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Root Colonization and ISR-mediated Anthracnose Disease Control in Cucumber by Strain *Enterobacter asburiae* B1

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Here, we show that an endophytic bacterial strain, *Enterobacter asburiae* B1 exhibits the ability to elicit ISR in cucumber, tobacco and *Arabidopsis thaliana*. This indicates that strain B1 has a widespread ability to elicit ISR on various host plants. In this study, *E. asburiae* strain B1 did not show antifungal activity against tested major fungal pathogens, *Colletotrichum orbiculare*, *Botrytis cinerea*, *Phytophthora capsici*, *Rhizoctonia solani*, and *Fusarium oxysporum*. Moreover, the siderophore production by *E. asburiae* strain B1 was observed under *in vitro* condition. In greenhouse experiments, the root treatment of strain B1 significantly reduced disease severity of cucumber anthracnose caused by fungal pathogen *C. orbiculare* compared to non-treated control plants. By root treatment of strain B1 more than 50% disease control against anthracnose on cucumber was observed in all greenhouse experiments. Simultaneously, under the greenhouse condition, the soil drench of strain B1 and a chemical inducer benzothiadiazole (BTH) to tobacco plants induced GUS activity which is linked with activation of PR promoter gene. Furthermore, in *Arabidopsis thaliana* plants the soil drench of strain B1 induced the defense gene expression of *PR1* and *PDF1.2* related to salicylic acid and jasmonic acid/ethylene signaling pathways, respectively. In this study, for the main focus on root colonization by strain B1 associated with defense responses, bacterial cells of strain B1 was tagged with the *gfp* gene encoding the green fluorescent protein in order to determine the colonization pattern of strain B1 in cucumber. The *gfp*-tagged B1 cells were found on root surface and internal colonization in root, stem, and leaf. In addition to this, the scanning electron microscopy observation showed that *E. asburiae* strain B1 was able to colonized cucumber root surface.

Keywords : *Arabidopsis thaliana*, cucumber plant, green fluorescent protein, GUS gene, induced systemic resistance, *PR1a*, *PDF1.2*

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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. However, for the first time, the group of Auburn University had reported the bacterial-induced ISR against anthracnose pathogen of cucumber in greenhouse using gram-negative bacteria (Wei et al., 1991). Induced systemic resistance (ISR) in plant is a broad-spectrum and phenotypically similar to pathogen-induced systemic acquired resistance (SAR). Eventhough, the ISR pathway functions independently of salicylic acid (SA) but requires responsiveness to jasmonate and ethylene (van Wees et al., 1999). Preciously, 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). Ethylene is involved in the expression of the pathogen-inducible genes *Hel* (encoding a hevein-like protein) (Potter et al., 1993), *ChiB* (encoding a basic chitinase) (Samac et al., 1990), and *Pdf1.2* (encoding a plant defensin) (Penninckx et al., 1996), all with potential antifungal activity. Jasmonate has been shown to activate the *Hel*, *ChiB*, and *Pdf1.2* genes as well (Penninckx et al., 1996; Thomma et al., 1998). For full expression of *Pdf1.2*, both ethylene and jasmonate are required, indicating that these hormonal signals act in concert (Penninckx et al., 1998). Jasmonate is also implicated in the regulation of other pathogen-induced genes. *Lox1* and *Lox2* (encoding two lipoxygenases) (Bell and Mullet, 1993; Melan et al., 1993) control a feed-forward loop in jasmonate synthesis, but may also cause irreversible membrane damage leading to plant cell death (reviewed by Siedow, 1991). *Pal1* (encoding phenylalanine ammonia-lyase) controls the synthesis of phenylpropanoids such as lignin, and of SA in *Arabidopsis* (Mauch-Mani and Slusarenko, 1996), and has been demonstrated to be jasmonate-inducible (McConn et al., 1997).

Apart from rhizosphere colonizing bacteria, a number of

endophytic bacteria have been shown to increase plant growth promotion and protection against fungal infection by promoting marked host metabolic changes including production of phytoalexins (van Peer et al., 1991), accumulation of pathogenesis-related proteins (Zdor and Anderson, 1992), and deposition of structural barriers (Benhamou et al., 1996). In PGPR activity, among various groups of bacterial strains, many species of the genus *Enterobacter* have been described as effective biological control agents antagonistic to many fungal phytopathogens (Chernin et al., 1995; Wisniewski, et al., 1989) and plant growth promotion (Kampfer et al., 2005; Shoebitz et al., 2007). In the present study, an endophytic bacteria, *E. asburiae* B1 belongs to the genus *Enterobacter* was taken into account based on its performance on plant growth promotion through induced systemic resistance under *in vitro* condition. Here, the aim of the study was to evaluate the potential of an endophytic bacterium, *E. asburiae* B1 isolated from wild plants against cucumber anthracnose disease and its resistance mechanisms through transgenic tobacco and Arabidopsis plants. Also, the colonization of strain B1 with cucumber was examined with help of green fluorescent protein (GFP) gene marker and scanning electron microscopy (SEM). In previous studies, *E. asburiae* strains have been reported that is a frequent inhabitant of the rhizosphere of cotton, bean and cucumber (Hallman et al., 1998). Moreover, *E. asburiae* strain has significant role in P-solubilization (Gyaneshwar et al., 1999) and plant growth promotion by detoxification of metal ion (Kavitha et al., 2008). Besides, *E. asburiae* has been reported that it accounted for induced systemic resistance against wildfire disease in tobacco (Park and Kloepper, 2000). Also, *E. asburiae* has been examined as a potential hydrogen-producing bacterium (Shin et al., 2007).

Materials and Methods

Isolation and identification of bacterial strain. Rhizobacteria were collected from wild plants in Korea. After shaking the excised roots to remove all but tightly-adhering soil root segments (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 plated on nutrient agar (NA) (Fang, 1998). A total of 130 bacterial isolates were selected based on differences in colony morphology and pigment production. Purified strains were stored on Nutrient medium at 4°C (data not shown). Among them, in this study, only one bacterial strain which shows induced systemic resistance on different host plants was taken. This strain was tentatively identified by 16S rDNA analysis and was maintained at −80°C in tryptic soy broth (TSB) with glycerol (20%) for long-term storage. For preparing bacterial suspension, culture from −80°C was grown on tryptic 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.

Antibiosis. To test for *in vitro* antibiotic activity, green bean agar (GBA) and potato dextrose agar (PDA) plates were used to test for antibiosis against major fungal pathogens, *Colletotrichum orbiculare*, *Botrytis cinerea*, *Fusarium oxsporum*, *Phytophthora capsici*, and *Rhizoctonia solani*. Tested fungi were applied as mycelia plugs from the edge of a pregrown colony and were placed onto PDA plate in the edge of each PDA plate (two mycelia plugs/plate). A sterile assay paper disk (8 mm in diameter Advantec Co. JAPAN) was placed in the middle of each PDA plate and bacterial suspension of strain B1 (10^{10} cells/ml, 50 μl/disk) was applied to each disk. Plates were incubated at 18°C, 20°C and 28°C, for *C. orbiculare*, *B. cinerea* and *F. oxysporum*, *P. capsici*, *R. solani*, respectively, for 7 days. The inhibition zone was observed and the diameter was recorded. There were five replications per treatment.

Siderophore production test. Siderophore production by *E. asburiae* strain B1 was examined according to modified universal Chrome azurol S (CAS) assay method (Milagres et al., 1999).

***gfp*-tagged strain B1.** Strain B1 was grown on Luria Bertani medium for 6 h and bacterial cells were collected by centrifugation and washed three times in cold sterile distilled water. The final cell concentration was approximately 10^{10} cells/ml. To 100 μl of the concentrated cell suspension, 200 ng of purified *gfp* delivery plasmid DNA (pGFP uv) was added, then the mixture was transferred to an electroporation cuvette. The electroporation conditions used were 2.5 kV/cm, 25 mF, and 200 Ω, which were provided by a Gene Pulser electroporation device (Bio-Rad Laboratories, Hercules, Calif.). After electroporation, the cell suspension was diluted in 1 ml of LB medium and incubated for 40 min at 28°C before it was plated onto LB medium plates amended with Ampicillin (50 μg/ml). The presence of the *gfp* gene was confirmed by PCR (Kieran et al., 2004) and UV-light along with wild type strain B1.

Gnotobiotic root colonization studies. Strain *E. asburiae* B1::GFP was cultured on LB medium (Luria and Burrous, 1955) containing ampicillin (50 μg/ml) for 48 h. Seeds of cucumber were surface-sterilized by immersion in 1% aqueous sodium hypochlorite for 2 min and thoroughly washed with distilled water. Surface sterilized seeds were inoculated with strain *E. asburiae* B1::GFP before or after

seed germination. Seeds treated with 10 mM $MgSO_4$ served as a control. The seeds were transferred to sterilized growth pouches with half-strength Hoagland's N-free nutrient solution (Hoagland and Boyer, 1936). The growth pouches (3 seeds per growth pouch and 3 growth pouches for each treatment) were incubated in a growth cabinet at 28°C with a 12/12-h light/dark cycle. Growth pouches were harvested at 7 days growth. The pattern of root colonization by strain B1 at 7th day was observed on three different roots at different locations on the root (top-adjacent to the seed, mid region and region near the root tip) by fluorescent microscopy (Carl Zeiss AG, Germany).

For checking internal colonization, a second set of experiments was performed in order to enumerate the number of colony forming units (CFU) of bacteria colonizing the roots of cucumber using the dilution plating technique. Roots with plant obtained at 7th day after treatment was suspended in 10 ml of 10 mM $MgSO_4$ and vortexed vigorously for 30s in order to remove bacteria adhering to the root surface. Cucumber root, stem and leaf bits were surface sterilized and ground with sterile water. Then, they were suspended in 10 ml of 10 mM $MgSO_4$. Ten-fold dilutions were performed and plated onto LBA medium containing ampicillin (50 μg/ml) and incubated at 28°C for 48 h in order to determine the internal colonization of strain B1 in cucumber.

Tissue processing for scanning electron microscopy (SEM). Samples (1 cm long) from cucumber root under gnotobiotic condition, 3 days inoculated with bacterial suspension of strain B1 for 1h and control were examined under scanning electron microscope. The general method to prepare specimens for scanning electron microscopy was used. Samples were fixed in 3% glutaraldehyde in a 0.1 M sodium phosphate buffer (pH 7.0) for 1.5 hrs. The fixed samples were rinsed four times with distilled water and post-fixed with the buffered 2% OSO_4 for 90 minutes. The double fixed samples were rinsed two times with distilled water and then dehydrated with an ethanol series in five steps (50%, 75%, 90%, 95%, and 100%) for 40 minutes. The dehydrated samples were dried with liquid CO_2 by using critical point drier (Hitachi HCP-2) for 30 minutes after double treatments with a needle to the specimen stubs affixed with the both adhesive tape and were coated with gold ions in the ion coater (Eiko IB 3). Electron microscopy was conducted at 20 kV with a Hitachi S-570 scanning electron microscope. Three samples per treatment were examined.

Cucumber bioassay. The fungal pathogen used for the challenge inoculation was *C. orbiculare*, causal agent of anthracnose disease on cucumber. This fungal pathogen was maintained on green bean agar (GBA) plates at 22°C. Cucumber, which is highly susceptible to fungal pathogen tested, was used. Seeds of cucumber were surface-sterilized by immersion in 1% aqueous sodium hypochlorite for 5 min and thoroughly washed with distilled water. For the test of induced systemic resistance, cucumber seeds were planted to a depth of approximately 1 cm in 10-cm^2 plastic pots (one seed per pot) containing soilless Flora Guard (TKS 2 INSTANT, Kultur substrate) growing medium and kept in the greenhouse with daily watering. Cucumber roots were drenched with 50 ml (10^8 cells/ml) of bacterial suspension of B1 or 0.1 mM benzothiadiazole (BTH) or 10 mM $MgSO_4$ solution at cotyledon stage. For challenge inoculation, inoculum of *C. orbiculare* was prepared by suspending spores from an 8-day-old culture grown on green bean agar (Goode, 1958) in sterile water to give a concentration of 10^5 spores per ml. *C. orbiculare* was sprayed onto entire plants using air sprayer and sprayed to run-off. All plants from experiments were placed into a humidity chamber for 24 h immediately after inoculation. Seven days after pathogen challenge, the second leaf of each plant was assessed for anthracnose and the percent diseased leaf area of each was recorded.

Tobacco bioassay and GUS activity. Seeds of *Nicotiana tabacum* L. cv. *Xanthi*-nc, which were genetically engineered with a 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 hypochloride solution followed by 3 min in 70% ethanol and thoroughly washed in sterile distilled water. After seedlings rose in Murashige and Skoog salt (MS) medium (GIBCO/BRL), each seedling was transplanted to 10-cm^2 plastic pots containing soilless Flora Guard (TKS 2 INSTANT, Kultur substrate) growing medium and kept in the greenhouse with daily watering. Six week-old tobacco plants were soil drenched around plant stem with 50 ml bacterial suspension of strain B1 at concentration of 10^8 cells/ml or 0.1 mM BTH or 10 mM $MgSO_4$. 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 GUS assay.

Quantitative determination of GUS activity was accomplished by fluorometric GUS assay (Jefferson, 1987). For MUG assays, 10 mg leaf tissue from each plant was homogenized in 30 μl of extraction buffer (50 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 0.1% sarcosyl, 10 mM β-mercaptoethanol) and centrifuged at 13,000 g at 4°C. Thirty microlitre of the supernatant was added to 30 μl of GUS assay buffer (1 mM 4-methylumbelliferyl-β-D-glucuronic acid, (MUG) in extraction buffer)

and incubated at 37°C for 1 h. The reaction was stopped by adding 940 μl of 0.2 M Na_2CO_3 100 μl aliquots were measured with excitation at 365 nm and emission at 455 nm in a GENios fluorometer (Tecan, Austria). Background fluorescence was determined by carrying out the reaction in the absence of MUG, and this value was subtracted from each sample. As a calibration standard, 4-methylumbelliferone (MU) was used (Wilson et al., 1992), and, therefore, GUS activity was expressed as nM of MU/10 mg of sample/h.

RT-PCR. In this study, *Arabidopsis thaliana* wild type (Col-0) and transgenic line (nahG which was not able to express *PR* genes derived from parental *A. thaliana* ecotypes) were used. They were obtained from the Ohio State University Stock Center, Ohio State University, Columbus. NahG transgenic plants encode salicylate dehydrogenase and degrade SA (Delaney et al., 1995). *A. thaliana* seeds were surface-sterilized (2-min, 70% ethanol soaking followed by a 20-min, 1% sodium hypochlorite soaking) rinsed (four times) in sterile, distilled water, placed on petri dishes containing half-strength Murashige and Skoog salt (MS) medium (GIBCO/BRL) containing 0.8% agar and 1.5% sucrose, adjusted to pH 5.7, and vernalized for 2 days at 4°C in the absence of light. Seedlings then were placed in growth cabinets set to a 12-h-light/12-h-dark cycle under 40-W fluorescent lights; the temperature was maintained at 22°C±1°C with a relative humidity of 50-60%. Two-week-old seedlings were transferred to 60 ml pots containing a potting soil mixture that had been autoclaved twice for 1 h with a 24 h interval. Before transfer of the seedlings to pot, the potting soil was supplemented with either a suspension of strain B1 or an equal volume of a solution of 10 mM $MgSO_4$. Plants were cultivated in a growth chamber with a 9 h day (200 μE/m2.s at 24°C) and a 15 h night (20°C) cycle at 70% relative humidity. Plants were watered on alternate days and once a week supplied with a modified half-strength Hoagland's nutrient solution (Pieterse et al., 1996).

After two weeks, Arabidopsis plants were soil drenched with bacterial suspension of strain B1 for second time. One week later, leaf tissues were collected for RNA analysis. Total RNA was extracted by homogenizing at least 2 g of frozen tissue in an equal volume of extraction buffer (0.35 M Glycine, 0.048 M NaOH, 0.34 M NaCl, 0.04 M EDTA, 4% (w/v) SDS). The homogenates were extracted with phenol and chloroform and the RNA was precipitated with LiCl, as described (Sambrook et al., 1989). RT-PCR was performed according to Kishimoto et al. (2005) with Ex *Taq* polymerase (Takara Biomedicals, Japan). The reaction mixture contained 0.1 μg of cDNA, 10 pMol each of forward and reverse primers, 250 nM dNTPs, and 0.5U of Ex *Taq* polymerse in 20 μl of buffer solution. The PCR was carried out in a MJ Research (PTC-100, USA): 94°C for 5 min followed by 94°C for 1 min, 57°C for 1 min for 25 cycles, followed by a final 72°C extension for 10 min. Primers for the defense genes are *PDF1.2* (Plant defensin) primers F-5'-TGCGGTAACACCGAACCATAC-3'; R-5'-CGACAGTTGCATTGGTCCTCT-3'; *PR-1a* (Pathogenesis-related protein) primers, F-5'-AACCGCCAAAAGCAAA-CGCA3' R-5'-TCACGGAGGCACAACCAAGTC-3. Amplified PCR products were analyzed with 1.2% agarose gel and gels were documented (LAS-3000, Fuji photo film Co. LTD., Japan).

Statistical analysis. Each experiment had three 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

Identification of strain B1. From isolation, a bacterial colony was selected and designated B1. B1 was identified by sequence analysis of a 1500 bp sequence of its 16S rDNA gene. strain B1 was observed to possess 99% similarity to *E. asburiae* in the NCBI Nucleotide database (data not shown).

Antifungal activity. Strain B1 did not exhibit antibiosis against the tested all five fungal plant pathogens. *C. orbiculare*, *B. cinerea*, *F. oxysporum*, *P. capsici* and *R. solani* grew toward strain B1 and eventually overgrew the B1 (Fig. 1).

Siderophore production by strain B1. Formation of orange halo zone around the well poured with cell free culture supernatant of strain B1 grown in TSB and basal medium containing 0.5 μM and 20 μM $FeSO_4$ solution, in CAS agar plate confirmed the production of siderophore by *E. asburiae* B1 (Fig. 2).

Construction of *gfp* expressing strain B1. After electroporation, strain B1 was selected from a group of *gfp* mutant based on its high-level green fluorescence intensity among non *gfp* mutant colonies since wild type *E. asburiae* strain B1 was naturally resistant to ampicillin. In addition, the colony morphology, and growth patterns in TSA medium was similar to those of the wild type. No white colonies appeared after repeated transfers of the tagged strain onto non-selective media. Thus, the integration of *gfp* into the chromosome appeared to be stable. Purified bacterial cells of *gfp*-tagged and wild type of strain B1 were streaked on LB medium containing ampicillin. After growth, they were

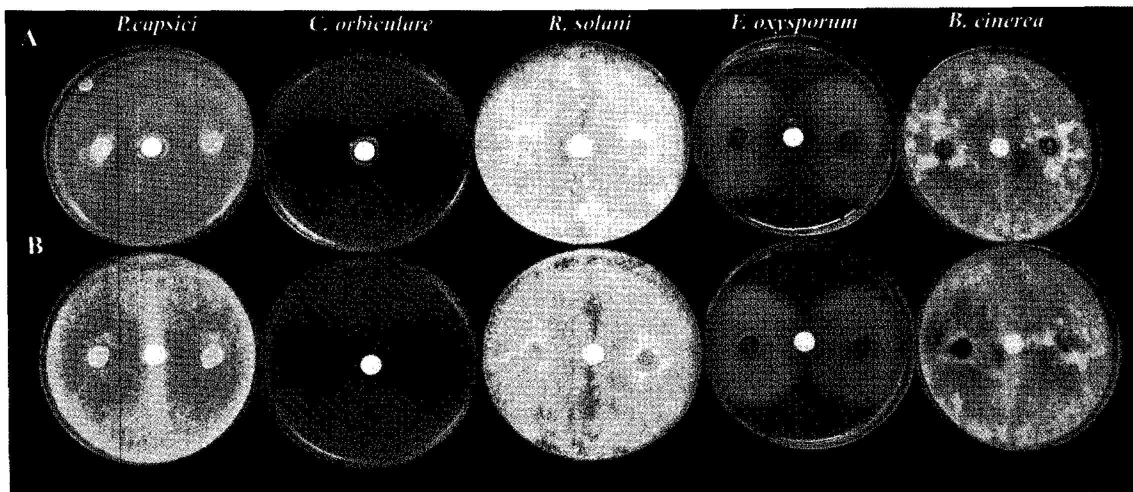

Fig. 1. Antifungal activity test. (A) *Enterobactor asburiae* strain B1 and (B) Control.

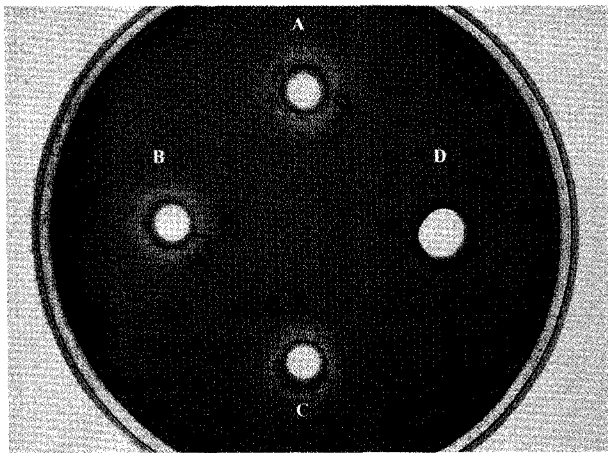

Fig. 2. Siderophore production test. A, B, C and D indicate the cell free culture supernatant of *Enterobactor asburiae* B1 grown in tryptone sucrose broth, 0.5 mM, 20 mM and $FeSO_4$ solution (Control).

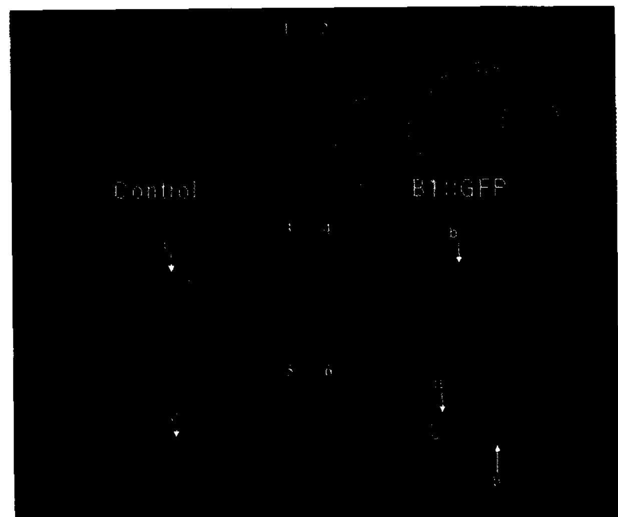

Fig. 3. Comparison of bacterial cells of wild type B1 with *gfp*-tagged cells under UV-light and cucumber root colonization by *gfp*-tagged bacterial cells of *E. asburiae* B1. (1: cucumber root colonization by bacterial cells of wild type strain B1 under UV-light; 2: root colonization of *gfp*-tagged bacterial cells of strain B1. 3: Cucumber root surface; 4 and 5: Root colonization of B1; 6: Bacterial cell attachment of root cell, a: plant cell, b: bacterial cell, c: individual cell death or HR).

compared under UV light for strong fluorescent activity and *gfp*-tagged bacterial cells were brighter than wild type. Similarly, *gfp*-tagged bacterial cells were given root treatment to cucumber and checked for their strong root colonization *in vitro* condition (Fig. 3).

Fluorescent microscopy. With help of fluorescent microscopy, qualitative image analysis of colonization patterns by strain *E. asburiae* B1::GFP on cucumber roots was done. No colonization pattern was found on root surface in seeds inoculated with bacterial suspension before germination. Only colonization pattern of *E. asburiae* B1 was found in seeds inoculated after germination. Strain *E. asburiae* B1::GFP was found on all three segments of cucumber roots as macro colonies and many cells were observed with specific attachment by strain *E. asburiae* B1-GFP (Fig. 3).

Enumeration of strain *E. asburiae* B1-GFP from cucumber grown under gnotobiotic condition. Bacterial population of strain B1 colonized in the internal and external plant parts was examined in cucumber grown under gnotobic conditions in growth pouches by serial dilution method. Seeds treated before germination showed no bacterial cells onto LBA medium containing 50 ng/ml ampicillin in serial dilution from extraction of root, stem and leaf parts. Presence of bacterial cells of strain B1 was observed only in cucumber inoculated after seed germination and the number of B1-GFP cells was counted to 6×10^4 from root surface. In internal colonization, the bacterial populations

Table 1. Cucumber protection against anthracnose by treatment of *E. asburiae* B1 under greenhouse condition

Treatments[a]	Total number of anthracnose spot/leaf(% protection)[c]				
	Exp.1	Exp. 2	Exp. 3	Exp. 4	Mean[b]
Control	276.7(00.0%)	289.0(00.0%)	282.7(00.0%)	327.5(00.0%)	294.0
BTH	47.3*(82.9%)	26.3*(90.8%)	50.8*(84.4%)	46.8*(83.4%)	42.8*
B1	89.7*(67.5%)	71.8*(75.1%)	154.0*(52.9%)	131.7*(53.4%)	111.8*
LSD(P=0.05)	33.7	30.8	40.0	37.7	42.0

[a]Treatment included *Enterobacter asburiae* B1, and two controls: SAR control (that was treated with 0.1 mM benzo (1, 2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) and a disease control (that was not treated with bacteria).
[b]Mean of four experiments. *indicates significant different according to Student's least significant difference (P=0.05) test.

Table 2. *Enterobactor asburiae* B1-mediated induction of β-Glucuronidase (GUS) activity related to *PR-1a* gene in tobacco under greenhouse condition

Treatments[a]	Gus activity (n Mol Mμ[b]/10 mg FW/hr)				
	Exp.1	Exp. 2	Exp. 3	Exp. 4	Mean[c]
Water	404.33	455.0	345.88	291.5	374.2
Control	1216.0	1583.66	1413.77	1352.0	1391.4
BTH	21235.22*	14312.0*	27395.33*	40438.8*	25845.4*
B1	3261.44*	2855.0*	3361.33*	23854.7*	8333.2*
LSD(P=0.05)	242.0	3640.22	224.7	8542.2	11691.9

[a]Leaves of *Xanthi*-nc tobacco plants at the six week of growth stage were given root treatment with 50 ml of a 10^8 cells/ml of bacterial suspension of *E. asburiae* strain B1; controls were soil drenched with the same volume of 10 mM $MgSO_4$ or 0.1 mM BTH. At 7 day after soil drench, upper three leaves were detached and processed for analysis of GUS activity.
[b]MU, 4-methylumbelliferone.
[c]Mean of six replications. *indicates significant different according to Student's least significant difference (P=0.05) test.

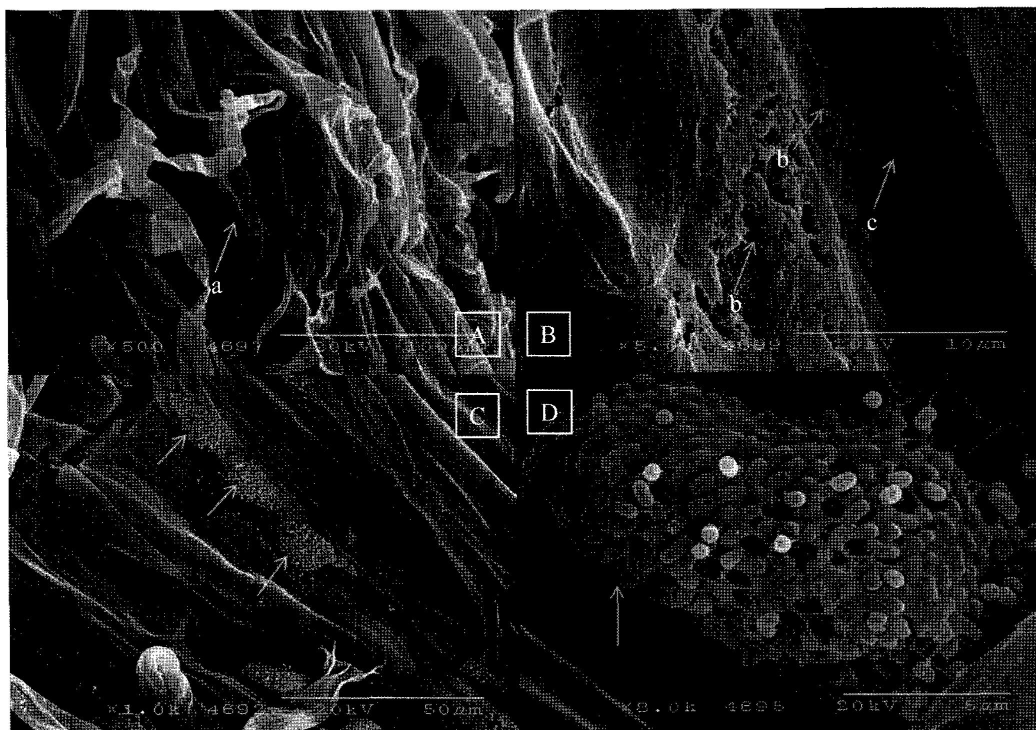

Fig. 4. Scanning electron micrographs. (A: cucumber root surface of non-treated control plant; B: B1 cells covered by exopolysaccharides (EPS) of strain B1 on root surface; C: Arrows indicate macrocolonies with aggregation of bacterial cells of strain B1 on root surface; D: Arrow indicates an enlarged macrocolony of B1 bacterial cells, a- root surface, b- bacterial cell of strain B1, c- EPS layer) 3 days after inoculation germinated seed 1 hour as soaking of bacterial suspension (1×10^7 cell/ml) of *Enterobacter asburiae* B1.

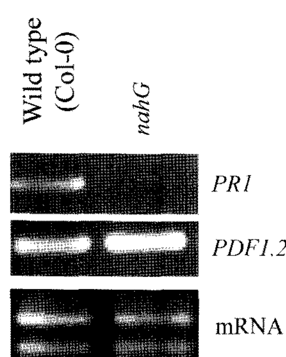

Fig. 5. Induction of *PR1* and *PDF1.2* in *Arabidopsis thaliana* by soil drench with bacterial suspension of strain B1 at 7 DAT in absence of pathogen.

were recorded 8.6×10^4, 8×10^4 and 3×10^4 from root, stem and leaf, respectively. This represents the increase in bacterial numbers on 7th day when compared to the number of bacteria present on the seeds (5.4×10^3 CFU/ml) at the time of inoculation.

Colonization of bacterial cells of strain B1 on cucumber root tissue scanning electron micrographs (SEM). Examination of cucumber root surface after inoculation with bacterial cells of *E. asburiae* B1 revealed that the bacteria abundantly colonized the root surface (Fig. 5A, B, C, and D). In cucumber plants treated with strain B1, bacterial cells were densely attached with root surface of cucumber and bacterial cells were covered with exopolysaccharides (EPS). And also, many macro colonies were found to be scattered on the root surface and in non-treated control plants, colonization of bacterial cells was not observed.

Cucumber anthracnose disease. Sunken brown lesions developed at the infection site of susceptible cucumber leaves 4 days after challenge. Later, the lesions expanded and coalesced quickly into a large sunken lesion, leading to the distortion of cucumber leaves. However, the lesions on leaves treated with strain B1 developed more slowly resulting in smaller lesions than those observed on strain B1-untreated plants. The background level of disease obtained in the disease control treatment varied among the replications; however, the same trends for treatment effects on disease occurred in all replications. Data in Table 1 are from four experiments. strain B1 treatment induced significant disease protection compared with the water treated control to varying degrees against *C. orbiculare* (52.9 to 75.1% of disease suppression). By root treatment with chemical inducer, BTH, there was significant reduction of cucumber anthracnose (82.9 to 90.8% of disease suppression) compared to non-induced control plants.

GUS assay. Under greenhouse condition, the root treatment of strain B1 to tobacco plants induced the GUS gene expression significantly in all four experiments compared to water treated control plants (Table 2). In chemical inducer (BTH) treated tobacco plants, the GUS gene expression was two or three fold compared to strain B1 treated tobacco plants. In all four experiments, the GUS gene expression was more or less equal in water treated plants. But, in strain B1 and BTH treated tobacco plants, the GUS expression was not equal. However, the same trends of treatment effects on plants occurred in all experiments.

RT-PCR. To gain more insight into the defense mechanisms involved by strain B1-mediated ISR on cucumber and tobacco in the absence of pathogen, we conducted the gene expression studies with Arabidopsis gene-specific primers of defense-related genes *PR-1* and *PDF 1.2*. strain B1 treated wild type Arabidopsis Col-0 showed systemic induction of both *PR-1* and *PDF1.2*. In transgenic plant *nahG*, the gene expression of *PDF1.2* was recorded and no effect on the expression level of the SA-induced PR-1 gene was found. And, 10mM $MgSO_4$ treated Arabidopsis plants showed none of the gene expression of *PR-1* and *PDF1.2* (Fig. 5).

Discussion

In this study, the results clearly provide evidence that the disease resistance against anthracnose in cucumber is associated with induced systemic resistance (ISR) by soil drenching with *E. asburiae* B1. In antibiosis study, strain B1 not showing antimicrobial activity is related to bacterial-mediated ISR against fungal pathogen, *C. orbiculare* which causing anthracnose in cucumber (Wei et al., 1991). And also, it has been reported that antagonistic activity of PGPR strain is not a direct mechanism to control disease (Jetiyanon and Kloepper, 2002). In this study, leaf infiltration with cell free culture supernatant of strain B1 on cucumber did not induce disease resistance against tested fungal pathogen, *C. orbiculare* causing anthracnose disease (unpublished data). Thus, this result indicates that there is no compelling evidence for an overall ISR signal produced by bacteria (Haas et al., 2002; Leeman et al., 1996; Van Loon et al., 1998; van Wees et al., 1997). Meanwhile, according to Ryu, et al. (2004) the volatile organic compounds by PGPR strains also play a key role in disease control. However, very recently, the influence of abiotic factors on production of elicitor involved in systemic resistance induction in different plants has been observed (Ongena et al., 2008).

In this study, seed treatment of strain B1 did not protect the cucumber plants from anthracnose (data not shown) and

induced systemic resistance against anthracnose is associated with soil drenching of strain B1 in cucumber. However, in a previous study, seed treatment with PGPR strain has protected cucumber against anthracnose by induced systemic resistance (Wei et al., 1991). This difference in the treatment on induce systemic resistance may be the result of bacterial colonization in plants by applied PGPR strain. In a previous study, no *E. asburiae* strains were recovered from inoculated plants by seed treatment (Musson et al., 1995). Furthermore, the soil drench with strain B1 induced GUS gene expression which related to *PR-1a* gene in transgenic tobacco. This result is not agreed with the concept of Pieterse et al. (1998) on ISR by a PGPR strain. However, some PGPR strains have involved in induction of GUS gene promoter related to *PR-1a* gene and protected tobacco against *P. syringes* pv. *tabaci* (Chen et al., 1999; Park and Kloepper, 2000; Schneider and Ullrich, 1991; Zdor and Anderson, 1992). According to Ran et al. (2005), rhizobacteria may have more than one mechanism of eliciting ISR, making the significance of SA produced by rhizobacteria in the elicitation of ISR enigmatic. However, in the present study, *E. asburiae* B1 is found to be involved in the production of siderophore under *in vitro* condition. Also, early studies reported that rhizobacteria produced siderophores have implicated in the production SA and involved in the induction of systemic resistance against fungal and viral diseases in different plants (Leeman et al., 1996; Maurhofer et al., 1998). Moreover, Press et al. (1997) has reported that in cucumber or tobacco, bacterial-mediated ISR system by production of SA is depends on the iron concentration.

Besides, strain B1 induced defense genes *PR-1* and *PDF1.2* related to SA and JA/ET (jasmonic acid/ethylene), respectively in wild type Arabidopsis plants (Col-0) in the absence of pathogen, simultaneously. Precisely, according to Pieterse and van Loon (1999) onset of resistance in plants, SA and JA/ET have been implicated in the signal transduction pathways leading to SAR and ISR, respectively and both of them are regulated by distinct signaling pathways in Arabidopsis. Thus, the present study may demonstrate that SA-dependent SAR pathway is fully compatible with JA-dependent ISR pathway (van Wees et al., 2000). Meanwhile, the tagging bacterial strain B1 with the *gfp* gene enabled us to both microscopically count green fluorescent cells and localize the specific green fluorescing bacteria on cucumber roots. In microscopic study, seed treatment (pregermination) with *gfp*-tagged strain B1 did not show the colonization pattern, but only in seeds treated after germination. Similarly, in a previous study with *E. asburiae,* no strains were recovered from inoculated plants by seed treatment (Musson et al., 1995). The high GFP fluorescence intensity of the strain B1 mutant allowed us to visualize the root surface of cucumber by fluorescent microscopy. At the same time, the internal colonization could study through serial dilution followed by crushing plant specimens. In the microscopy study, we could see some specific attachments of *gfp*-tagged B1 cells with more bright on cucumber root cells compared to rest of the cells. This interaction between bacterial and plant root cells are supposed to be related to some elicitors for induction of defense system in host plants such as lytic enzymes (Takeuchi et al., 1990) or accumulation of exopolysaccharide (EPS) (Desaki et al., 2006) or HR-like cell death (Vleesschauwer et al., 2009). But, in a previous study, *E. asburiae* strain did not induce marked cellular alterations upon internal colonization, but they did hydrolyze wall-bound cellulose Hallmann et al. (1997). In SEM, the aggregation of bacterial colonies of *E. asburiae* B1 on cucumber root surface could be seen very clearly along with EPS layer covered the bacterial cells of strain B1 attached on the root surface. Commonly, the polysaccharides derived from Gram-negative bacteria are representative of typical PAMP (pathogen-associated molecular pattern) molecules and associated with programmed cell death (PCD). They have been reported to induce defense-related responses, including the suppression of the hypersensitive response, the expression of defense genes and systemic resistance in plants (Desaki et al., 2006).

In conclusion, evidences are provided in this study that the root treatment with PGPR strain, *E. asburiae* B1 reduces disease incidence of cucumber anthracnose through induced systemic resistance and systemic resistance was confirmed by the induction of *PR* promoter gene in tobacco and *PR1* and *PDF1.2* genes in Arabidopsis to soil drench with strain B1. In this study, interestingly, strain B1 has elicited defense system on different host plants and this result is not agreed with van Wees (1997) who has demonstrated that rhizosphere pseudomonads are differentially active in eliciting ISR in related plant species. Moreover, in a previous study, *E. asburiae* JM-22 did not induce protection in cucumber to soil drench but induced GUS expression in tobacco (Park and Kloepper, 2000). But, in this study, *E. asburiae* strain B1 has induced systemic resistance in cucumber, tobacco and Arabidopsis plants to soil drench. This indicates that strain B1 must possess multi-defense mechanisms on different host plants by soil drench. However, this study has to be continued for analyzing mechanisms involved in ISR.

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