 500 μg mL^{-1} of L-troptophan Sigma™ (Patten & Glick, 1996). Flasks were then incubated at 37°C and 150 rpm on an orbital shaker and samples were taken after 24, 48 and 120 h after inoculation. Optical density (OD) at 630 nm was recorded as an indicator of growth and an aliquot of each flask was centrifuged (12,000 rpm) to remove bacterial cells. One millilitre of supernatant was mixed with 4 mL of Salkowski reagent (150 mL of 18 M H_2SO_4, 250 mL DW, 7.5 mL of 0.5 M $FeCl_3{\cdot}6H_20$) and absorbance at OD 535 nm was measured after 20-min incubation (Patten & Glick, 1996). IAA concentration was estimated from a standard curve spiked with IAA (Sigma I-2886) and was expressed as microgram per millilitre (Sarwar & Kremer, 1995).

Phosphate-solubilising activity of the selected LABs was detected by plate assay using Pikovskaya (PVK) agar (Pikovskaya, 1948) which results in clear halo formation. A pure colony from fresh culture of each LABs was stab inoculated in duplicate into PVK agar media using a sterile wooden stick (Puritan, Guilford, ME). The diameter of the resulting clear halo was measured after 24-, 48-, 72- and 96-h incubation of plates at 37°C. Control plates were inoculated with sterile MRS broth.

Statistical analysis

Statistical analysis was conducted using the Statistical Analysis System (SAS; SAS Institute, Cary, NC). The greenhouse and field experiments on disease severity were conducted in a completely randomised block design. Disease severity data were log transformed and subjected to analysis of variance using general linear model (GLM) procedures, and means were separated using least significant difference (LSD) tests. The greenhouse and field experiments on plant growth promotion were arranged in a randomised block design with three replications. Analysis of variance was conducted using GLM procedures and means were separated with LSD tests at $P < 0.05$.

Results

Antagonistic effect of LABs

All three LAB strains tested showed inhibitory effect against *X. campestris* pv. *vesicatoria* (Figure 1), as determined by clear zone on MRS agar plates. The antagonistic activity of the LABs persisted for different time intervals (Figure 1).

Effect of LABs on reduction of bacterial spot disease in pepper

All LABs significantly reduced the severity of foliar disease in greenhouse conditions. The greatest disease reduction was shown by strain KLF01 (73.9% in comparison to the control). Strains KLC02 and KPD03 showed 57% and 62% disease reduction, respectively (Table 1). The efficacy of disease control by the LABs was evaluated under field conditions at two sites, Site A and Site B (Table 1). In Site A, strain KLC02 showed the highest reduction (86.7%), while at Site B, KLF01

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reduced disease severity the most (94.5%). All LABs significantly ($P < 0.05$) reduced disease severity in the field experiments.

Effect of LABs on plant growth-promoting parameters under greenhouse and field conditions

The greenhouse assay revealed that LABs significantly ($P < 0.05$) increased root length, shoot length, root fresh weight and chlorophyll in pepper plants (Table 2). However, the root dry weight, shoot fresh weight and dry weight did not differ significantly ($P > 0.05$) from untreated plants.

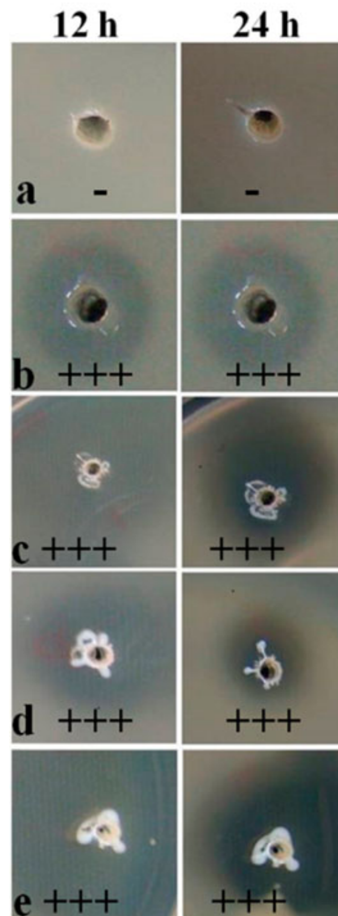

Figure 1. (Colour online) Agar well diffusion method to observe antibacterial activity of the KLF01, KLC02 and KPD03 LAB strains against *Xanthomonas campestris* pv. *vesicatoria*, at different time intervals with treatments (a) control, (b) streptomycin, 200 ppm, (c) KLF01, (d) KLC02 and (e) KPD03. –, no inhibition (<1 mm); +, weak inhibition (<5 mm); ++, mild inhibition (=5 mm); +++, strong inhibition (>10 mm). Distilled water (DW) was used as the control. The antibacterial activity was assayed by observing inhibitory zones in the background of the LABs at different time intervals after 12 and 24 h of incubation.

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Table 1. Efficacy and consistency of foliar severity reduction of bacterial spot on pepper by LABs in greenhouse and field experiments.

	Greenhouse[b]		Field[c]			
Treatments[a]	Reduction[e], %		Chuncheon	Reduction[e], %	Hongcheon	Reduction[e], %
Control	14.2 ± 3.1[d] a		39.7 ± 8.3 a		23.8 ± 9.5 a	
CH	4.5 ± 2.6 b	63.7	11.8 ± 4.7 b	70.2	5.0 ± 1.3 bc	78.8
KLF01	3.5 ± 1.4 b	73.9	5.8 ± 1.7 bc	85.4	1.3 ± 0.1 c	94.5
KLC02	5.5 ± 4.1 b	57.5	5.3 ± 2.0 c	86.7	2.2 ± 0.3 c	90.7
KPD03	5.0 ± 3.3 b	62.1	8.7 ± 2.0 bc	78.1	2.5 ± 0.9 c	89.7

[a]Treatments were applied to pepper plants for 3 consecutive weeks before pathogen inoculation.
[b]Foliar disease severity under greenhouse conditions was assessed as lesions/leaflets. Eight to ten days after inoculation with *X. campestris* pv. *vesictoria* three to six pepper leaflets per plant were sampled arbitrarily and lesion numbers were determined for individual leaflets. Foliar disease severity was assessed 10 days after inoculation with the pathogen.
[c]Foliar disease severity under field (Chuncheon and Hongcheon) conditions was assessed as lesions/leaflets. 50 pepper leaflets per plant were sampled arbitrarily and lesion numbers were determined for individual leaflets. Disease severity was assessed 10 days after inoculation with the pathogen.
[d]Means ± standard error in a column followed by the same letter are not significantly different according to LSD at $P < 0.05$. Log transformed data were subjected to analysis of variance using GLM procedure. However, untransformed data are presented here.
[e]Disease reduction (%) compared with the untreated control.

Shoot and root length in pepper plants increased by 4–23% after treatment with LABs, except in case of shoot length treated with KLC02. Of the three strains, KLF01 was associated with the greatest increase in shoot length (23%) and chlorophyll content (15%). There was no effect on dry shoot and root weight in pepper plants treated with LABs.

In the field experiment, LABs-treated plants showed significantly ($P < 0.05$) increased shoot length (Table 3) in first observation (2 weeks after the first treatment) but in second observation (1 week after the third treatment), only KLF01-treated plants showed this increase. Maximum and minimum shoot lengths were 135 and 106 cm, respectively, in pepper plants treated with LABs 2 weeks after the first treatment, and 138 and 124 cm, respectively, in plants 1 week after the third treatment (Table 3). A 3–16% increase in shoot length was observed in treated pepper plants compared to untreated plants. Similarly, chlorophyll content was enhanced by 11–19% in plants treated with LABs. Strain KLF01 was associated with the greatest increase in shoot length, chlorophyll content shoot and root fresh weight. The greatest increases in height were observed in plants treated with strain KLF01: approximately 27% at the first observation and 12% at the second observation. The greatest increase in chlorophyll content was found in plants treated with strains KPD03 (19%) and KLC02 (15%) at the first and second observation, respectively. An overall increase of 15–47% was observed in marketable pepper harvest (Table 3).

Root colonisation, siderophores, IAA and phosphate-solubilising activity of LABs

All three LABs resulted in significant growth promotion and were able to successfully colonise the roots. Simple staining of the root tip initially fixed in 4% glutaraldehyde

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Table 2. Effects of LABs on different growth parameters of pepper under greenhouse conditions.

Treatments[a]	Shoot length (cm)	Root length (cm)	Shoot fresh weight (g)	Shoot dry weight (g)	Root fresh weight (g)	root dry weight (g)	Chlorophyll (SPAD units)
Untreated control	59.33 ± 0.80 b[b]	24.10 ± 0.67 b	40.67 ± 0.67 a	6.67 ± 0.88 a	7.67 ± 0.33 b	1.17 ± 0.17 a	34.63 ± 0.72 c
KLF01	73.33 ± 1.76 a	27.60 ± 0.57 a	43.17 ± 0.88 a	7.50 ± 0.76 a	12.00 ± 0.29 a	1.33 ± 0.17 a	39.87 ± 1.46 bc
KLC02	62.00 ± 1.15 b	29.57 ± 0.81 a	42.67 ± 0.88 a	8.17 ± 0.60 a	8.83 ± 0.67 a	1.17 ± 0.17 a	38.97 ± 0.64 ab
KPD03	72.67 ± 1.20 a	27.70 ± 0.46 a	45.67 ± 3.48 a	7.50 ± 0.58 a	8.83 ± 0.44 a	1.33 ± 0.17 a	36.47 ± 0.23 a

[a]Plants were treated with bacteria strains, at cell concentrations at 100× times dilutions and water (untreated control).
[b]Means ± standard error within a column followed by the same letter are not significantly different according to LSD test at $P < 0.05$. These experiments were performed with three replicates.

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Table 3. Effect of LABs on height, chlorophyll content and fruit production of pepper plant under field conditions.

	Height (cm)		Chlorophyll (SPAD units)		Number of pepper fruit[b]	
Treatments[a]	First observation	Second observation	First observation	Second observation	Harvest-1	Harvest-2
Untreated control	106.97 ± 2.48[c] c	124.07 ± 2.24 b	54.10 ± 2.05 b	55.90 ± 2.00 b	13 ± 1 c	17 ± 1 c
KLF01	135.87 ± 2.73 a	138.93 ± 3.84 a	62.90 ± 0.26 a	62.53 ± 1.60 a	17 ± 0 ab	20 ± 1 b
KLC02	126.97 ± 2.85 b	135.50 ± 3.28 ab	63.03 ± 0.23 a	64.17 ± 1.13 a	15 ± 1 bc	24 ± 1 a
KPD03	121.83 ± 1.58 b	128.83 ± 0.90 b	64.73 ± 1.15 a	62.90 ± 1.67 a	18 ± 1 a	25 ± 0 a

[a]Plant roots and leaves were treated with bacteria strains and water (untreated control).
The first treatment was performed on 1 July following second on 15 July, third treatment in 21 July and fourth treatment in 28 July.
The first observation was recorded 1-week post inoculation with the third treatment and second observation was recorded after 2-weeks post inoculation with the fourth treatment.
[b]Numbers of marketable pepper fruits. Fruits were harvested on 24 July and on 28 August.
[c]Means ± standard error within a column followed by the same letter are not significantly different according to LSD test at $P < 0.05$. These experiments were carried out with three replicates.

was visualised by light microscopy. Motile rod- and cocci-shaped bacteria were observed colonising the root surface (Figure 2(A)). When the root segment was viewed through 100× light microscopy, we observed rod-shaped KLF01 and cocci-shaped KLC02 and KPD03. Such characteristics were not observed in control roots (Figure 2(A)).

The LABs produced siderophores on CAS agar plates (Figure 2(B)) confirmed by a change in colour of plates from blue to yellow as a result of siderophores sequestering and binding iron from the medium. This ability was increased as long as incubation time was increased in this study. Among the three strains, KPD03 showed the strongest effect.

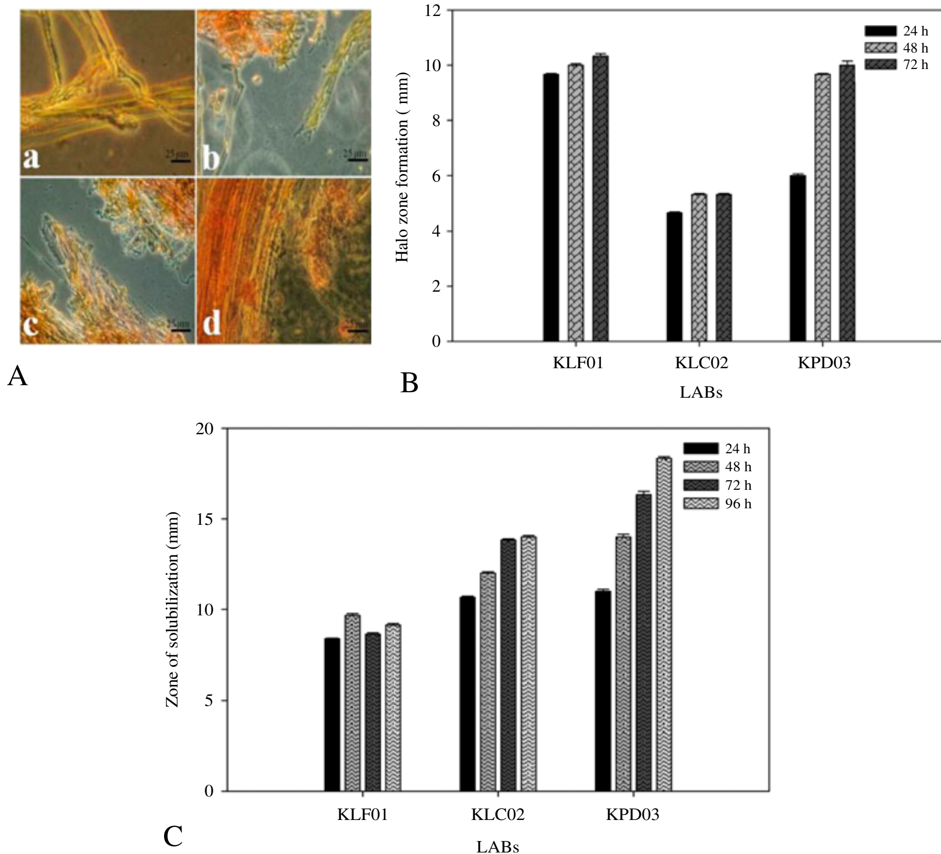

Figure 2. (Colour online) (A) Phase contrast micrographs showing colonisation by LABs: (a) non-inoculated pepper seedlings control, seedlings inoculated with (b) KLF01, (c) KLC02, and (d) KPD03 on the roots of 2-week-old pepper plants. LABs showed colonisation on their root surface, especially at sites of lateral root. (B) Graphic representation of CAS assay performed with LABs. Colour change in the CAS agar was observed at 24, 48 and 72 h, respectively. Experiments were carried out in three replicates. DW was used as control. (C) Graphic representation of detection of halo zone in PVK agar at different time intervals 24, 48, 72 and 96 h, respectively. Experiments were carried out in six replicates. DW was used as the control.

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Table 4. Production of indole-3-acetic acid (IAA) by LABs in the presence of various concentrations of tryptophan.

LABs	Tryptophan concentration (μg/mL)	IAA production (OD at 530 nm unit) 24 h	48 h	120 h
KLF01				
	0	0.26 ± 0.002	0.41 ± 0.003	0.03 ± 0.006
	50	0.30 ± 0.001	0.41 ± 0.001	0.34 ± 0.001
	200	0.33 ± 0.002	0.47 ± 0.001	0.39 ± 0.002
	500	0.37 ± 0.001	0.56 ± 0.000	0.48 ± 0.001
KLC02				
	0	0.38 ± 0.001	0.34 ± 0.001	0.36 ± 0.005
	50	0.34 ± 0.001	0.33 ± 0.001	0.35 ± 0.005
	200	0.37 ± 0.010	0.35 ± 0.001	0.38 ± 0.002
	500	0.40 ± 0.002	0.46 ± 0.033	0.41 ± 0.002
KPD03				
	0	0.28 ± 0.001	0.40 ± 0.000	0.32 ± 0.000
	50	0.30 ± 0.009	0.39 ± 0.002	0.33 ± 0.014
	200	0.33 ± 0.002	0.42 ± 0.000	0.34 ± 0.007
	500	0.30 ± 0.002	0.49 ± 0.001	0.38 ± 0.001

Note: IAA indole acetic acid ± standard error.
The experiments were performed with three replicates (n = 3).

LABs also produced IAA in the presence of different concentrations of tryptophan (Table 4). The highest amount of IAA was produced by KLF01 at 48 h of incubation in presence of 500 μg mL^{-1} tryptophan followed in decreasing order by KPD03 and KLC02.

In addition, LABs solubilised tri-calcium phosphate on PVK agar medium. Strain KPD03 showed the highest solubilising effect, rendering an 18.5-mm diameter clear zone (Figure 2(C)).

Discussion

In previous studies, we reported that LABs (KLF01 and KLC02) could be used against different plant pathogenic bacteria, including *Ralstonia solanacearum* (Shrestha et al., 2009a) and *Pectobacterium carotovorum* subsp. *carotovorum* (Shrestha et al., 2009b) as effective biological control agents. In the present study, we further investigated whether LABs have biocontrol activity against bacterial spot disease caused by *X. campestris* pv. *vesicatoria*, and their plant growth-promoting effects in pepper under greenhouse and field conditions. Although various control measures using chemicals, resistant mutants and antibiotics (Fayette et al., 2012; McCarter, 1992; Moss et al., 2007) are used against *X. campestris* pv. *vesicatoria*, frequent occurrence of resistance to those control agents in different races of this pathogen has led to concerns about adequate control methods (Bouzar et al., 1999; Dahlbeck & Stall, 1979; Gassmann et al., 2000; McCarter, 1992; Mirik, Aysan, & Cinar, 2007; Stall & Thayer, 1962). The reduction in efficacy of bactericides, chemicals and antibiotics due to resistant pathogen strains is forcing the development of alternative strategies for combating bacterial diseases in vegetable crops. The use

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of LABs as a biological treatment against bacterial spot caused by *X. campestris* pv. *vesicatoria*, as reported here, may be an effective method for controlling this pathogen.

Based on greenhouse conditions, strains KLF01 and KPD03 showed better growth-promoting effects than KLC02 in pepper plants, while KLC02 showed better results in the field. Inconsistencies between greenhouse and field experiments may be a result of different abiotic and biotic factors, including environmental parameters (e.g. soil texture and nutrient content), root colonisation, competition and production of antagonistic metabolites. *Lactococcus lactis* isolated from organic agricultural soil was also reported to show plant growth-promoting activity in cabbage (Somers et al., 2007) and some LABs demonstrated growth-promoting effects on cucumber, and tomato seedlings (Lutz et al., 2012). Treatment of *C. annuum* L. var. *annuum* with mixtures of plant growth-promoting rhizobacterial strains might be considered as a potential means of biological control for promoting growth and for protecting plants from bacterial spot disease under both greenhouse and field conditions (Hahm et al., 2012). Rhizosphere competence is an important prerequisite for an effective biological control. Soil-borne diseases have been controlled using beneficial bacteria that are indigenous to the rhizosphere of plants (Thomashow, 1996). The root-colonising capacity of the LABs studied here may also be cause of their plant growth-promoting effects (Figure 2(A)). Root-colonising capacity of LABs defines their capacity to control bacterial soft rot caused by *Pectobacterium* (Shrestha et al., 2009b). LABs have been isolated from soil (Chen, Yanagida, & Shinohara, 2005), vegetables surfaces (Trias, Baneras, Montesinous, & Badosa, 2008) and the rhizosphere, suggesting that they may have the ability to colonise plant roots. Root colonisation by *Bacillus* and *Pseudomonas* has been reported in previous studies (Espinosa-Urgel, Kolter, & Ramos, 2002; Kloepper et al., 2004). In addition, these LABs are reported to be natural colonisers of fresh fruit and as a useful antagonist of several bacterial and fungal species in different food products (Batish, Roy, Lal, & Grower, 1997). Although lactic acid bacteria are usually considered to be non-motile, *Lactobacillus ghanensis* sp. nov. isolated from Ghanian cocoa (Nielsen et al., 2007) and *Lactobacillus sucicola* sp. nov. isolated from oak trees (Irisawa & Okada, 2009) have been reported as a motile, consistent with the motility shown by the LABs used in the present study.

Many rhizobacteria produce siderophores (Glick, 1995), which can limit the growth of plant pathogens via iron deprivation (Neilands & Leong, 1986). Many earlier studies attempting to examine siderophore production in LABs were unsuccessful (Imbert & Blondeau, 1998; Pandey, Bringel, & Meyer, 1994). However, the genome of two *Lactococcus lactis* strains isolated from vegetables showed non-ribosomal peptide pathways, suggesting that LABs can produce siderophores like other bacterial strains. In addition, genes involved in iron transport were reported in these LABs (Duhutrel et al., 2010). Previously, there was doubt that the change in colour of CAS agar induced by LABs was due to low pH (Pandey et al., 1994). Thus, before performing the assays we adjusted the pH to neutral (7.0) in all experiments. Several studies have demonstrated that production of siderophores by plant growth-promoting bacteria was the most effective mechanism in phytopathogens controls. Therefore, such siderophore production shown by LABs supports their antibacterial action against *X. campestris* pv. *vesicatoria*.

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Phosphate-solubilising bacteria play an important role in plant nutrition by increasing phosphorus uptake and are used as plant growth promoters Phosphate-solubilising activity of the three LABs examined here was evident (Figure 2(C)), and may have contributed to the increase in plant height, weight and chlorophyll content of plants treated with LABs.

We have also shown that these gram-positive lactic acid bacteria can produce and secrete significant amounts of IAA (Table 4), a phytohormone that is important in promoting growth. This phyothormone is reported to enhance both rapid (e.g. increases in cell elongation) and long-term (e.g. cell division and differentiation) responses in plants (Cleland, 1990; Hagen, 1990). The production of auxins in the presence of a suitable precursor such as L-tryptophan also plays a pivotal role in plant growth promotion. IAA may function as an important signalling molecule in the regulation of plant development. These findings suggest that plant growth promotion of LABs is due to their ability to produce IAA. Different strains of IAA-producing *Lactobacillus acidophilus* have been reported to render antagonistic effect towards selected food-contaminating bacteria (Klewicka & Libudzisz, 2004).

Numerous plant growth-promoting rhizobacteria have been used and proven to be an effective biological control agents (Bhattacharyya & Jha, 2012). It is well known that promotion of plant growth can be achieved directly by enhancing uptake of minerals and nutrients or by regulating plant hormones such as IAA, cytokinin and ethylene (Mia, Shamsuddin, Wahab, & Marziah, 2010; Zhang et al., 2010). Our results show that LABs facilitate plant uptake of phosphate and production of IAA, which helps in root elongation leading to plant growth. Lactic acid compounds produced by rhizobacteria *Pseudomonas putida* were found to be responsible for promoting plant growth in *Asparagus officinalis* L. (Yoshikawa, Hirai, Wakabayashi, Sugizaki, & Iwamura, 1993), suggesting that similar mechanism may be present for the LABs examined in the present study. Different bacterial strains showing plant growth-promoting effects are equally effective in triggering induced systemic resistance, an innate plant immune system against different phytopathogens (Raupach, Liu, Murphy, Tuzun, & Kloepper, 1996; Vallad & Goodman, 2004; Van Loon, Bakker, & Pieterse, 1998).

The findings of the present study suggest that LABs have great efficacy for increasing growth of pepper plant. However, field experiments that examine the mechanisms behind this growth-promoting effect are required. Moreover, our results show that LABs can improve plant growth and suppress bacterial spot in pepper as well as bacterial wilt (*R. solanacearum*) in pepper and tomato and soft rot (*P. carotovorum* subsp *carotovorum*) in cabbage. LABs can suppress both soil-borne (Shrestha et al., 2009a, 2009b) and air-borne bacterial pathogens (this study), suggesting their potential ability to be used as effective biological control agents. Therefore, the application of LABs can be further developed as a stable approach for managing bacterial spot and promoting growth in pepper plant.

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