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Evaluation of *Bacillus subtilis* Native Strains for Plant Growth Promotion and Induced Systemic Resistance in Tomato and Red-pepper

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Abstract

Bacillus subtilis strains isolated from different regions of Korea were screened for their plant growth promotion and induced systemic resistance (ISR) in tomato and red-pepper. The plant growth promotion on red-pepper and tomato revealed maximum plant height (22.73 cm) on red pepper treated with *B. subtilis* strain JE 21-1 and 30.18cm in case of tomato treated with *B. subtilis* strain JE 8-1. There was also significant improvement in root and shoot dry weight in both the plants. The strain JE 21-1 showed better promise for all growth parameters in red-pepper and tomato when compared to other strains and positive check BTH. Different strains screened in square plate method also revealed maximum plant height and leaf width, and suppressed anthracnose on red pepper in case of strain JE 21-1 at 10^6 and 10^7 cells/ml when compared to other strains. In all the bacterial inoculations the population was significantly high when compared to untreated check. In plant growth promotion with respect to fruit length and weight, fruit length was maximal in treating with JE 9-4 and ES 2-2, while fruit weight was maximal in treating with JE 3-6, ES4-2, ES2-2 and JE 21-2 on red pepper. In case of tomato, comparatively better fruit weight was in JE 21-1, ES 3-3 and JE 10-2 when compared to BTH and untreated control. The soft rot disease caused by *Pectobacterium carotovorum* SCC1 was completely suppressed in case of transgenic tobacco harboring GUS gene related to PR1a and increased the level of salicylic acid significantly in combined application of JE 9-4 on par with BTH. Thus, this study clarified some potential *Bacillus subtilis* strains for plant growth promotion and ISR in red-pepper and tomato.

Key words *Bacillus subtilis*, BTH, Plant Growth Promotion, Disease Suppression

Introduction

Plant rhizosphere is the buffering ecological niche where interactions of microbes, such as bacteria, fungi and protozoa, are produced due to the availability of rich and diverse microbial food source (Bais *et al.*, 2006). In modern agriculture, the relative thrust is on the role of rhizosphere microorganisms that stimulate plant growth termed as plant growth-promoting rhizobacteria (PGPR) that significantly support the growth promotion, induced resistance and also improve the crop yields in the greenhouses and fields conditions (Kloepper *et al.*, 2004). The genus *Colletotrichum* comprises large number of plant pathogenic fungi and saprophytes covering very wide host range causing diseases commonly known as anthracnose in more than 197 plant species including crops, weeds, and trees. In Korea, these

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groups of fungal pathogens cause serious economic damage on red pepper, cucumber, strawberry, grapevine and apple (Park and Kim, 1992).

Anthracnose and soft rot are the major production constraints in cucumber cultivation in Korea (Kim and Nam, 1999; Kim *et al.*, 2007). The complexity of the soil ecosystem is a constraint that makes biological control a challenging task by introduced antagonists (Pierson and Weller, 1994). Phytophthora blights and rots caused by oomycete fungi such as *Phytophthora capsici*, *Phytophthora infestans* and *Phytophthora medicaginis* cause significant economic damage on many important crops such as red peppers, potatoes, tomatoes and alfalfas.

Among various groups of plant-associated microorganisms, some microorganisms are attracted by scientists based on their performance on plant growth promotion by exerting its antibiotic or induced systemic resistance (ISR) activities and these microorganisms are called as plant growth promoting rhizobacteria (PGPR) (van Loon and Glick, 2004). In plant growth promotion by bacteria, both gram positive and negative bacterial species have been reported. Preciously, salicylic acid (SA) controls the induction of genes encoding pathogenesis related proteins (PRs) of the families PR-2 (β-1,3-glucanases), PR-5 (thaumatin-like proteins), and PR-1 with unknown biochemical properties (Uknes *et al.*, 1992). The PGPR are one group among the various groups of plant-associated microorganisms that can elicit plant defences (van Loon and Glick, 2004). Although commercialization of PGPR is mainly proceeding with *Bacillus* spp. rather than pseudomonads, the preponderance of research on PGPR as elicitors of growth promotion or ISR employs PGPR strains, which are fluorescent pseudomonads. The most widely studied groups of PGPR groups are colonizing the root surfaces and the closely adhering soil interface, the rhizosphere (Kloepper, *et al.* 1991). Most of the PGPR strains protect the plant through direct mechanisms by production of bacterial allelochemicals, including iron-chelating siderophores, antibiotics, biocidal volatiles, lytic enzymes, and detoxification enzymes (Raupach *et al.*, 1996). So far there is limited information on role of native strains on plant growth promotion and inducing disease resistance in tomato and red-pepper under Korean conditions. Hence, we studied in detail influence of different native strains of *B. subtilis* in imparting the plant growth promotion and inducing resistance in tomato and red-pepper for major economically important diseases affecting the Korean agriculture. The results obtained on these aspects are discussed in this paper.

Materials and Methods

Microbial isolates

Rhizobacteria were collected from different regions of South Korea from either turf soil, rhizosphere soils of Dunae in Kangwon province, Umsung in Chungbuk province, Chungju in Chungbuk province and Suwon in Gyeonggi province. After shaking the excised roots (1 cm long) were agitated in 50 ml of sterilized phosphate-buffered saline (PBS, pH 7.3) for 5 min. Diluted soil samples (10^{-5} and 10^{-6}) were placed on trypticase soy agar (TSA). A total of 62 bacterial isolates from soil and rhizosphere of various plant roots were selected based on differences in colony morphology and pigment production. The selected strains which based on antibiosis and induce systemic resistance were tentatively identified by 16S rDNA analysis and were maintained at -80°C in trypticase soy broth (TSB) with glycerol (20%) for long-term storage. For preparing bacterial suspension, culture from -80°C was grown on trypticase soy agar (TSA) for 24 h at 28°C, and single colonies were transferred to TSB and incubated 24 h at 28°C with shaking at 150 rpm. Bacteria were pelleted with centrifugation for 5 min at 8,000g and resuspended in 10 mM $MgSO_4$ to give concentration of 1×10^8 cells/ml.

Selection assay for candidate bacterial strains

Red-pepper (*Capsicum annuum* L.) cv. Hanbyul at first-branch stage was used in this study. Red-pepper seeds were sown in a plastic tray (55 cm × 35 cm × 15 cm) containing steam-sterilized soil, sand and compost (1:1:1, v/v/v). Seedlings at the two leaf stage were transplanted to plastic pots (5 cm × 15 cm × 10 cm) containing the same soil mix. Complex fertilizer was applied to plants after transplanting. Pepper plants were raised in a growth chamber under 16 hr day illuminations at 27±2°C.

Seeds of tomato cv. Seokwang were surface-sterilized by immersion in 1% aqueous sodium hypochlorite for 1 min and thoroughly rinsed in sterile distilled water prior to being allowed to germinate in petri dishes containing sterile, moist cotton. Plates were incubated at 25°C in the dark for 48 h. Germinated seeds were carefully removed from the cotton and sown in a mixture of peat-perlite-vermiculite (1:1:1) at a density of four seedlings per 6 cm pot. Tomato seedlings of the pot were grown on a green-house bench at room temperature. Three-week old red-pepper and tomato seedlings were infiltrated with a cell suspension of bacillus strains (10^8 cells / ml) carefully with a sterile syringe. Control plants were treated similarly with sterile deionised water only. Four days later, plants were challenge inoculaed by spray with spore or cell suspension of *C. acutatum* and *P. carotovorum* SCC1 respectively. Twenty plants were used for each treatment and the experiment was repeated twice. After seven days of challenge inoculation, plants were observed for lesions area and numbers per leaf of each treatment.

For the cubic plate assay of ISR against *P. carotovora* SCC1, the selected bacillus strains EXTN-1, JE3-6, JE8-1 and JE10-2 were soil drenched as cell suspension of 10^7 cfu/ml concentration on 25 days-old seedling tomato plant. The BTH and water treatment served as positive and negative control in green house. Seven days after challenge inoculation of *P. carotovora* SCC1, the diseased plant leaf was recorded as percent lesion area.

Challenge inoculation with anthracnose and soft rot pathogens

Anthracnose pathogen, *C. acutatum* was grown in potato dextrose agar medium for 5 days and *P. carotovorum* SCC 1 was grown on Luria Britania Agar medium for 48 hrs. Ten sterile distilled water was poured on the medium grown the anthracnose fungi and soft rot bacteria to harvest the suspension of conidia and bacterial cells, respectively. The conidial concentration was adjusted to 2.5×10^5 conidia per ml in case of *C. acutatum* and 1.0×10^8 cells per ml in case of SCC1. The conidial and cell suspension of *C. acutatum* and *P. carotovorum* SCC1 were run off on the red-pepper and tomato plant leaves respectively. The plants inoculated with the conidia and cell suspension of the pathogens were kept in a humid chamber maintaining 100% RH for 24 h and then transferred to the greenhouse condition at 25°C for a week.

Plant growth promotion

Red-pepper and tomato plants were infiltrated at cotyledon stage and also soil drenched with cell suspension of different strains of *Bacillus* which were isolated from cucumber rhizosphere. The BTH and water treatment served as positive and negative control in green house. Growth promotion was measured at 14 days after treatment. Later challenge inoculated with pathogenic strain.

Tobacco bioassay and GUS activity

Seeds of *Nicotiana tabacum* L. cv. *Xanthi-nc*, which were genetically engineered with PR-1a promoter::GUS reporter gene, were kindly provided by J. Ryals (Novartis Agricultural Biotechnology Research Unit, Research Triangle Park, NC). GUS reporter gene was correlated to the PR-1a gene (Uknes *et al.*, 1993). Seeds were surface-sterilized with 1% sodium hypochlorite solution followed by 3 min dipping in 70% ethanol and thoroughly rinsed in sterile distilled water. After the seed germination on Murashige and Skoog salt (MS) medium (GIBCO/BRL) in 24 well cell culture plate, each seedling was transplanted to 10 sq.cm plastic pots containing soil less Flora Guard (TKS 2 INSTANT, Kultur substrate) growing medium and kept in the greenhouse with daily watering. 4 week-old tobacco plants were soil drenched with 50 ml bacterial suspension of each strains at concentration of 10^8 cells/ml. One week after the administration of the bacterial or chemical treatment or 10 mM $MgSO_4$, three leaves from each plant were collected and carried to lab aseptically for quantitative determination of GUS activity. GUS detection was accomplished by fluorometric GUS assay from Jefferson, 1987 and Park & Kloepper, 2000.

Statistical analysis

Each experiment had six replications and each replication consisted of 6 plants. Data were analyzed with SAS JMP software (SAS Institute, USA) (SAS, 1995). Significant

differences in treatment means on each sample data were determined using LSD at P=0.05.

Results

In the present investigation, we studied the role of different isolates of *B. subtilis* isolated from different ecological niche for their plant growth and ISR activity in red-pepper and tomato in Korea. In case of red-pepper, highest plant height (23.29 cm) was recorded in JE 9-4 followed by 22.72 cm in JE3-5 and 22.66 cm in JE 21-2 and these treatments are statistically on par with each other. The plants recorded more than (or above) 20 cm plant height in different bacterial strains when compared to BTH (22.68 cm) and untreated control (19.96 cm). The bacterial strains supported better plant height and on par with positive check and significantly superior to untreated check (Table 1). The maximum root length (12.63 cm) was recorded in JE 9-4 which was statistically on par with JE 21-1 (12.13 cm) and positive check (12.49 cm). In case of root dry weight, maximum root dry weight was recorded in JE 3-5 followed by JE 8-1 and JE 21-2. The treatment with different strains of *Bacillus* also supported maximum shoot dry weight (0.0382 g/plant) per plant in JE 8-1 followed by JE 3-5 (0.03592 g/plant) and JE 21-2 (0.03498 g/plant). In case of tomato, maximum plant height was recorded in JE 8-1 (30.18 cm) followed by 28.2 cm in JE 21-2 and JE 9-4 (27.93 cm). The root length also significantly influenced by treatment with different *Bacillus* strains. The maximum root length (11.91 cm) was recorded in JE

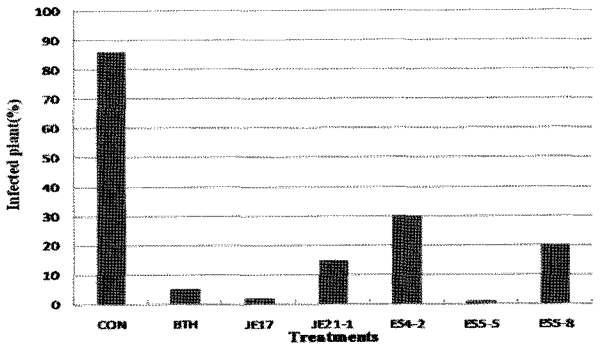

Fig. 1. Induced systemic resistance of tobacco plants against *Pectobacterium carotovorum* SCC1 by treatment of selected strains.

Table 1. Growth characteristics of red-pepper and tomato seedlings treated with selected strains in cubic plate assay

Treatment	Red-pepper				Tomato			
	Root length (cm)	Plant height (cm)	Root dry weight (g)	Shoot dry weight (g)	Root length (cm)	Plant height (cm)	Root dry weight (g)	Shoot dry weight (g)
Control	9.92	19.96	0.0064	0.0324	10.15	27.61	0.0177	0.085
BTH	12.49	22.68*	0.0072	0.0342	10.11	27.46	0.0142	0.0665
EXTN-1	10.01	20.57	0.0064	0.0328	10.28	27.41	0.0167	0.0635
JE 3-5	11.74	22.72*	0.0078	0.0359	9.52	27.06	0.0073	0.0695
JE 3-6	9.69	20.25	0.0066	0.0331	10.60	27.66	0.0119	0.0610
JE 8-1	11.36	22.50*	0.0074	0.0380	11.91	30.18	0.0158	0.0775
JE 9-4	12.63	23.29*	0.0072	0.0345	9.47	27.93	0.0173	0.0625
JE 10-2	9.54	20.1	0.0060	0.0324	8.24	25.13	0.0970	0.0535
JE 21-1	12.13	22.73	0.0056	0.0304	9.02	28.20	0.0762	0.0770
JE 21-2	11.95	22.66	0.0074	0.0350	9.35	26.75	0.0688	0.0650
ES 2-1	10.95	21.42	0.0054	0.0321	9.1	26.78	0.0684	0.0685
ES 2-2	9.73	20.06	0.0057	0.0324	8.94	25.27	0.0782	0.0610
ES 3-3	10.45	20.18	0.0043	0.0273	9.7	25.29	0.0071	0.0590
ES 4-2	11.27	20.15	0.0048	0.0245	8.83	24.76	0.0087	0.0625
LSD (P=0.05)	4.429	2.116	0.0011	0.0043	1.419	1.419	0.0027	

Asterisks (*): indicates significant difference from control
LSD=Least Significant Difference (probability=0.05)

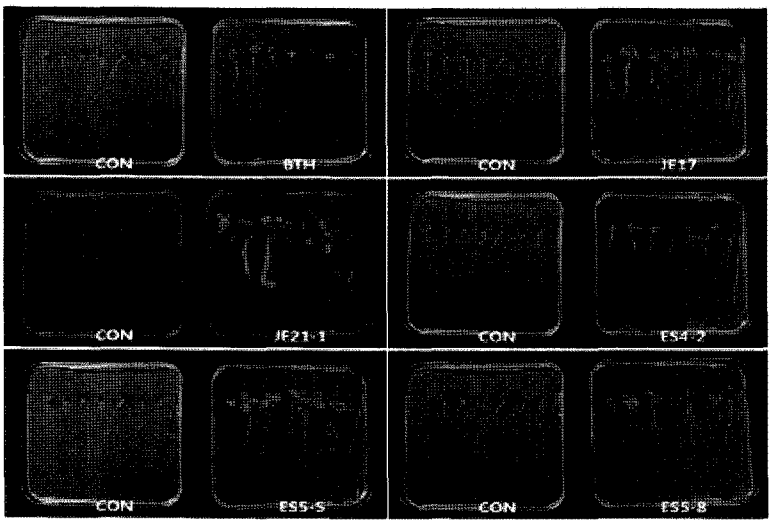

Fig. 2. Efficacy of selected strains on plant growth promotion and rhizosphere colonization in tomato.

8-1 strain which were statistically on par with EXTN-1 (10.28 cm) and BTH (10.11 cm). There was no significant influence on root and shoot dry weight by treatment with different *Bacillus* strains (Table 1 and Fig. 2).

In the cubic plate assay against anthracnose of red-pepper, different strains were selected at green house condition. All the strains recorded better plant height, leaf width in the treatment with 1×10^6 cells per ml when compared to 1×10^5 and 1×10^8 cells/ml. Plant height was better than positive checks BTH (19.5 cm) and EXTN-1 (21.8 cm). The untreated check recorded least plant height (19.3 cm). The leaf width was also >100 mm in all the strains except JE 3-5, JE 21-2 and ES 2-2. However, there was no significant difference with respect to leaf width in different *Bacillus* strains. The leaf width was less than 100 mm in case of positive check BTH (99.9 mm) and also untreated check (99.8 mm). The study indicated that treatment with *Bacillus* strains significantly improved the leaf width which in turn indicates more leaf area for photosynthetic activity. The number of anthracnose lesions per leaf was minimum in JE 21-2 (43.6) when compared to EXTN-1 (97.6 lesions/leaf) and BTH (45.5 lesions/leaf). The maximum disease pressure was recorded in untreated check (110.4 lesions/leaf) (Table 2).

Maximum fruit length (26.3 mm) was recorded in JE9-4 followed by 24.6 mm in ES 2-2 when compared to positive checks, EXTN-1 (22.4 mm), BTH (15.2 mm) and Control

Table 2. Plant growth promoting effects selected strains on red-pepper and tomato in green house assay

Treatment	Red-pepper		Tomato
	Fruit length (mm)	Fresh weight (g/fruit)	Fresh weight (g/fruit)
Control	21.2	0.0243	0.0242
BTH	15.2	0.0250	0.0183
EXTN-1	22.4	0.0259	0.0313
JE 3-5	17.5	0.0179	0.0607
JE 3-6	24.1	0.0310*	0.0520
JE 7-4	19.7	0.0233	0.0293
JE 8-1	21.0	0.0281	0.0336
JE 8-5	21.8	0.0275	0.0260
JE 9-3	20.8	0.0242	0.0381
JE 9-4	26.3*	0.0262	0.0421
JE 9-6	17.7	0.0248	0.0675
JE 10-2	20.2	0.0227	0.0867*
JE 17	17.1	0.0222	0.0464
JE 21-1	17.1	0.0226	0.1389*
JE 21-2	19.9	0.0284*	0.0132
CJ 1-3	15.7	0.0190	0.0599
ES 1-4	22.7	0.0240	0.0542
ES 2-1	19.7	0.0249	0.0677
ES 2-2	24.6	0.0287*	0.0146
ES 3-2	17.3	0.0236	0.0671
ES 3-3	24.4	0.0297*	0.0998*
ES 4-2	16.8	0.0227	0.0436
ES 5-4	17.4	0.0228	0.0489
ES 5-5	22.3	0.283	0.0501
ES 5-8	19.5	0.267	0.0537
LSD (P=0.05)	4.19	0.0039	0.0451

Asterisks(*): indicates significant difference from control.
LSD=Least Significant Difference (probability=0.05)

Table 3. PR1a promoter expression on tobacco plant by treatment of selected *Bacillus strains*

Treatment	PR1 GUS expression (nM MU/10 mg F.W/h)
Water	266.3
Control	3516.3
BTH	53366.0
JE 9-3	6056.3
JE 9-4	46288.0
JE 9-6	10675.3
JE 10-2	11476.7
ES 3-3	7113.0

(21.2 mm). The fresh weight of fruits was maximum (0.0310 g/fruit) in JE 3-6 followed by 0.0297 g/fruit in ES 4-2 and 0.0287 in ES 2-2 strains. The fresh fruit weight recorded in different *B. subtilis* treatments was better than EXTN-1, BTH and significantly superior to untreated control (Table 3).

In order to study the induced systemic resistance ability of these bacterial strains, we studied their effect on tobacco plant labelled with PR1 a for expression of GUS activity, effect on soft rot incidence and plant growth promotion. Among 22 strains screened, maximum fresh weight of 0.13893 g was shown in JE 21-1 followed by 0.09977 g in ES 3-3 and 0.08666 g in JE 10-2 when compared to positive checks EXTN-1 (0.03131 g), BTH (0.01827 g) and untreated check (0.0242 g) in square plate assay. Further, we studied induced systemic resistance of selected strains on tobacco. The incidence of soft rot was below 10% in case of JE 17, ES 5-5 and also positive check BTH. The incidence of soft rot was about 20-30% in case of ES 5-8 and ES 4-2, respectively. The maximum soft rot incidence (85%) was recorded in untreated control (Fig. 1 and Fig. 3). The maximum GUS activity (53366 nM MU/10 mg.F.W/hr) was recorded in BTH which was on par with JE 9-4 indicating higher induced systemic resistance. In other strains like JE 10-2, JE 9-6, JE 9-3 and ES 3-3, the GUS activity was relatively low. The minimum GUS activity was recorded in untreated control (Table 3).

Discussion

Exploitation of natural defense in host plant has been a long term goal of modern agriculture. Research towards this has lead to the development of biotic and abiotic agents that can induce systemic resistance in tobacco plant against invading pathogen. A positive correlation between different *B. subtilis* strains for plant growth promotion and induced

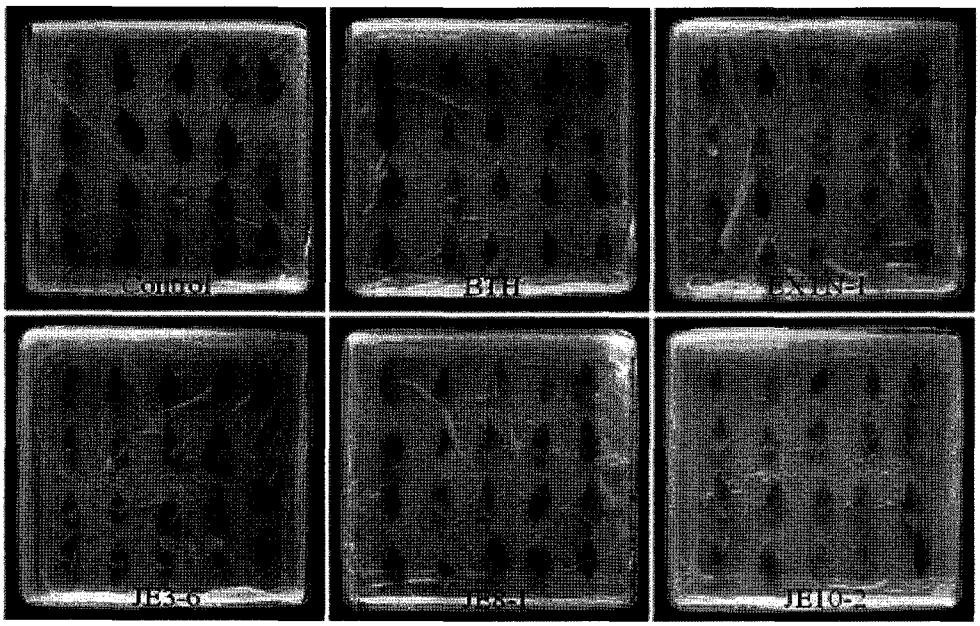

Fig. 3. Induced systemic resistance of tomato plants against *Pectobacterum carotovorum* SCC1 by treatment of selected strains in cubic plate assay.

resistance to infection of *C. acutatum* and *P. carotovorum* in red-pepper and tomato was confirmed by (i) plant growth promotion by different strains of *Bacillus* reflected by significant increase in plant height, root length, root dry weight and shoot dry weight of red-pepper and tomato when compared to BTH and untreated control. (ii) The suppression of anthracnose lesions on leaves treated with JE 21-2 when compared to chemical elicitor BTH, EXTN-1 and untreated control where the disease pressure was maximum. (iii) The different bacterial strains also supported maximum fruit length and fruit weight in case of JE 9-4 and ES 2-2. (iv) The study also demonstrated the role of *Bacillus subtilis* strains JE 21-2 and JE 8-1 in inducing the systemic resistance against two major pathogens *C. acutatum* and *P. carotovorum* in red-pepper and tomato apart from plant growth promotion. These positive aspects brought the importance and scope for use of *Bacillus subtilis* strains developed for plant growth promotion. (v) The host defense was triggered by expression of higher GUS activity in JE 9-4 followed by JE 9-6 on par with BTH and also significantly reducing the soft rot infection in tobacco and also supporting the better fruit weight.

Jeun *et al.* (2004) reported the role of two bacterial strains in managing anthracnose of cucumber and red-pepper. However, in the present study we identified for the first time role of native bacterial strains as potential elicitors of induced resistance again two major pathogens and also identified their role in plant growth promotion in red-pepper and tomato. More recently, the role of introduced *Bacillus* strains in ISR and PGPR was also reviewed by Kloepper *et al* (2004) in different crops and the role of hyaluronic acid from *Streptomyces spp.* as potential ISR agent in cucumber, tomato against major economically important diseases has been established by Park *et al.*, 2007. Thus, present investigation demonstrated the role of native *Bacillus* strains for effective plant growth promotion and induced systemic resistance against two major pathogens of red-pepper and tomato. Thus, this gives a wider scope for use of diverse strains of *Bacillus subtilis* for commercial organic soils in green house conditions for plant growth promotion, induced systemic resistance in different agro-climatic conditions for sustainable cultivation of red-pepper and tomato in Korea.

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토마토, 고추의 생육촉진 및 병 저항성 의 농업적 활용을 위한 토착 *Bacillus subtilis*의 생물활성 평가

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요 약 한국의 여러 지역에서 수집 분리 동정한 *Bacillus subtilis*의 토마토, 고추에 대한 생육촉진 및 병 저항성 활성을 평가하였다. 선발된 *B. subtillis* JE 21-1와 JE8-1 균주는 토마토 및 고추의 생육을 크게 증진 시켰다. *B. subtillis* JE21 균주 처리는 무처리 혹은 0.1 mm BTH 처리와 비교하여 고추와 토마토의 초장, 잎의 크기 등 생육을 촉진하고 고추 탄저병의 발생을 억제시켰다. ES2-1 균주는 고추 근권에 정착력이 우수하며 1.2×10^5 cfu/g root 의 밀도를 나타내었다. 생육촉진은 과실의 크기와 무게로 평가하였는데 JE9-4, ES2-2 균주를 처리하였을 때 과실크기가 향상되었고, JE 3-6, ES4-2, ES2-2 및 JE 21-2를 처리하였을 때 고추과실의 무게가 높았다. PR1a::GUS 유전자 함유 담배(Xanthi nc)에 선발된 바실러스균을 선 정착시키고 무름병균인 *Pectobacterium carotovorum* SCC를 접종하여 병 저항성 발현을 조사한 결과 JE9-4의 PR1::GUS 발현량이 가장 많았다.

색인어 바실러스 서브틸리스, 식물생육촉진, 유도저항성, 고추, 토마토