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Pseudomonas sp. LSW25R, antagonistic to plant pathogens, promoted plant growth, and reduced blossom-end rot of tomato fruits in a hydroponic system

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Abstract A Gram-negative rhizobacterial isolate (LSW25) antagonistic to *Pseudomonas corrugata,* a vein necrosis pathogen of tomato*,* and promotes the growth of tomato seedlings was isolated from surface-sterilised tomato roots. A spontaneous rifampicin-resistant mutant (LSW25R) was selected to facilitate its tracking, and identified as *Pseudomonas* sp. and named as *Pseudomonas* sp. LSW25R (LSW25R), based on its sequences of the internal transcribed spacer (ITS) region and 16 S rRNA gene. LSW25R inhibited mycelial growth of 12 other plant fungal pathogens such as *Botrytis cinerea* on V8 agar plates. By using a scanning electron microscope, LSW25R colonised not only the root surface around the natural aperture of tomato radicles but also under epidermal cells like endophytic bacteria. LSW25R successfully colonised the roots of tomato, eggplant and pepper seedlings, significantly promoted the fresh weight, height and dry matter of tomato plants at 10^8 cfu·ml^{-1}, and increased the plant growth of eggplants and peppers at 10^4 cfu·ml^{-1}, suggesting that the optimal population density of LSW25R for growth promotion varies from species to species. Moreover, densities of LSW25R inside roots and the lowest leaf of tomato plants were $>9.3\times10^3$ cfu·g^{-1}. Although the growth promotion of tomato by LSW25R was observed under N- or Ca-deficient conditions as well as a standard nutrient condition, the uptake of calcium was increased only under the standard nutrient condition. In a hydroponic system, LSW25R not only successfully colonised the rhizosphere during cultivation due to its broad spectrum of antifungal activity and endophytic colonisation, but also reduced blossom-end rot of tomato fruits presumably through increasing calcium uptake.

Keywords Calcium · Blossom-end rot (BER) · Hydroponic · Plant growth-promoting rhizobacteria (PGPR) · *Pseudomonas* sp. LSW25R · Tomato

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Introduction

In the rhizosphere, complex communities of soil microorganisms exist, some of which are plant growth-promoting rhizobacteria (PGPR) that stimulate plant growth in fields (Kokalis-Burelle et al. 2002) and/or under greenhouse conditions (Gagne et al. 1993). Many studies have demonstrated that certain PGPR strains, particularly those belonging to the genera *Bacillus* and *Pseudomonas*, proliferate not only on and around plant roots but also inside root tissues (van Peer et al. 1990). PGPR have been reported to stimulate plant growth by synthesising hormones such as indole acetic acid (Gravel et al. 2007) and/or increasing nutrient availability (e.g., phosphate solubilisation) (Kucey et al. 1989). Fluorescent pseudomonads can also suppress various soil-borne plant diseases (Weller 1988). This disease suppressiveness has been attributed to their rhizosphere competency (Rosales et al. 1995) and ability to induce systemic resistance against pathogens (Kessmann et al. 1994). Despite the potential of PGPR as environment-friendly growth stimulants and protectants against diverse pathogens, their use in commercial production has been limited, mainly due to the inconsistency in efficacy. Studies on the population dynamics of introduced PGPR strains demonstrated that their population densities on roots tended to decrease over time (Baker 1987), suggesting that the poor root colonisation might be a major cause underpinning inconsistent results obtained under field conditions.

Tomato (*Lycopersicon esculentum*) is one of the leading crops hydroponically cultivated in Korea. One advantage of hydroponic cultivation is that a grower can tightly control and optimise plant nutrition and the physiological environment, resulting in higher yield and improved product quality (Jones 2000). A frequently encountered problem with tomato plants hydroponically-grown in greenhouses is blossom-end rot (BER), which is a physiological disorder that causes rotting of tissues at the distal end of the fruit. Because BER of tomato is often caused by calcium deficiency (Ho and White 2005), application of a high concentration of calcium fertiliser is a cultural practice frequently utilised to control the disease. BER can also occur under suboptimal growth conditions such as drought (Nishio and Morita 1991) and high salinity (Ehret and Ho 1986), even when the calcium concentration is sufficient for fruit development under normal conditions.

In the present study, a newly isolated PGPR strain of *Pseudomonas* sp. isolated from tomato root was evaluated for its effect on mycelial growth of plant pathogenic fungi, the growth of tomato, mineral uptake, and incidence of BER.

Materials and methods

Isolation of rhizobacteria , antagonistic to *Pseudomonas corrugata* and promoting tomato growth

Fifty grams of root samples were collected from ten tomato plants yielding the largest amount of fruits among the plants grown in replanted soil for 10 years in Aomori Prefecture Field Crop and Horticulture Experimental Station, Aomori, Japan. After washing off surface soil particles with running tap water, the roots were surface-sterilised using 100 ml of 70% ethanol for 3 min in 250 ml flask, and then 100 ml of 1% sodium hypochloride (NaClO) for 10 min. After washing with sterile water, the root samples were macerated with 50 ml of sterilised distilled water; 10-fold serial dilutions of samples were prepared and an aliquot (0.5 ml) from each dilution spread on King's B agar plates (King et al. 1954). After 24 h, a cell suspension of *P. corrugata* (3×10^8cfu ml^{-1}), grown in King's B medium at 28°C with shaking at 150 rpm, was plated by spray-inoculation, and the plates incubated for 24 h at 28°C. *Pseudomonas corrugata*, causal agent of tomato pith necrosis grows well on King's B agar plates, which makes it easy to select antagonistic bacteria such as *Pseudomonas* spp. Thirty-eight bacterial isolates that showed a clear inhibition zone against *P. corrugata* were selected. To determine whether any resulting bacterial isolates promote tomato growth, the following assay was conducted. Seedlings of tomato cv. 'Momotaro T93' (Takii Seed Co., Japan) were grown in plastic pots (10 cm diam) with autoclaved replant soil successively cultivated with tomato (*L. esculentum*) for 10 years. Tomato seedlings at 10 days after sowing were cut under the cotyledon, dipped into individual bacterial suspensions (10^8cfu ml^{-1}) for 2 h, and transplanted in sterilised replant soil in order to select endophytic

bacteria which promote plant growth. The fresh weights of ten plants of tomato in each treatment were assessed 20 days after transplanting. This experiment was repeated three times.

Isolation of antibiotic resistant mutants of LSW25 for tracking

To monitor the bacterial population of LSW25 in the rhizosphere, we attempted to make antibiotic-resistant mutants. A bacterial suspension of LSW25 was inoculated in 50 ml of nutrient broth supplemented with 5 mg kg^{-1} rifampicin, and then incubated for 7 days (28°C, 150 rpm). When the culture turned turbid, 500 μι of bacterial suspension was re-inoculated in nutrient broth amended with 10 mg kg^{-1} of rifampicin. This serial culturing step with increasing concentration of rifampicin was repeated until bacteria could grow in the presence of 200 mg kg^{-1} rifampicin. In order to test resistance against other antibiotics, LSW25 was inoculated in 5 ml nutrient broth individually supplemented with 50 mg kg^{-1} ampicillin, streptomycin, kanamycin, neomycin, chloramphenicol or tetracycline, and cultivated for 24 h at 150 rpm, 28 . Bacterial cell concentration was measured using a spectrophotometer (at 550 nm).

Scanning electron microscopy

Scanning electron microscopy (SEM) was used to investigate patterns of bacterial colonisation on tomato radicles. Tomato seeds were dipped for 1 h into cell suspensions of LSW25R (10^8 cfu ml^{-1}), and kept on a moistened filter paper in a Petri-dish without light. Germinated seeds were sampled 3 days after treatment. Fixation, staining, dehydration, air-drying, and coating were conducted as described previously (Viret et al. 2004). The specimens were examined with a scanning electron microscope (JSM-5410LV; JEOL Ltd., Japan).

Identification of LSW25R

To determine species identity of LSW25R the following characters were analysed: (i) carbon source utilisation profiles, (ii) fatty acid composition, and (iii) sequences of 16 S rRNA (Borneman et al. 1996), and 16 S/23 S rRNA intergenic spacer (ITS) region (Martin-Laurent et al. 2001). Carbon source utilisation by LSW25R and reference organisms was analysed using Biolog microplates (Biolog GN MicroPlate; Biolog, Hayward, CA, USA). Fatty acid methyl esters (FAME) extracted from LSW25 were analysed with the Sherlock Microbial Identification System Version 2.11 (MIDI Inc, Newark, DE). Quantities of individual FAME were expressed as percentages of the total FAME (Lee et al. 2005). Both analyses were performed more than three times. The 16 S rRNA-encoding gene was amplified via polymerase chain reaction (PCR) by using primers (530F, 5′-TGA CTG ACT GAG TGC AGC MGC CGC GG-3′, and 1494R, 5′-TGA CTG ACT GAG GYT ACC TTG TTA CGA CTT-3′) under the following conditions (Borneman et al. 1996): 2 min at 96°C, 34 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s and additional 2 min incubation at 72°C , and the bacterial ITS region was amplified by PCR using primers (38r, 5′-CCG GGT TTC CCC ATT CGG-3′, and 72f, 5′-TGC GGC TGG ATC TCC TT-3′) under the following conditions (Martin-Laurent et al. 2001): 5 min at 94°C , 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, and additional 10 min incubation at 72°C. Amplified DNA fragments were sequenced using an automated 3730XL DNA sequencer (ABI /Hitachi, Tokyo, Japan).

Inhibition of mycelial growth of plant pathogenic fungi by LSW25R

Because bacterial antagonism is sometimes dependent on media composition (Nielsen et al. 1998), three media were used to investigate the ability to inhibit several fungal pathogens such as potato dextrose agar (PDA, Difco), yeast extract dextrose agar (YDA, Difco) and V8 agar ar (20% V8 juice, 0.3% $CaCO_3$, 1.7% agar). *Mycosphaerella melonis,* the causal agent of oriental melon stem black rot, *Sclerotina sclerotiorum* causing eggplant sclerotinia rot, *Rhizoctonia solani* causing rice sheath blight, *Verticillium dahliae* causing eggplant verticillium wilt, *Didymella bryoniae* causing oriental melon black rot, *Bipolaris coicis* causing leaf blight, *Alternaria solani* causing eggplant early blight, *Botrytis elliptica* causing lily blight spot, *Sclerotinia sclerotiorum* causing stock sclerotinia rot, *Sclerotinia* sp. causing lettuce sclerotinia rot, *Botrytis cinerea* causing tomato grey mold, *Rhizoctonia solani* causing damping-off, *Phytophthora capsici* causing pepper root rot, *Magnaporthe grisea* causing rice blast and

Phytophthora infestans causing tomato late blight were challenged with LSW25R. All of these fungal pathogens, isolated from fields and preserved by Gyeonggi Province Agricultural Research and Extension Services (GARES), Hwaseong, Republic of Korea, were used. Mycelial plugs (5 mm diam) of fungus were inoculated onto the centre of agar plates and incubated for 2 days at 25°C, and then 2 ul of culture solution of LSW25R was placed on plates 4 cm apart from the fungal plugs with the control, and the plates were incubated for 5 days at 25°C. Nutrient broth served as a negative control. In order to determine ability of the bacterial strains to inhibit fungal growth, mycelial growth rates of the cultures challenged with the bacteria were compared with growth rates of cultures that were not challenged [Growth inhibition rate=(diameter of mycelium from control — diameter of mycelium challenged with bacteria)/diameter of mycelium from control×100]. A completely randomised design with five replications per treatment was used (3 media×16 plant fungal pathogens and control×5 replications).

Enumeration of LSW25R

Bacterial populations of LSW25R were investigated with 10-fold serial dilution methods during the experiments described above. Five plants of each treatment were excavated, and 20 g of root from each plant with soil or perlite particles were sampled and shaken in 100 ml of sterile water for 30 min at 200 rpm, and the solution serially diluted 10-fold with sterile water. An aliquot (0.1 ml) from individual dilutions was plated on King's B agar medium supplemented with rifampicin (100 mg l^{-1}) and ampicillin (50 mg l^{-1}). After incubation at 28°C for 24 h, the number of bacterial colonies was counted. To determine the endophytic population of LSW25R, tomato roots and leaves were collected 10 days after soil treatment with LSW25R. Roots were surface-sterilised with 70% ethanol for 5 min followed by 1% sodium hypochloride for 1 min. After sterilisation, samples were ground with a pestle and mortar and plated on King's agar plate as described above. To determine the efficacy of the sterilisation procedure, roots were collected as the control 24 h after soil treatment with LSW25R and treated as described above. Each experiment was conducted with three replications.

Bacterial treatment and growth response of Solanaceous vegetables to LSW25R

After culturing LSW25R in King's B broth for 48 h, at 28°C by shaking at 150 rpm, bacterial cells were collected by centrifugation at 5,000 rpm for 10 min. The cell concentration was adjusted to desired levels using sterile distilled water. Seeds of tomato cv. Momotaro T93 (Takii Seed Co., Japan), pepper cv. Youmyeong (Nongjin Seed Co. Korea) and eggplant cv. 'Heuklyonggang' (Singenta Co., Korea) were sown in 72 cell plug trays filled with a mixture of perlite and peatmoss (7:3). A completely randomised design with three replications (plug tray), with each replication consisting of ten plants collected from each tray, was used. Seedlings were fertilised with one-half strength Yamazaki formula nutrient solution (NO_3-N:7, NH_4-N:0.67, PO_4-P:2, K:4, Ca:3, Mg:2 milliequivalent l^{-1}) and a full strength micro-nutrient solution (Fe: 2.0, B:0.2, Mn:0.2, Zn:0.02, Cu:0.01, Mo:0.005, Cl:1.0 mg l^{-1}). The plug trays were drenched with 10 ml of cell suspensions of LSW25R at 10^4, 10^6 and 10^8 cfu ml^{-1} after 15 days, and nutrient broth served as a negative control. Plant growth parameters such as leaf length, leaf width, stem diameter, and fresh weight were observed 30 days after sowing.

Growth of tomato seedlings under different nutrient conditions in the presence of LSW25R

Seeds of tomato cv. Momotaro T93 were sown in plug trays filled with a mixture of perlite and peatmoss (7:3), and the seedlings were fertilised as described above. Seedlings were transplanted into 10 cm diam pots filled with vermiculite 15 days after sowing. After transplanting, the pots were drenched with 50 ml of a cell suspension of LSW25R (10^8 cfu ml^{-1}), and subsequently seedlings were fertilised with nutrient solutions deficient in a specific ion. Nutrient broth served as a negative control. The Yamazaki nutrient solution for tomato was used as the control. Each of the following elements, including nitrogen, phosphorus, and calcium, was removed from the normal control, and the concentration of other elements was adjusted to the same milliequivalent l^{-1}. A completely randomised design with three replications, with each replication consisting of 20 plants, was used. Plant growth parameters of 20 plants

collected from a tray, such as leaf length, leaf width, stem diameter, and fresh weight were observed 16 days after transplanting.

Tomato cultivation in a hydroponic system

This experiment was carried out in a greenhouse from June 1998 to January 1999 at GARES , Hwaseong, Republic of Korea. Tomato seeds (Momotaro T93) were sown in 10 cm diam pots filled with a mixture of perlite and peatmoss (7:3). Seedlings were fertilised with one-half strength Yamazaki formula nutrient solution. At the eight-leaf stage, the seedlings were transplanted into the perlite medium in a re-circulating hydroponic system with 30 cm space between plants. On the same day, 20 ml of bacterial cells (LSW25R, 1×10^8 cfu ml^{-1}) was applied to each transplanting hole. Nutrient broth served as a negative control. The hydroponic system was built in a greenhouse using a styrofoam bed (7 m long×30 cm wide) filled with perlite, and each plot consisted of a pump with a return line, and trickle irrigation. Twenty-five plants were grown in each replication. Tomato plants were fertilised using full strength Yamazaki nutrient solution with micro-nutrients as described above. The nutrient solution was irrigated at a rate of 2 l $plant^{-1}day^{-1}$. All lateral shoots were removed, and a single stem was trained. All fruits were harvested weekly under the 4th truss, and classified into marketable, BER and non-marketable (cracked, damaged, diseased) fruits. Soluble sugar contents (°Brix) of tomato fruits were measured with a refractometer (PR-1, Atago Co., Japan) at every harvest. Population sizes of LSW25R in the rhizosphere of tomato plants were measured three times during cultivation as described above. The experiment was carried out in a randomised complete block design with three replications.

Analysis of the content of inorganic elements in tomato leaves

Tomato leaves were sampled and dried for 3 days at 80°C. Triplicate dried samples of 0.15 g were treated with a mixture of 90 ml HNO_3, 30 ml HCl and 3 ml $HClO_4$ in a block heater, and heated to about 500°C until the solution became clear. After filtering individual mixtures through filter paper, they were analysed for individual elements. The Kjeldahl method (Bremner and Breitenbeck 1983) was used to analyse the total N, and the amounts of P, K, Ca and Mg were determined by inductively coupled plasma-optical emission spectrometry (XM2, GBC, Australia) as previously described (Sapkota et al. 2005). The analysis was carried with three replications.

Results

Isolation and identification of a plant growth-promoting rhizobacterium (PGPR) antagonistic to *P. corrugata*

A total of 38 isolates of Gram-negative bacteria collected from roots of tomato plants showed a clear inhibition zone against *P. corrugata* on King's B agar plate. Among these isolates, one isolate, designated as LSW25, highly promoted tomato growth compared with other isolates. The fresh weight of tomato plants treated with LSW25 was as much as 2.7 times heavier than that of untreated plants (nutrient broth alone). To facilitate tracking of LSW25 in inoculated plants, a spontaneous mutant of LSW25 resistant to rifampicin (named as LSW25R) was selected by successively isolating bacterial cells growing in the presence of increasing concentrations of rifampicin. LSW25R also exhibited ampicillin resistance but could not grow in the presence of other antibiotics such as streptomycin, kanamycin, neomycin, chloramphenicol, and tetracycline. This spontaneous mutant retained the antibiotic resistance even after successive transfers on King's B agar without antibiotics. Sequences of 16 S rRNA from LSW25R exhibited 99% identity to the corresponding region in several *Pseudomonas* species, such as *P. lurida*, *P. tolaasii*, *P. veronii,* and *P. trivialis,* and the ITS sequence was 98% identical to that of *P. fluorescens* strains. However, results from Biolog and fatty acid analysis did not match with the identity of the profile of any *Pseudomonas* species >90%. Therefore, we tentatively identified LSW25R as a strain of *Pseudomonas* sp.

LSW25R inhibited the mycelial growth of plant pathogens

Antagonistic ability of LSW25R varied depending on the test medium. However, LSW25R inhibited the mycelial growth of most plant pathogens tested

except *Rhizoctonia solani* on V8 agar plates (Table 1). Generally, the inhibition of fungal growth was greater on V8 agar than on the other media.

Plant growth promotion induced by LSW25R

Upon inoculation with 10^8 cfu ml^{-1}, LSW25R persistently colonised the rhizosphere of tomato, eggplants and peppers. Furthermore, the density of LSW25R in sterilised tomato roots was 3.5×10^5 cfu g^{-1} and that of the lowest leaf was 9.3×10^3 cfu g^{-1}. As the concentration of inoculated bacterial cells increased, the bacterial population on roots increased in all three crops. LSW25R appeared to increase the height and fresh weight of tomato plants as the bacterial concentration increased with the inoculation of 10^8 cfu ml^{-1} being most effective. In contrast, the growth-promoting effect was highest at a much lower dose (10^4 cfu ml^{-1}) in eggplants and peppers and appeared to decrease as the bacterial concentration increased (Table 2).

Table 1 Inhibition rate of mycelial growth of plant pathogenic fungi on several media by *Pseudomonas* sp. LSW25R

Plant fungal pathogens	Growth inihibition rate (%)[a]		
	PDA	YDA	V8
Mycosphaerella melonis	37±10.6	0	42±13.5
Sclerotinia sclerotiorum	0	0	27±5.8
Rhizoctonia solani	0	0	0
Verticillium dahliae	14±5.7	0	5±3.3
Bipolaris coicis	11±7.2	0	20±4.2
Alternaria solani	30±13.2	28±9.4	24±12.6
Botrytis elliptica	0	0	45±11.4
Sclerotinia sclerotiorum	0	0	17±4.8
Sclerotinia sp.	0	0	31±9.3
Botrytis cinerea	0	0	37±12.7
Phytophthora capsici	10	0	19±6.6
Magnaporthe grisea	0	0	23±10.3
Phytophthora infestans	40±7.2	34±13.2	35±6.1

[a] Growth inhibition rate=(diameter of mycelium from control plate- diameter of mycelium challenged with bacteria)/ diameter of mycelium from control plate×100

PDA : potato dextrose agar, YDA : yeast extract dextrose agar, V8 agar : 20% V8 juice, 0.3% $CaCO_3$, 1.7% agar

Colonisation of LSW25R on tomato radicles

To investigate locations of colonisation by LSW25R, the tomato roots of 3 day-old plantlets were examined by SEM. Clusters of bacteria colonised the surface of the epidermis in high-density around the natural aperture of the root (Fig 1, A), and on grooved lines of the epidermal cells in the root (Fig 1, B). Moreover, it was observed that LSW25R colonised under the epidermal cells around natural apertures (Fig 1C and D, white arrows).

Increase in mineral uptake and reduction in BER by LSW25R

LSW25R increased plant height, leaf length, leaf width and fresh weight (Table 3), and also increased concentrations of calcium and iron in leaves of tomato seedlings cultivated in nutrient solution (Table 4). However, it did not increase uptake of phosphorus, potassium and magnesium of tomato. Since BER of tomato is often associated with calcium deficiency, we hypothesised that increased Ca uptake in response to the LSW25R treatment might decrease BER. This hypothesis was supported in a pot test (Table 4); BER incidence in tomato plants treated with LSW25R was significantly lower (by 61%) than that of non-treated control plants. Although the marketable yield of tomato was increased by 25% upon LSW25R treatment, the difference to that of control plants was not significant. Moreover, LSW25R increased the sugar contents of fruits (Table 5). Consistent with the data summarised in Table 4, analysis of mineral elements in tomato leaves showed that calcium uptake increased in tomato plants treated with LSW25R (Table 6). The population of LSW25R was stable in the rhizosphere of tomato throughout cultivation (Fig. 2).

Discussion

LSW25R was selected as a PGPR which increased fresh weight of tomato plants among antagonistic bacteria by a stem-cut and dipping method. However, even though tomato roots were treated with a bacterial suspension of LSW25R without a stem-cut in three vegetable crops, LSW25R could successfully colonise the rhizosphere and promoted plant growth (Table 2).

Table 2 Effect of *Pseudomonas* sp.LSW25R on the seedling growth of tomato, hot pepper and eggplant

Plant	Treatment[a] (cfu g^{-1})	Plant height (cm)[b]	Stem diameter (mm)[b]	Fresh weight (g)[b]	% dry matter[c]	Colonisation on root[c] (cfu g^{-1})
Tomato	10^8	44.2 a	4.8 a	46.3 a	6.3 a	2.5×10^5 a
	10^6	41.7 ab	4.6 a	32.1 b	5.3 b	1.8×10^3 b
	10^4	39.3 bc	4.6 a	35.8 b	5.8 ab	8.0×10^3 b
	control	35.7 c	4.7 a	30.7 b	5.7 ab	–
Eggplant	10^8	18.6 b	2.6 bc	14.0 c	7.0 ab	1.0×10^5 a
	10^6	20.4 a	2.8 b	17.0 b	6.5 b	2.5×10^4 b
	10^4	20.8 a	2.9 a	20.0 a	7.1 a	3.5×10^3 b
	control	18.2 b	2.5 c	14.2 c	6.9 ab	–
Pepper	10^8	22.5 b	2.7 b	9.7 b	6.4 b	1.8×10^5 a
	10^6	20.7 c	2.8 b	9.9 b	6.6 b	6.0×10^4 ab
	10^4	24.7 a	3.0 a	13.3 a	7.2 a	6.0×10^3 b
	control	21.6 bc	2.8 ab	10.1 b	6.7 b	–

[a] Suspensions of LSW25R at different concentrations were individually applied

[b] Means in columns followed by the same letters are not significantly different by Duncan's multiple range test at $P=0.05$

[c] Bacterial cells washed off from roots were plated on the King's B agar with added ampicillin (50 mg kg^{-1}) and rifampicin (100 mg kg^{-1})

Furthermore, densities of LSW25R inside the tomato root or lowest leaf were $>9.3\times10^3$ cfu g^{-1}, and it colonised under the root epidermal surface (Fig 1C and D) suggesting its endophytic colonisation behaviour. Endophytic bacteria (Quadt-Hallmann et al. 1997) may have several beneficial effects on plants, such as growth promotion and biological control of pathogens (Nejad and Johnson 2000; Lodewyckx et al.2002). In general, although populations of introduced bacteria gradually decrease after application (Lee et al. 2000), populations of introduced endophytic bacteria were stably maintained at the level of $10^3 - 10^5$ cfu g^{-1} of plant roots in most plant species investigated (Quadt-Hallmann and Kloepper 1996). In

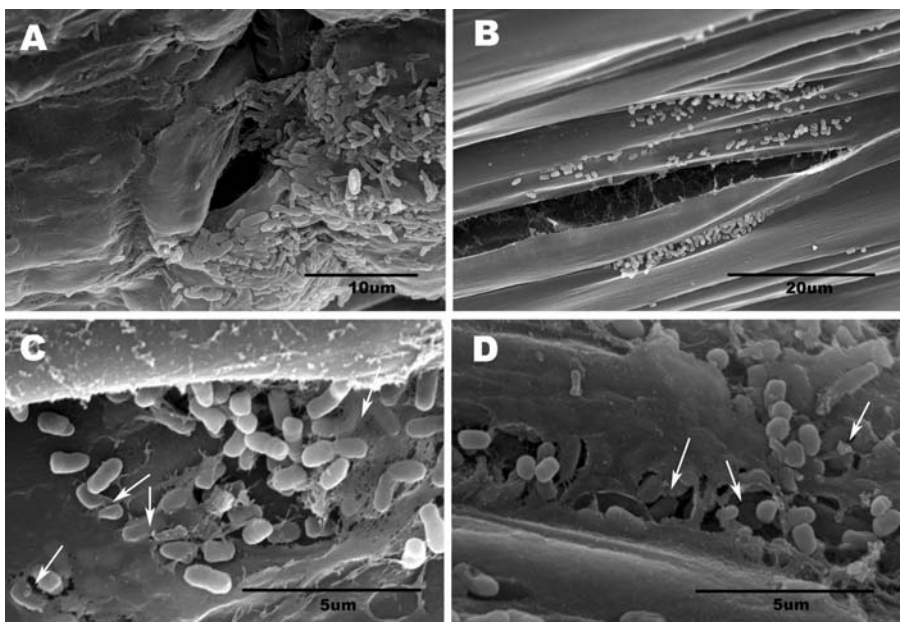

Fig. 1 Colonisation of *Pseudomonas* sp. LSW25R on tomato radicle. **a**, **b** Clusters of LSW25R were localised on surface of root around root apertures. **c**, **d** Bacterial cells of LSW25R were observed under the epidermis of root (white arrows). SEM

Table 3 Effect of *Pseudomonas* sp. LSW25R on the growth of tomato seedlings fertilised with nutrient solutions deficient in a specific element

Deficient Nutrient Treatment[a]		Plant height (cm)[b]	Leaf length (cm)[b]	Leaf width (cm)[b]	Fresh weight (g/plant)[b]
Standard[c]	Control	49 b	21 b	12 b	11.0 b
	LSW25R	56 a	24 a	14 a	15.0 a
N	Control	42 b	19 b	10 a	7.3 b
	LSW25R	48 a	25 a	11 a	9.3 a
P	Control	50 a	21 a	12 a	9.8 a
	LSW25R	51 a	19 a	12 a	10.3 a
Ca	Control	55 a	21 a	13 a	12.5 b
	LSW25R	55 a	23 a	13 a	14.5 a

[a] A bacterial suspension (10^8 cfu ml^{-1}) was applied

[b] Means within columns followed by the same letters are not significantly different according to Fisher's protected least significant difference test at $P=0.05$

[c] Yamazaki formula nutrient solution for tomato (NO_3-N: 7, NH_4-N:0.67, PO_4-P:2, K:4, Ca:3, Mg:2 milliequivalent l^{-1}) with full strength micronutrients (Fe 2.0 mg kg^{-1}, B 0.2 mg kg^{-1}, Mn 0.2 mg kg^{-1}, Zn 0.02 mg kg^{-1}, Cu 0.01 mg kg^{-1}, Mo 0.005 mg kg^{-1}, Cl 1.0 mg kg^{-1})

agreement with these reports, population sizes of LSW25R were stably maintained in the tomato rhizosphere throughout the entire duration of cultivation in a hydroponic system (Fig. 2). Successful colonisation of the rhizosphere by introduced beneficial bacteria usually requires that the bacteria should be well adapted to the rhizosphere and also have some selective advantages over many indigenous organisms colonising the rhizosphere. The broad-spectrum antimicrobial activity of LSW25R may be one such advantage (Table 1).

Growth of tomato, eggplant and pepper was promoted significantly upon treatments with LSW25R, but the optimal inoculum dose was different among them

Table 4 Effect of *Pseudomonas* sp. LSW25R on the mineral composition of leaves of tomato plants grown with nutrient solutions deficient in N, P, or Ca

Deficient nutrient	Treatment[a]	Mineral elements[b]					
		Total N	K_2O	CaO	MgO	P_2O_5	Fe
Standard[c]	Control	3.2 a	7.5 a	3.0 b	0.9 a	1.3 a	0.07 b
	LSW25R	3.4 a	8.0 a	3.6 a	1.1 a	1.3 a	0.12 a
N	Control	1.2 a	4.3 b	2.2 a	0.6 a	1.4 a	0.07 b
	LSW25R	1.2 a	5.2 a	2.5 a	0.8 a	1.3 a	0.10 a
P	Control	2.9 a	5.8 b	3.3 a	1.2 a	0.3 a	0.07 b
	LSW25R	2.8 a	6.6 a	3.5 a	1.2 a	0.3 a	0.12 a
Ca	Control	3.3 a	6.7 a	2.6 a	0.7 b	1.6 a	0.09 a
	LSW25R	3.5 a	7.0 a	3.0 a	0.9 a	1.5 a	0.06 b

[a] A bacterial suspension (10^8 cfu ml^{-1}) was applied

[b] Content of individual elements are shown in percent concentration per dry weight of sample with the exception of Fe (mg kg^{-1}). Means within columns followed by the same letters are not significantly different according to Fisher's protected least significant difference test at $P=0.05$

[c] Yamazaki formula nutrient solution for tomato (NO_3-N:7, NH_4-N:0.67, PO_4-P:2, K:4, Ca:3, Mg:2 milliequivalent l^{-1}) with full strength micronutrients (Fe 2.0 mg kg^{-1}, B 0.2 mg kg^{-1}, Mn 0.2 mg kg^{-1}, Zn 0.02 mg kg^{-1}, Cu 0.01 mg kg^{-1}, Mo 0.005 mg kg^{-1}, Cl 1.0 mg kg^{-1})

Table 5 Effect of *Pseudomonas* sp. LSW25R on the yield and prevalence of blossom-end rot (BER) of tomato cultivated in a hydroponic system

Treatment[a]	Amount of tomato fruits (g/plant)[b]			Sugar content (°Brix)
	Total yield	Marketable yield[c]	BER[c]	
Control	3,495 a	2,688 (100) a	569 (100) a	6.9 b
LSW25R	3,781 a	3,368 (125) a	223 (39) b	7.2 a

[a] A bacterial suspension (10^8 cfu ml^{-1}) was applied

[b] Means within columns followed by the same letters are not significantly different according to Fisher's protected least significant difference test at $P = 0.05$

[c] Relative index (percentage) compared with that of the control

(Table 2). In tomato, the increase in plant height and fresh weight was the highest with the treatment of 10^8 cfu ml^{-1}, and this effect increased as the inoculum concentration became higher. This positive dose-response relationship is generally attributed to a better colonisation of the rhizosphere by introduced micro-organisms (Raaijmakers et al. 1995). The population size of LSW25R in the tomato rhizosphere was 2×10^5 cfu g^{-1} after a treatment with 10^8 cfu ml^{-1}, which is 30–130 times higher than that resulting from lower doses of LSW25R. Similar patterns of colonisation were observed in eggplant and pepper (Table 2). However, in contrast to tomato, the growth of eggplant and pepper was not promoted at the highest dose (10^8 cfu ml^{-1}) but was significantly enhanced at lower doses (10^4 – 10^6 cfu ml^{-1}), suggesting that the optimal population density of LSW25R for growth promotion varies from species to species. In an earlier study, some PGPR strains exerted detrimental effects on root growth at high inoculation doses (Kapulink et al. 1985).

Table 6 Effect of *Pseudomonas* sp. LSW25R on the mineral composition in tomato leaves cultivated in a hydroponic system

Treatment[a]	Mineral elements (%)[b]				
	K_2O	CaO	MgO	P_2O_5	Total N
Control	4.3 a	4.8 b	3.2 a	1.8 a	2.3 a
LSW25R	3.9 b	5.6 a	2.7 a	1.6 a	2.2 a

[a] A bacterial suspension (10^8 cfu ml^{-1}) was applied.

[b] Macro- and micro-nutrients were analysed in dried leaves after grinding, and means within columns followed by the same letters are not significantly different according to Fisher's protected least significant difference test at $P = 0.05$.

Increased uptake of certain key nutrients might be a factor underlying growth promotion by LSW25R. Growth of tomato plants under N-deficient and normal conditions was promoted by LSW25R, whereas this effect was absent among plants grown under conditions deficient in calcium or phosphorus with the exception of fresh weight under the Ca-deficient condition (Table 3). The LSW25R treatment

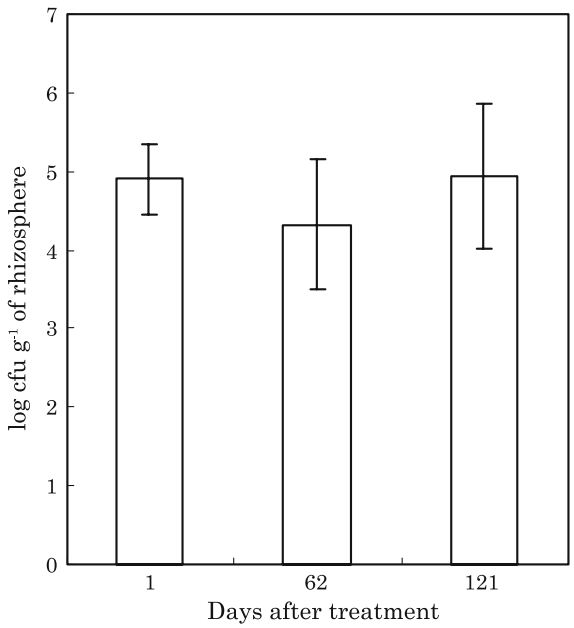

Fig. 2 Degrees of rhizosphere colonisation by *Pseudomonas* sp. LSW25R during the cultivation of tomato plants in a hydroponic system. Root samples were shaken in sterile water for 30 min at 200 rpm, and the resulting solutions were plated on King's B agar medium supplemented with rifampicin (100 mg l^{-1}) and ampicillin (50 mg l^{-1}), and after incubation at 28°C for 24 h, the number of colonies were counted (each error bar indicates the standard deviation)

increased iron uptake by tomato under all conditions with the exception of the Ca-deficient condition (Table 4). Iron is one of the essential nutrients of plants, and plant roots could absorb iron more easily as reduced ferrous (Fe^{2+}) ion than ferric (Fe^{3+}) ion, a form that commonly exists in soil and can be easily precipitated in iron-oxide forms (Salisbury and Ross 1992). The mechanism underpinning increased iron uptake caused by LSW25R remains to be characterised but may involve siderophores, which bind with Fe^{3+} to keep it from precipitation and facilitate uptake. Some PGPR produce siderophores, and many plant species can absorb bacterial Fe^{3+}-siderophore complexes (Bar-Ness et al. 1992).

BER is a physiological disorder that occurs on the fruit of tomato. Many factors including high salinity, inadequate xylem tissue development, low soluble soil calcium, high and low transpiration, accelerated growth rate (Marcelis and Ho 1999) and high temperature (Kreij 1996) contribute to this disorder. However, the main underlying cause is an insufficient amount of calcium in the blossom-end of fruit (Taylor and Locascio 2004). Calcium deficiency in this tissue is usually caused by poor distribution of calcium due to other internal needs, poor uptake, and/or the antagonistic effect of other elements such as K, Na or NH_4 (Keiser and Mullen 1993), but in glasshouse production, BER is rarely caused by insufficient calcium in feed. More often, BER occurs in plants with an adequate Ca supply because certain growth conditions inhibit adequate calcium uptake (Ho and White 2005). Therefore, BER can be reduced by maximising calcium uptake of roots. However, mechanisms of calcium uptake by root cells are poorly understood, and there are few reports showing treatments enhancing calcium uptake (Park et al. 2005). Calcium uptake was increased by LSW25R only under the normal nutritional condition (Table 4), and this observation led us to test the hypothesis that LSW25R could reduce the incidence of BER. In hydroponically-cultivated tomato plants, although there was no significant difference in the marketable yield of fruits upon LSW25R treatment, BER was dramatically reduced (by 61%) by the LSW25R treatment (Table 5). Analysis of leaves from the treated plants revealed that calcium uptake indeed increased in plants treated with LSW25R (Table 6), supporting the hypothesis.

To the best of our knowledge, this is the first report showing increase of Ca uptake and reduction of BER by *Pseudomonas* species. Certain strains of Gram-negative bacteria are highly efficient at dissolving calcium phosphates, which have very low solubility in water (Goldstein et al. 1999); whether this is the mechanism underlying increased calcium uptake by LSW25R remains to be investigated, but LSW25R showed potential as a means to reduce the incidence of BER in a hydroponic system.

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