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Systemic Resistance Induction by Bacterial strains Isolated from Jeju Island against Late blight caused by *Phytophthora infestans* in Tomato

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ABSTRACT

Plant growth promoting rhizobacteria (PGPR) has been known as a biological inducer of resistance against various plant pathogens in many crops. The efficacy of resistance induction against late blight disease by bacterial strains, which were isolated from Halla mountain, was tested in tomato plants. Furthermore, the resistance mechanism on the leaf surfaces of the plants expressing induced systemic resistance (ISR) was investigated using a fluorescence microscopy. Pre-inoculation with the bacterial strains TRH423-3, TRH427-2, KRY505-3 and KRJ502-1 among the isolated bacterial strains in rhizosphere to tomato plants caused systemic resistance against late blight disease by *Phytophthora infestans*. DL-3-amino butyric acid, which was used as a positive control in this experiment, also mediated systemic resistance against same disease. All selected bacterial strains did not show direct antifungal effect to *Phytophthora infestans* in vitro test, indicating non correlation between the efficacy of resistance induction and expressing antifungal activity of bacterial strains. As other effective bacterial strains showing efficacy of resistance induction, all selected bacterial strains promoted the growth of tomato plants. The fluorescence microscopical study revealed that there were no differences in germination rate and in appressorium formation of the fungal cysts on the leaf surfaces between untreated plants and bacterial strains pre-inoculated plants. However, the callose was more frequently formed at the penetration sites on the leaf surfaces of the plants either pre-inoculated with bacterial strains or pre-treated with BABA. The frequency of callose formation on BABA pre-treated plants was slightly lower compared with those on bacterial strains pre-inoculated. But the brightness of fluorescence at the penetration

sites was higher in BABA pre-treated plants, indicating more active defense response of host cells. Conclusionally, the bacterial strains isolated from rhizosphere showed efficacy of resistance induction against late blight disease and their resistance mechanism may be difference to that of BABA mediated resistance.

I. INTRODUCTION

Late blight disease, caused by fungal pathogen *Phytophthora infestans* (Mont.) de Bary, has been one of the greatest limiting factors for production, attacking potato and tomato world-wide including in korea. *Phytophthora infestans* can be very serious on tomato particularly when the weather is consistently cool and rainy and it is responsible for a large proportion of total monetary losses sustained by growers each growing season (Soylu et al., 2006). Late blight may kill the foliage and stem of tomato with most infection spread by airborne asexual sporangia at any time during the growing season (Shattock, 2002). It also attacks tomato fruits in the field, which rot either in the field or while in storage (Agrios, 2004a).

This disease's control is still heavily based on the multiple applications of chemical fungicides (Shattock, 2002). These kinds of fungicide such as metalaxyl, oxadixyl, fosetyl-Al, cymoxanil and mancozeb, and dimethomorph which have excellent treated effects against *P. infestans* were currently reported as chemical fungicides for the control to late blight disease in tomato (Korea Crop Protection Association, 2006). Recently, by increasing interest to safe products of crops, reduction of chemical control effects to resistant agent and environmental problem by chemical's remaining toxicity, the alternative methods of disease control is highly required. Additionally, culturing area (6,749ha) and yields (438,991ton) in 2005 of tomato have continuously been increased with well-being boom compared to culturing area (3,348ha) and yields (205,763ton) of that in 2001 (Ministry of Agriculture and Forest, 2005).

One of the potential methods may be using a systemic acquired

resistance (SAR) or induced systemic resistance (ISR)on crops. SAR is induced in response to avirulent pathogens causing necrotic lesions. It is generally effective against subsequent infection by a broad range of pathogens, including viruses, bacteria and fungi, and it can last for several weeks or even months (Madamanchi and Kuc, 1991; Sticher et al., 1997). The induction of PR protein is mediated via a salicylic acid dependent signaling pathway and their expression as well as the SAR phenotype can also be induced by exogenous application of salicylic acid or its synthetic functional analogues 2,6-dichloroisonicotinic acid (INA) or benzothiazole(BTH) (Agrios, 2004b; Kessmann et al., 1994; Ryals et al., 1996; Sticher et al., 1997). Recently, a SA-independent pathway leading to systemic resistance has been discovered. It is induced by plant growth promoting rhizobacteria, such as Pseudomonas fluorescence, independent of PR protein induction and mediated via jasmonate and ethylene signalling (Pieterse et al., 1996; Van Loon et al., 1998; Ye et al., 1995). This PGPR mediated resistance has been defined as induced systemic resistance(ISR) (Van Loon et al., 1998). This rhizobacteria that live in the plant rhizosphere and colonize the root system, have been studied as a plant growth promoting agent for increasing agriculture products and as a bio-control agent against plant diseases (Kloepper and Beauchamp, 1992; Liu et al., 1995).

The extent of protection of this agent has sometimes been observed to vary (Silva et al., 2004) and may depend upon the genotype physiological condition of the plants, as well as the nature of the inducing agent used (Tuzun, 2001). However, When applied to farming without these limitation, this resistance generally has multiple resistance range against a broad spectrum of pathogens, non-chemical resistance, a resistance effect without direct contact with plant and pathogen, long lasting effect of resistance and the advantage of friendly environmental control methods.

There have currently identified cytoplasmic defense reaction, cell wall defense structures such as thicken cell wall, deposition of callose papillae, histological defense structures and necrotic or hypersensitive defense reaction as a induced structural defense reactions by induced resistance plants, and studies about these reactions are conducted constantly (Agrios, 2004c).

Until now, the studies about resistance mechanism on the induced leaf surface of plants expressing induced resistance using a fluorescent microscopy were presented in a preceding communication (Kováts et al., 1990a; Kováts et al., 1990b; Jeun et al., 2000a; Jeun et al., 2000b; Jeun et al., 2004; Jeun et al., 2005; Lee et al., 2005). They have identified resistance mechanism, which activates callose accumulation against various pathogens through fluorescent microscopical observation of stained tissues on the leaves of induced plants in preceding experiments. However, it has very rarely reported in isolated bacterial strains from Jeju.

In this study, experiments were conducted in the greenhouse to screen isolated bacterial strains from Jeju-island for induction of systemic protection of tomato against late blight disease, by evaluating disease control efficacy of the bacterial isolates. Furthermore, the resistance mechanism on the leaf surfaces of the plants expressing induced systemic resistance (ISR) was cytologically investigated using a fluorescent microscopy. In the BABA pre-treated plants the autofluorescence was very strong at the penetration site. And, there were no difference in germination rate of in appressorium formation between untreated and both BABA pre-treated and selected bacterial strains pre-inoculated plants at 1-3 days after inoculation. However, the callose formation on the selected bacterial strains pre-inoculated plants at 3 days after challenge was significantly increased compared to those of control plants, indicating

an enhancement of defense reaction of the plants.

Ⅱ. MATERIALS AND METHODS

This study was carried out in four phases. In the first phase of this study, 126's bacterial strains were separated from various places in Jeju and screened to select effective strains against late blight disease. And then, only this selected strains were investigated once again. In the second phase, experiments of growth enhancement effect by this selected bacterial strains on tomato growth were conducted. In the next, selected bacterial strains were tested whether they additionally have antifungal activity against *Phytophthora infestans*. In the final phase, interactions of plant-pathogen against late blight disease on the tomato leaf surface were cytologically observed by using fluorescent microscopy.

1. Separation of bacterial strains culture conditions

Plants included with roots were collected from various areas of coast and mountain Halla in Jeju in 2004-2005. One g fresh weight of the roots were attached and the soils were removed from root by tap water. The washed roots were thoroughly homogenized with 1㎖ of sterilized water in a mortar by using pestle. After filtering with three sheets of cheesecloth, the filtrate was diluted 10 times with steriled water. Three hundred ㎕ of each dilute was taken three times and unfolded on tryptic soy agar medium (TSA). TSA medium was made of Difco™ Tryptic Soy Agar® (Becton, Dickson and Company, France) 39.5g and Agar 5g in 900 ㎖ distilled water and then was autoclaved at 121℃ for 15 minute. The TSA plates were incubated at 28℃ for 2 days and the plates containing 1 to 10 colonies were selected. The colonies were classified by visual criteria. By the streaking method every colony was further isolated on

TSA. After incubation at 28℃ for 2 days the well-developed cells were transferred to TSA. Each combined bacterial strains (∅ 2㎜) were mixed with 15% glycerol in TSB (600㎕) in an ependorf-tube and stored at -80℃ until be used. TSB medium was made of Difco™ Tryptic Soy Broth® (Becton, Dickson and Company, France) 39.5g in 900 ㎖ distilled water and then was autoclaved at 121℃ for 15 minute.

2. Plant

Tomato (*Lycopersicum esculentum* Mill.) seeds (Sunmyeong®, Nongwoobio, Korea) were germinated in a incubator maintaining 28℃ for 72 hour, and then, the germinated seeds (approximately 30 units) were planted in a plastic pot (7cm in diameter) filled with sterile commercial soil (Tuksimi®, Nongwoogreentec, Korea) containing 20% of Perlite (Parat® , Sam son, Korea). Individual seedlings of tomato were transplanted to another plastic pots (7㎝ in diameter) 7 days after planting. Plants were fertilized once a week with 667 ppm 30 ㎖ of the complex fertilizer - Choroc Nala® (N-P-K, 30-10-10, Bokyung Nongsan, Korea) after the first leaf was appeared. Plants of the 2-3 leaf stage were used for disease inhibition effect assay. For the fluorescent microscopical observation of infection structures, 4-5 leaf stage plants were used. Plants were maintained in the greenhouse and watered daily by overhead watering at 20-25℃ for about 30 days.

3. Pathogen

Phytophthora infestans (Mont.) de Bary KACC40718 was distributed from Korean Agricultural Culture Collection (KACC) and kept on the potato dextrose agar (PDA) medium at 15℃ (Daeil Engineering, Korea) and then was used in the following study. PDA medium was made of Difco™ Potato Dextrose Agar® (Becton, Dickson and Company, France)

39.5g and Agar 5g in 900㎖ distilled water and then was autoclaved at 121℃ for 15 minute (Daihan labtech, Korea).

Phytophthora infestans (Mont.) de Bary KACC40718 was grown on oatmeal agar medium for 14-15 days at 15℃ to induce formation of sporangium. Oatmeal agar medium (72.5g, Difco™ Oatmeal Agar® (Becton, Dickson and Company, France) 72.5g was heated on a gas range by boiling in 900㎖ distilled water and then was autoclaved at 121℃ for 15 minute. To prepare a challenge inoculation, 20㎖ distilled water was poured in the oatmeal agar medium plates on which the late blight pathogen was grown and then these plates were kept in a refrigerator at 4℃ for 1-2 hours until zoospores were released from sporangiums. The suspension containing zoospores was filtered through two times folded Miracloth (CALIBIOCHEM, Germany). In order to encyst the zoospores, the suspension were shaken using a voltax (G-560, SCIENTIFIC INDUSTRIES, USA). The concentration of the cysts was adjusted to 1.0 X 10^5 cysts / ㎖ using a hemacytometer (Hausser, USA) for the inoculation of tomato.

4. Induction of induced systemic resistance

Experiments were conducted in a greenhouse to screen the separated bacterial strains for induction of systemic protection in tomato against late blight disease. The separated bacterial strains were freshly grown on tryptic soy agar (TSA) medium at 28℃ for 48h. 50㎖ suspension of about 126 separated bacterial strains were pre-inoculated by soil drench at the concentration of 2.0×10^6 cfu / ㎖ per plant.

For positive control, 50㎖ of BABA (1mM) solution was drenched per plant in soil at 5 days before challenge inoculation with *P. infestans*. As a negative control, water was given instead of the separated bacterial strains.

5. Challenge inoculation and assessment of late blight disease

The cyst suspensions of *P. infestans* (1.0×10^5 zoospores / ㎖, 0.01 % Tween 20) were sprayed onto upper and lower leaf surfaces at 5 days after pre-inoculation with bacterial strains or pre-treatment with BABA. The inoculated tomato was kept in a dew chamber (Donga, Korea) maintaining 100% RH (Relative Humidity) in the dark for 48 h at 18℃ and then placed in a growth chamber (Dasol science, Korea) with a day/night temperature of 18/23℃ and 60 % RH.

Disease infected lesion area rate on the inoculated leaves were investigated 5 days after challenge inoculation of the pathogen by visually estimating the late blight lesions. The disease severity rate (%) on tomato was calculated by visible measurement (% of the infected lesion area to whole area in a second leaf of tomato). Protection efficacy against the disease was calculated according to Cohen (1994) described as protection (%) = 100 (1-x/y) in which x and y are disease severity values in treated and control plants after challenge inoculation, respectively. The bacterial strains showing a efficacy of induction resistance were selected and further the same experiment was 3 times replicated.

6. Effects of growth promotion on tomato by the selected bacterial strains

To investigate the growth promotion of whole plants by the selected bacterial strains, the fresh weight as well as dry were measured at 6-7 leaf stage of tomato pre-inoculated with the selected bacterial strains, pre-treated with BABA and untreated. The whole plants were obtained by washing the soil attached roots by tap water. Also, for the dry weight, each plant was put in the envelope with aluminum foil and dried in a dry oven for 5 days at 70℃. These experiments were replicated three times

separately and each contained 6 plants.

7. Antifungal effect of the selected bacterial strains against *P. infestans*

Antifungal activities of selected bacterial strains were evaluated in vitro on PDA medium against *P. infestans*. The selected bacterial strains were spotted on the middle of half side of plate and then a mycelial disc (5 mm in diameter) of the plant pathogen from the margin of growing culture was placed at the center of the opposite side. The antifungal activity was surveyed against *P. infestans* after incubation for 6 days at 20℃. Inhibition rate of hyphal growth by a strain showing antifungal activity to all of the tested plant pathogens was evaluated by following: inhibition rate (%) = [1-(length of fungal colony near the isolate / length of fungal colony opposite of fungal colony opposite of the isolate)] × 100

8. Sampling for observation of infection structures using a fluorescent microscope

Samples for cytological investigations were collected at 24h, 48h, 72h after the challenge inoculation from the second leaf of four plants per treatment at each time. The leaf tissues were stained according to the method described by Jeun et al. (2000a). Sites of challenge infections were cut out with a razor blade (0.5 × 0.5㎜^{2}). To preserve the cell structures, the sections were fixed with 2% glutaraldehyde (SIGMA, Germany) in 0.05M phosphate buffer solution (PBS, pH7.2) for 2h. Phosphate buffer solution, 0.05M, pH7.2 was made according to the method described by Jeun et al. (2000), The solution (2.07g $NaH_2PO_4 \cdot H_20$ was dissolved in 300㎖ distilled water) was added to another solution (6.23g $Na_2HPO_4 \cdot 2H_20$ was dissolved in 700㎖ distilled water) until pH reaches 7.2. After the fixation, the sections were washed in the PBS for 10min 3times each. To identify the plant cells under a fluorescence

microscope, the sections were stained for 20min with 5.0% (w/v) aniline blue (SIGMA-ALDRICH, Germany) following each the washing procedure. For staining of fungal structures the leaf disks were subsequently incubated for 20min in 0.02% Uvitex 2B (w/v, Diethanol) which contains a fluorochrome for β-glucans. The leaf disks were washed with the PBS three times for 10 min each, after respective staining. And then, these were mounted on glass slides in 50% glycerin. The infection structures of the late blight disease fungus were observed using fluorescent microscopy (Olympus) equipped with filter set 05 (BP 400-440, FT 460, LP 470). Total number of germinated cysts, appressorium formations, callose formations were investigated from the data showed on the 4 leaf discs detached from each 4 plants in the 3 separated experiments.

9. Data analysis

The experimental design employed in the greenhouse studies was a completely randomized design (CRB) with 4-5 replicates.

The lesion areas of the plant leave and enhancement of fresh and dry weight of tomato, the germination rate and frequency of appressorium formation of the fungus, and callose formation rate in the inoculated leaves were compared using a Duncan's Multiple Range Test (DMRT). Statistical analysis was performed with SAS (version 8.02; SAS Institute)

Ⅲ. RESULTS

1. Isolation of bacterial strains

From the rhizosphere of about forty plants, 126 bacterial strains were isolated from diverse seven locations, Jeju in 2004-2005. The isolated bacterial strains were showed various features such as colors, visible features and smells (color, yellow or pale yellow or orange; visible features, wet or dry; smells, bad smell or no smell). Of these separated bacterial strains, effective bacterial strains TRH423-3, TRH427-2, KRJ502-1, KRY505-3 were finally selected through screening of separated bacterial strains against late blight disease in tomato.

2. Protection effects of late blight disease by the selected bacterial strains

Disease development on the leaves of tomato, which has been pre-inoculated with the separated bacterial strains, pre-treated with BABA and untreated were estimated by visible measuring the necrotic area at 5 days after inoculation with *P. infestans*. The symptom of late blight diseases was visually identified at 4 or 5 days after inoculation. The infected lesions were visually observed since 3 days after challenge inoculation on the leaves of all plants. On the leaves of untreated and pre-inoculated effective bacterial strains TRH423-3, TRH427-2, KRJ502-1, KRY505-3 among the separated bacterial strains, differences of the lesion number clearly were not identified in the early stage of the disease (data not shown). However, the lesion area of untreated plants was rapidly spread out compare to those of pre-inoculated one (Fig. 2B, Fig. 2C and Fig. 2D). Of separated bacterial strains, these remainders

except effective bacterial strains were not indicated in a number of lesion as well as the development area of infected lesion (data not shown). On the other hand, BABA pre-treated plants were showed low lesion numbers and not spread out to around area of infected regions compare to those of the other treats at the first stage of infection (Fig. 2A). Consequently, we finally chose bacterial strains TRH423-3, TRH427-2, KRJ502-1, KRY505-3 as a effective bacterial strains against late blight disease by *P. infestans* through 3 separated experiments. These selected bacterial strains TRH423-3, TRH427-2, KRJ502-1, KRY505-3 showed significant differences in disease inhibition of tomato against late blight disease caused by *P. infestans* compared to the untreated plants. As a positive control, BABA pre-treated plants showed highly significant differences compare to those of pre-inoculated selected bacterial strains as well as untreated tomato. While the lesion area was 23% on untreated plants, those of the bacterial strains showed about 11% (Fig. 1). On the BABA pre-treated plants, the lesion area was only 6% (Fig. 1). The protection rate by these strains were highly indicated about 50-55%, although the figure lower than that of BABA (Table 1).

3. Growth enhancement of tomato by the selected bacterial strains

In fresh weigh of tomato, there were not significant differences in between the plants pre-treated with BABA and untreated plants. Of the selected bacterial strains, TRH427-2 and KRJ505-3 showed growth enhancement as well as significant differences in fresh weight. Also, TRH423-3 and KRJ502-1 enhanced fresh weight of tomato, although they didn't have significant differences (Fig. 3A). In dry weight of tomato, the pre-inoculated with selected bacterial strains and BABA pre-treated plants also enhanced the dry weight, although they were not significant differences (except TRH427-2) (Fig. 3B). Proceeding from what has been

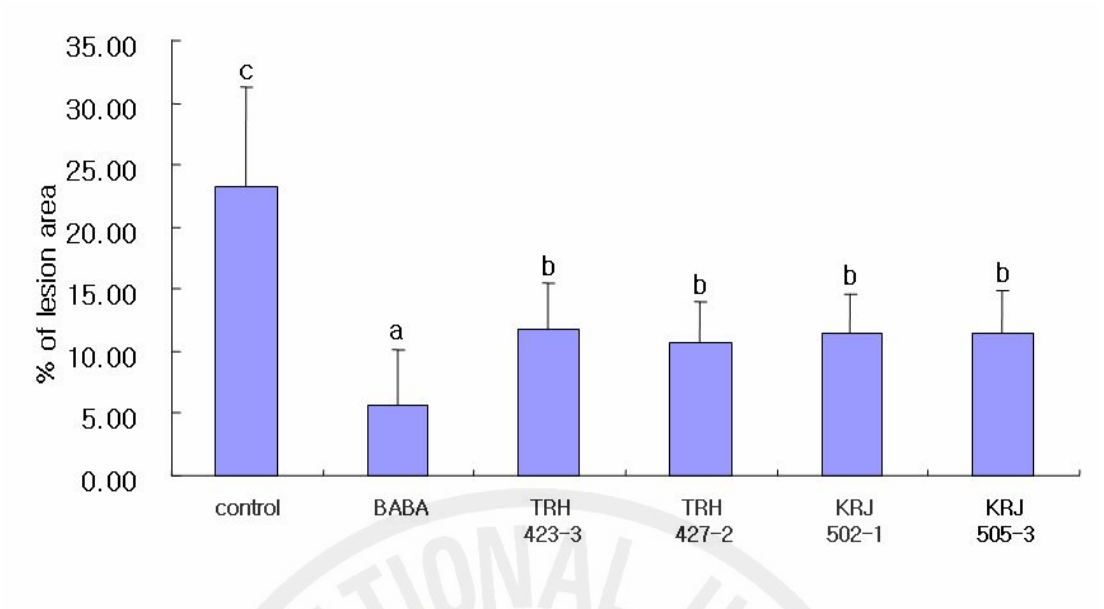

Fig. 1. Protective effects by pre-treatment with selected bacterial strains and DL-3-amino butyric acid (BABA) against late blight on tomato. The lesion area were measured 5 days after inoculation with *P. infestans* (1.0×10^5 zoospores / mℓ). The vertical bars indicate the standard deviation of the 3 separated experiments each containing 10 plants per treatment. Different letters on the columns are significantly ($P < 0.05$) different according to Duncan's multiple test.

Table 1. Protection rate on the second leaves of tomato plants pre-inoculated with selected bacterial strains or DL-3-amino butyric acid (BABA) pre-treated at 5 days after challenge inoculation with *Phytophthora infestans*

Treatment	Protection rate (%)[b]
Untreated	
BABA[a]	75.8
TRH423-3	49.5
TRH427-2	54.0
KRJ502-1	50.7
KRY505-3	50.6

[a] 50 ㎖ of BABA solution (1 mM) and 50 ㎖ of selected bacterial strains solutions (2.0×10^6 cfu / ㎖) per plant were drenched on the soil at 5 days before the challenge inoculation.

[b] Percentage rates were calculated by the formula, Protection (%) = 100 × (1 - x / y) in which x and y are % of lesion area on the leaves of treated and untreated plants, respectively.

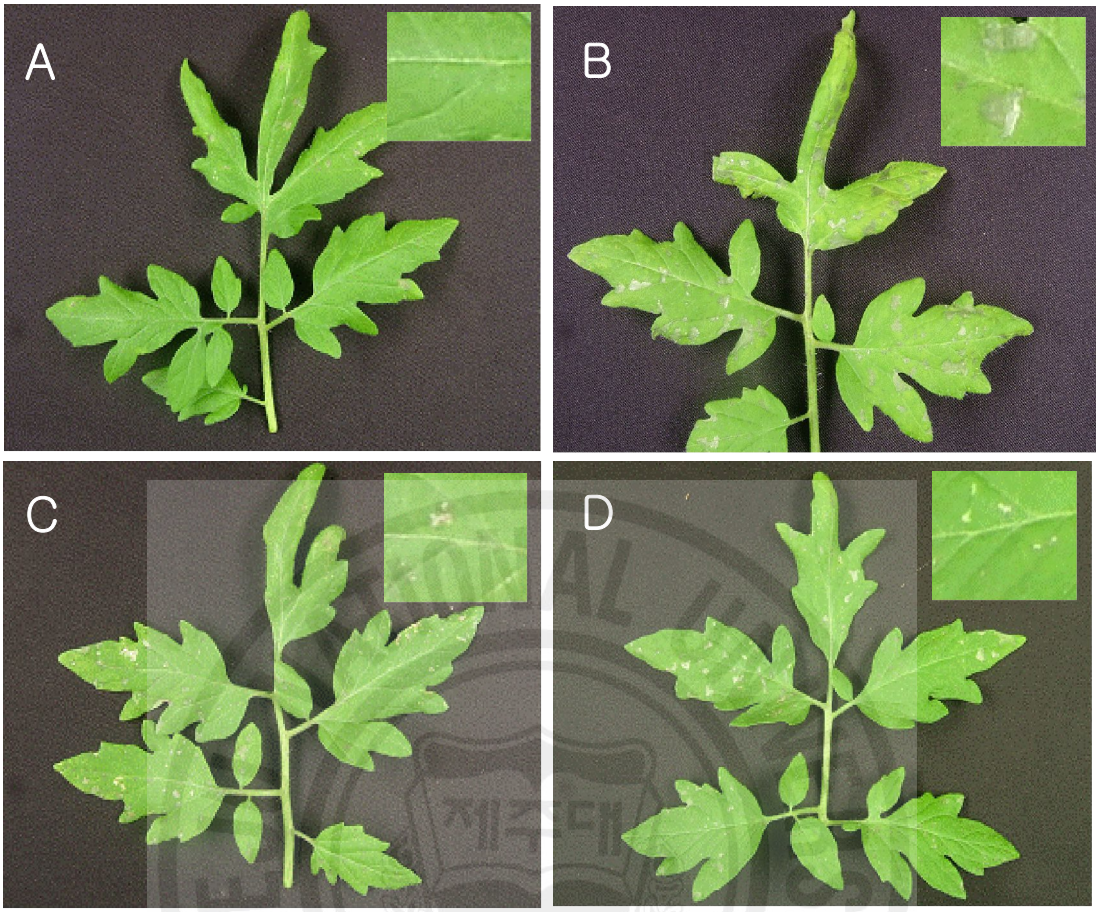

Fig. 2. Induction of systemically induced resistance in tomato against late blight disease. Second leaf of tomato pre-treated by soil drench with DL-3-amino butyric acid (1mM; 50㎖ / plant) (A) and the corresponding control leaf (B). Second leaves of tomato pre-inoculated with selected bacterial strains KRJ502-1 (C), TRH427-2 (D), respectively. The presented leaves were taken at 7 days after challenge inoculation with *Phytophthora infestans* at the concentration of 1.0×10^5 zoospores / ㎖.

said above, it should be concluded that these selected bacterial strains slightly increased growth of tomato.

4. Antifungal effect of selected bacterial strains against *P. infestans*

As results of these tests of selected bacterial strains, strain TRH423-3, TRH427-2, KRY505-3 had antifungal activity against *P. infestans* fungi in vitro test. Of these selected bacterial strains, TRH423-3, KRY505-3 inhibited effectively mycelial growth of *P. infestans* in the inhibition rate of about 62%, 45%, respectively. The strain TRH427-2 slightly inhibited mycelial growth of the *P. infestans* in the inhibition rate of about 27% (Table 2). However, the strain KRJ502-1 entirely didn't have antifungal activity to the *P. infestans* (Fig. 4 and Table 2).

5. Fluorescence microscopical observation of fungal development on the leaf surface

Using fluorescence microscope the resistance expression was examined both on the leaf surface and in the epidermal cell layer of tomato pre-inoculated with selected bacterial strains as well as pre-treated with BABA. On the leaf surfaces of untreated plants about 91.3% of total cysts were germinated and 82.8% of total cysts formed appressoria at 3 days after inoculation. Some cysts were germinated but failed to form appressoria. Most of the penetration sites were not intensively fluorescent, indicating no active defense reaction of the host cells (Fig. 8A).

On the leaf of plants pre-inoculated with selected bacterial strains, some penetration sites became fluorescent at 3 days after challenge inoculation, indicating the plant response to the fungal invasion of pathogen (Fig. 8B, 8C and 8D). However, there were no significant difference in callose formation at either 1 or 2 days after inoculation,

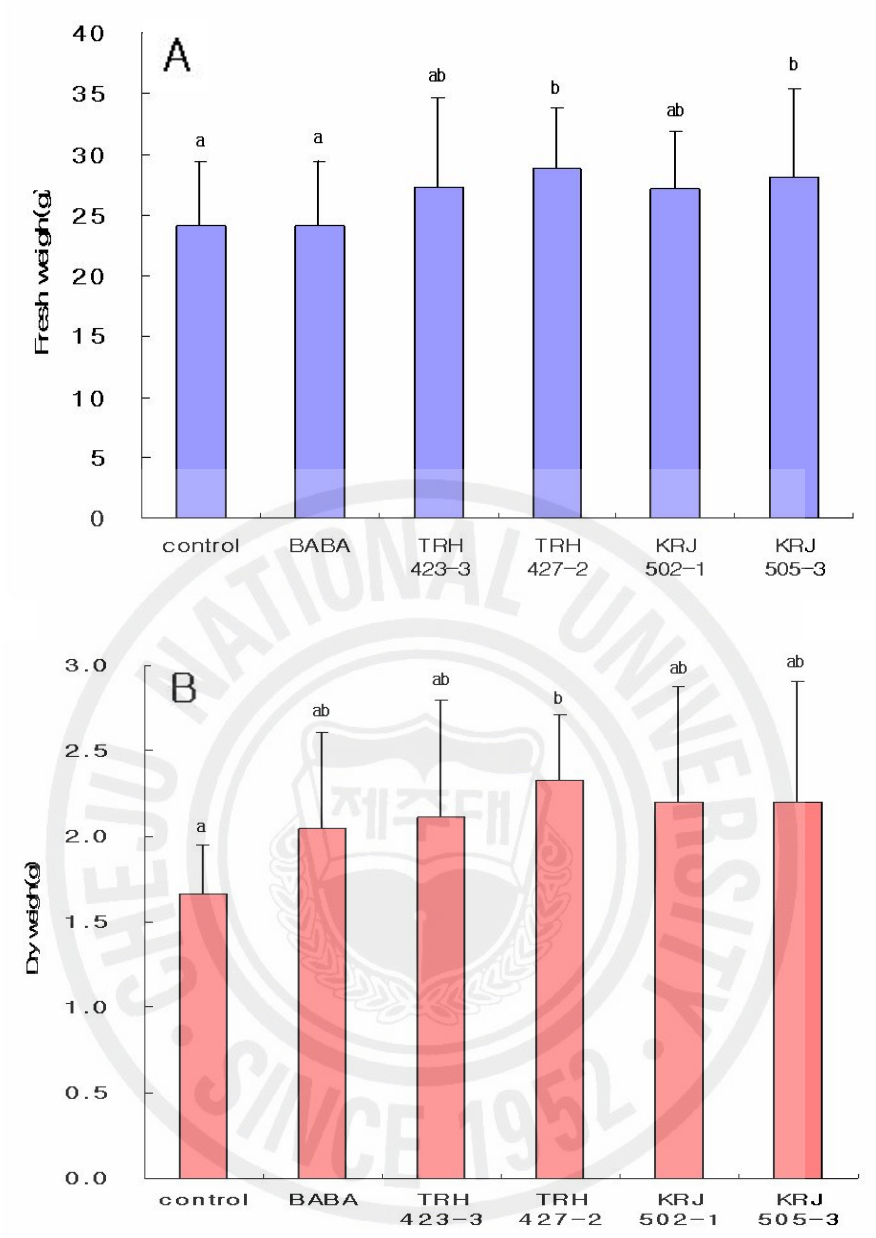

Fig. 3. Enhancement of the fresh (A) and dry (B) weight of tomato by selected bacterial strains TRH423-3, TRH427-2, KRJ502-1, KRY505-3. The vertical bars indicated the standard deviation of the 3 separated experiments each containing 6 plants per treatment. Different letters indicate significantly ($P < 0.05$) different according to Duncan's multiple range test.

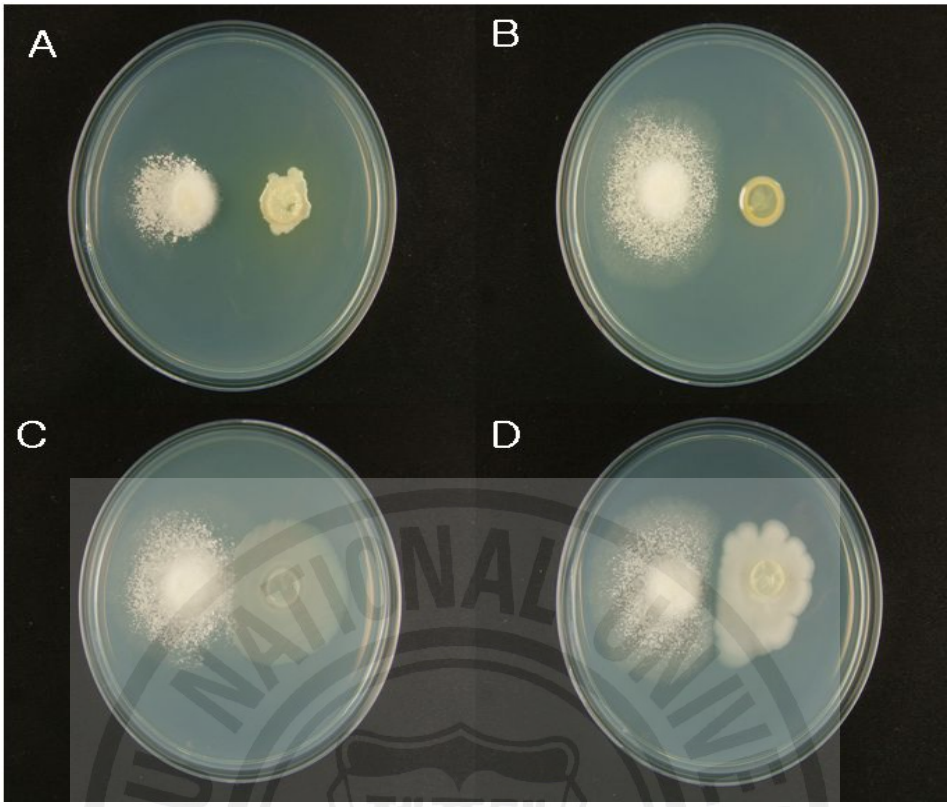

Fig. 4. Inhibition of hyphal growth of plant pathogen *Phytophthora infestans* by selected bacterial strain TRH423-3 (A), TRH427-2 (B) and KRY505-3 (D) in vitro test. The left of the plates represent *P. infestans* and the other site the selected bacterial strains. The inoculation-period either of the fungus or of the bacterial strains were same. The bacterial strain KRJ502-1 showed no inhibition of hyphal growth of *P. infestans* (C).

Table 2. Inhibition rates of hyphal growth of *Phytophthora infestans* by the selected bacterial strains TRH423-3, TRH427-2, KRJ502-1, KRY505-3

Inhibition rate of *P. infestans* by (%)[a]			
TRH423-3	TRH427-2	KRJ502-1	KRY505-3
61.8±11.7[b]	28.3±10.8	-	44.7±13.1

[a] Inhibition rate (%) = [1-(length of fungal colony near the strain/length of fungal colony opposite of the strain)] × 100

[b] Values represent means±standard deviation of two separated experiment, each containing three plates per treatment

[c] No antifungal activity

although pre-inoculated plants with selected bacterial strains were slightly increased in the callose formation compare to those of untreated one at 2 days after inoculation (Fig. 5C and 6C). Similarly, there were no difference in germination rate of in appressorium formation between untreated and both BABA pre-treated and bacterial strains pre-inoculated plants at 1, 2 or 3 days after inoculation (Fig. 5A, 5B, 6A, 6B, 7A and 7B).

However, callose was more frequently formed at the penetration sites on the BABA pre-treated as well as the selected bacterial strains pre-inoculated plants at 3 days after challenge inoculation compare to those of 1 or 2 days, whereas no difference were found on the leaves of control leaves (Fig. 5C, 6C and 7C). In the BABA pre-treated plants, the fluorescence by aniline blue was very strong at the penetration site (Fig 8E, 8F). Remarkably, the callose formation on the selected bacterial strains pre-inoculated plants at 3 days after challenge was significantly increased compared to those of control plants (Fig. 7C), indicating an enhancement of defense reaction of the plants.

The BABA pre-treated plants had similar results in the frequency of the fluorescent cells compared to those of the selected bacterial strains pre-inoculated plants (Fig. 7C), although disease severity on the leave of BABA pre-treated plants was highly lower than those of the selected bacterial strains pre-treated plants (Fig. 1).

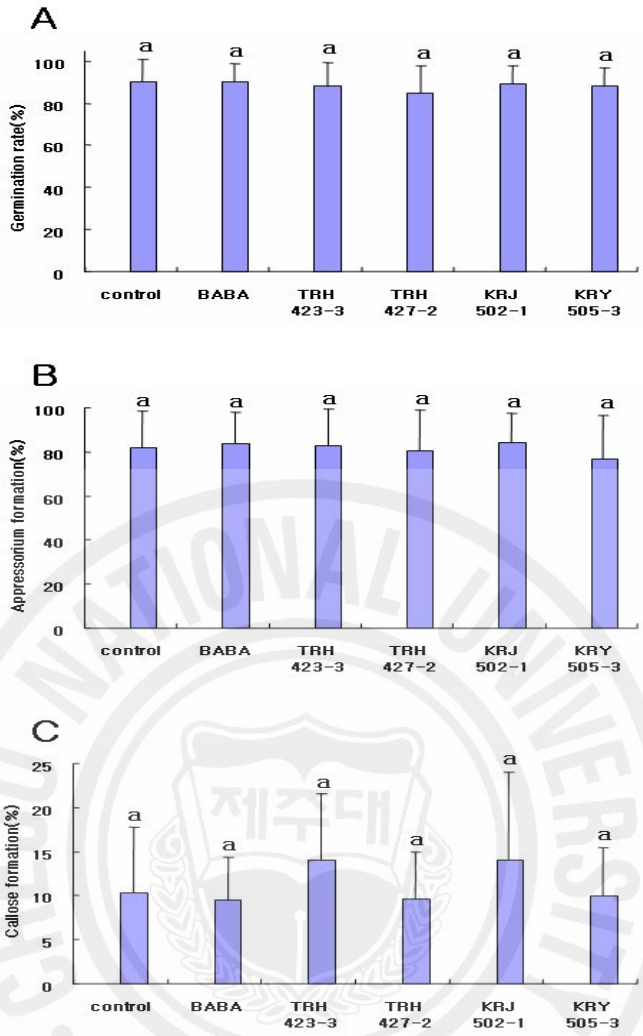

Fig. 5. Frequency of cyst germination, appressorium formation of *P. infestans* and callose formation of the plant cells on the leaves of tomato, pre-inoculated with selected bacterial strains TRH423-3, TRH427-2, KRJ502-1, KRY505-3, pre-treated with BABA and untreated plants. The leaves were attached at 1 day after challenge inoculation with *P. infestans* (1.0 X 10^5 cysts / mℓ). The vertical bars indicate the standard deviation of the 3 separated experiments each containing 4 leaf disc from 10 plants per treatment. Different letters indicate significantly ($P < 0.001$) different according to Duncan's multiple range test.

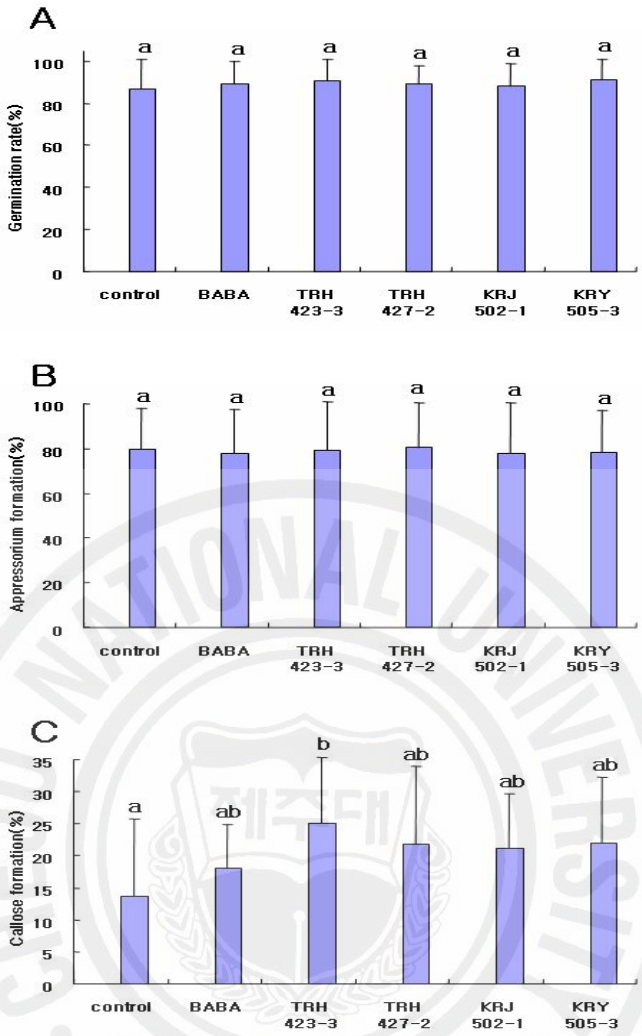

Fig. 6. Frequency of cyst germination, appressorium formation of *P. infestans* and callose formation of the plant cells on the leaves of tomato, pre-inoculated with selected bacterial strains TRH423-3, TRH427-2, KRJ502-1, KRY505-3, pre-treated with BABA and untreated plants. The leaves were attached at 2 days after challenge inoculation with *P. infestans* (1.0 X 10^5 cysts / mℓ). The vertical bars indicate the standard deviation of the 3 separated experiments each containing 4 leaf disc from 10 plants per treatment. Different letters indicate significantly ($P < 0.001$) different according to Duncan's multiple range test.

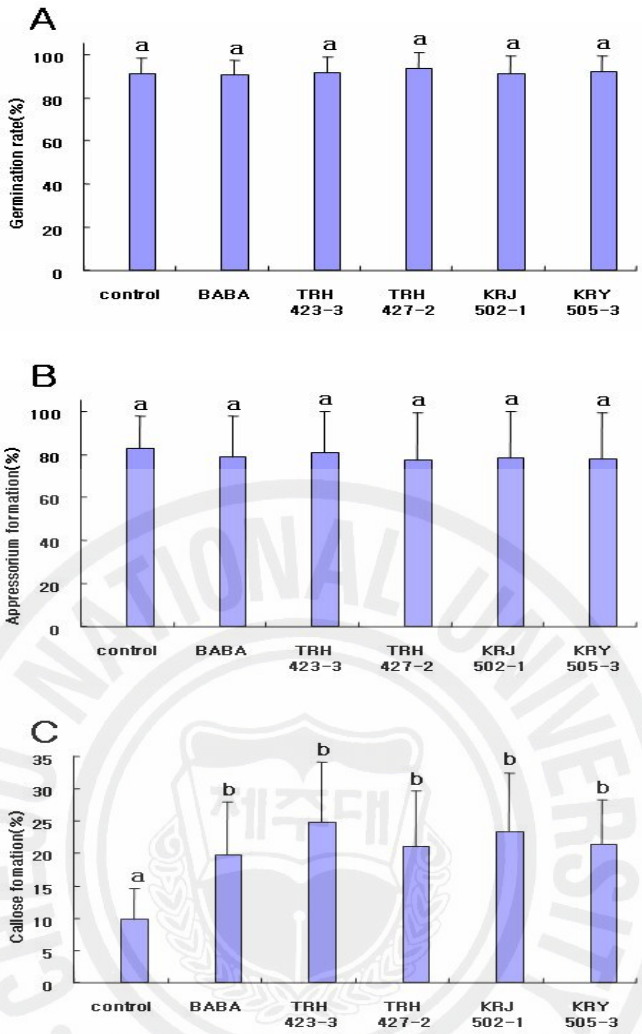

Fig. 7. Frequency of cyst germination, appressorium formation of *P. infestans* and callose formation of the plant cells on the leaves of tomato, pre-inoculated with selected bacterial strains TRH423-3, TRH427-2, KRJ502-1, KRY505-3, pre-treated with BABA and untreated plants. The leaves were attached at 3 days after challenge inoculation with *P. infestans* (1.0 X 10^5 cysts / mℓ). The vertical bars indicate the standard deviation of the 3 separated experiments each containing 4 leaf disc from 10 plants per treatment. Different letters indicate significantly ($P < 0.001$) different according to Duncan's multiple range test.

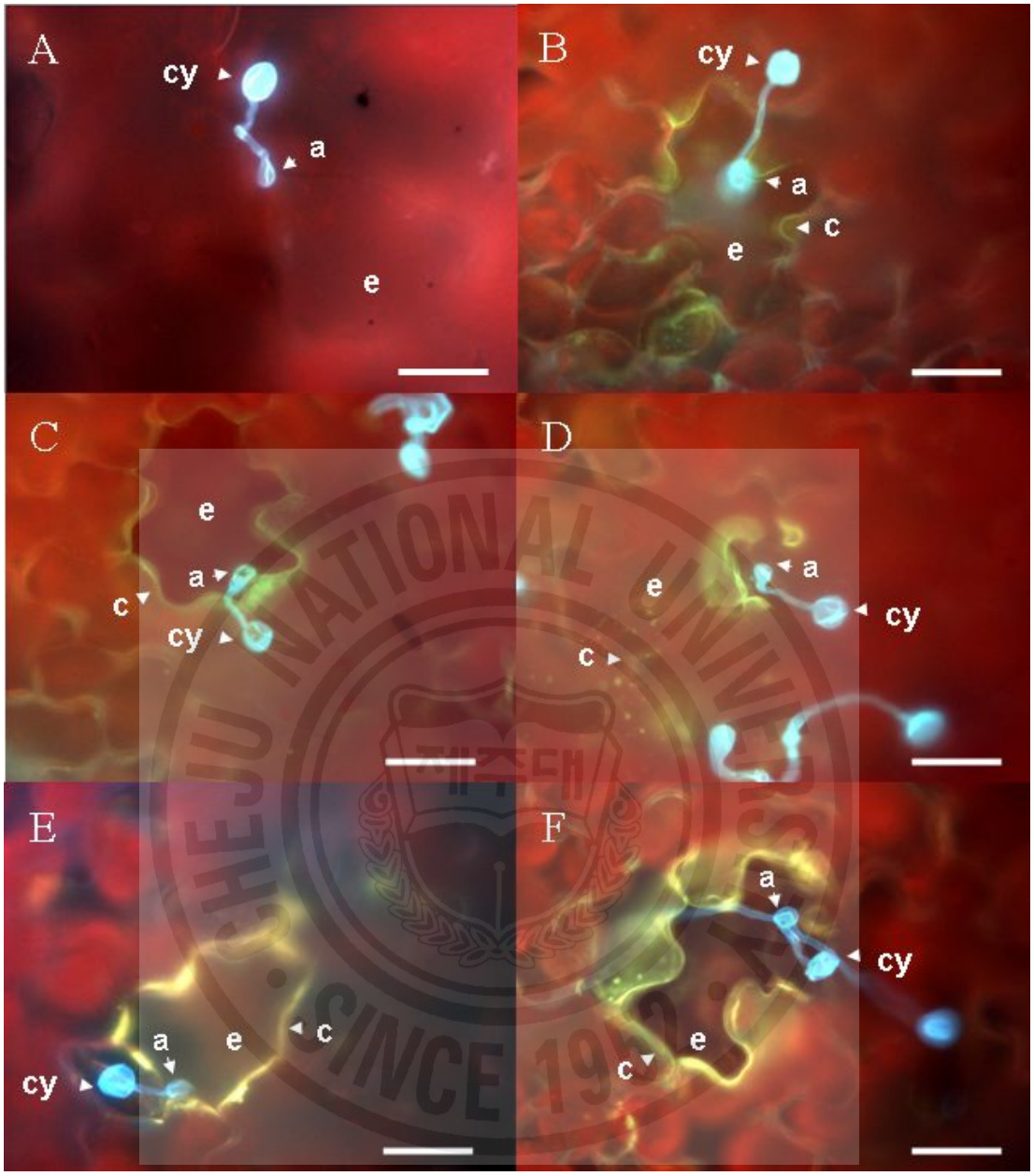

Fig. 8. Fluorescent microscopical observations of infection structures and resistance response on the leaves of the tomato pre-inoculated with selected bacterial strains (B, TRH423-3; C, TRH427-2; D, KRJ502-1), pre-treated BABA (E, F), and untreated (A) at 3 days after challenge inoculation. Abb. : a, appressorium; c, callose; cy, cyst; e, epidermal cell (bars = 20 μm).

Ⅳ. DISCUSSION

The use of plant growth promoting rhizobacteria (PGPR) for the control of various fungal pathogens has been reported (Kleopper et al. 1996; Jeun et al. 2004; Van Loon et al. 1998). This concept has exploited the commercial potential of the PGPR strains and resulted in the commercialization of a number of microbial bio-control products. In addition to disease control, rhizobacterial strains were found to increase the plant growth after inoculation (Kloepper et al., 1980).

Although the isolated bacterial strains were showed various features such as colors, visible features and smells, there were no correlations between features of those and resistance induction effect. However, isolated bacterial strains generally were identified around mountain areas more effective than around coast area like previous experiment's results. (Kim and Jeun, 2006)

The lesion area was decreased by approximately 50% in the plants pre-inoculated with selected bacterial strains (Table 1). On the other hand, DL-3-amino butyric acid (BABA) is well known as an activator in many plants for induced resistance (Cohen, 2002; Jeun and Park, 2003; Zimmerli et al., 2000). In this study the pre-treatment with BABA could be caused the effective induction of systemic resistance (Fig. 1). About 76% of lesion area was decreased by the application of BABA (Table 1). Based on this result it can be suggested that an abiotic activator may induce resistance more effectively compare to those of a biotic inducer such as selected bacterial strains (Jeun et al., 2004; Kim and Jeun, 2006). Similar results have been observed in other study e.g. the pre-inoculation with plant growth promoting rhizobacteria (PGPR) caused 40% reduction

of lesion number of anthracnose in cucumber plants, while about 85% of lesion number was decreased by the application of BABA (Jeun et al., 2004).

In the present study, the pre-inoculated with the selected bacterial strains enhanced slightly the growth of tomato (Fig. 3). Among the selected bacterial strains, only the TRH427-2 enhanced growth of tomato with significant difference. Although the others were no significant data, the weight of plants was almost increased by the pre-inoculated plants in all of the experiments (Fig. 3). Similar findings were reported by Kim and Jeun (2006) that the drench of PGPR strains promoted the plant growth besides effectively controlling potato late blight (*P. infestans*). These results indicated that the growth of plant might be improved by the interaction between the selected bacterial strains and root of plants. Plant growth promotion by rhizobacteria could be due to the production of growth hormones, Indole-3-Acetic Acid (IAA) & Gibberelinc Acid (GA), suppression of deleterious organisms and promotion of the availability of uptake of mineral nutrients (Kloepper et al., 1980). However, the exact growth enhancement mechanisms of PGPR to tomato have been not clearly illustrated.

Unlike mechanisms of systemic acquired resistance (SAR), mechanisms of induced systemic resistance (ISR) have been reported direct anti-fungal activity against pathogen. In this test to identify direct anti-fungal activity of selected bacterial strains against *P. infestans*, the selective strains (except KRJ502-1) showed the antifungal activity to *P. infestans*. Therefore, these 3 bacterial strains may be expected to control tomato late blight disease not only through inducing systemic resistance but also through directly anti-fungal activity in tomato. Such rhizosphere bacteria have been reported as a growth promoter as well as a resistance inducer in many host-pathogen interactions (Gamo and Ahn,

1991; van Loon et al., 1998). They were defined as plant growth promoting rhizobacteria (PGPR; Kloepper et al., 1980). Therefore, these four selected bacterial strains were considered as plant growth promoting rhizobacterias (PGPR).

In this cytological study it was attempted to illustrate the resistance mechanism mediated by pre-inoculation with the selected bacterial strains, and to compare with those of resistance by chemical BABA. Generally, the cyst of *Phytophthora infestans* germinates on the surface of leaves of tomato under optimal condition of humidity and temperature (Jeun et al., 2000a; Kovats et al., 1991b). The rate of cyst germination may be a criterion of expressing resistance in much plant-fungal pathogen interactions (Kovats et al., 1991a). In this study there were no differences in germination rate and appressorium formation rate between among the selected bacterial strains pre-inoculated, BABA pre-treated, and non-treated susceptible plants (Fig. 5A, 6A and 7A), indicating on role of germination and appressorium in expressing resistance. Like some other fungi, the *Phytophthora infestans* forms an appressorium, which is structurally differentiated from a cyst, in order to penetrate the host cell walls. Because *Phytophthora infestans* can mostly penetrate into the host cells with formation of on appressorium, the plant may acquire resistance against late blight by suppression of the appressorium formation. Indeed, the reduction of appressorium formation had been demonstrated in the resistance expressing leaves of cucumber plants (Kovats et al., 1991a). However, in this study appressorium formation did not suppressed on the leaves of plants pre-inoculated with the selected bacterial strains (Fig. 5B, 6B and 7B). Nevertheless, resistance against tomato late blight disease was triggered by the pre-inoculation with the selected bacterial strains (Fig. 1 and Table 1). This result indicates that some resistance mechanisms, other than the suppression of appressorium formation, may

be involved in the expression of resistance induced by the selected bacterial strains.

Many callose formation sites were observed at the penetration sites on the leaves of the plants pre-inoculated with selected bacterial strains compared to those of untreated tomato at 2 or 3 days after the challenge inoculation (Fig. 6C and 7C). Although it was not significant at 2 days after challenge inoculation, the callose formation of selected bacterial strains pre-inoculated plants was higher than that of untreated tomato at 2 or 3 days after challenge inoculation (Fig. 6C and 7C). The fluorescent cells indicate the active defense reaction against fungal attack similar to the callose formation of the host cells. The enhanced callose formation has been well known as a resistance mechanism in many host-parasite interactions (Sticher et al., 1997; Stroömberg and Brishammar, 1993; Kovats et al., 1991b). Similar results were observed in the other study, in which the callose formation was enhanced on the leaves of cucumber plants pre-inoculation with PGPR (Jeun et al., 2004).

On the bases of the results of cytological observations, it is suggested that the callose formations at the penetration site may be play an important role for expressing a resistance against late blight in the tomato pre-inoculated with selected bacterial strains. In BABA pre-treated plants, protection rate indicated high figure compare to those of PGPR pre-inoculated plants, however, showed lower rate of callose formation than those of the PGPR pre-inoculated plants. These different resistance expressions may be caused the different protection values by selected bacterial strains and BABA pre-treated plant. It could be also involved in the other defense responses such as the production of anti-fungal substance phytoalexins (Siegrist et al., 2000), the accumulation PR-proteins (Hwang et al.,1997; Jeun, 2000b), and encoding of enzymes involved in the metabolism of reactive oxygen species (Lamb and Dixon,

1997). To confirm this hypothesis, further investigations are required at the biochemical level.

Ⅴ. 適 要

식물 생장촉진 근권세균 (PGPR)은 많은 작물에 있어서 다양한 병원균에 대한 저항성을 유도하는 생물적 유도인자로서 알려져 왔다. 본 실험에서는 제주의 한라산에서 분리된 근권세균에 의한 토마토 역병에 대한 저항성 유도의 효과가 실험되었으며, 또한 더 나아가서 이러한 유도저항성 (ISR)을 나타내는 식물의 잎 표면에서의 저항성기작이 형광현미경을 이용하여 조사하였다. 분리된 근권세균 중 TRH423-3, TRH427-2, KRJ502-1, KRY505-3은 *Phytophthora infestans* 에 의한 토마토역병에 대해 전신적 저항성을 유도하여 병 발생정도가 감소하였다.

한편, 본 실험에서 긍정적 대조구로 사용된 DL-3-amino butyric acid (BABA)에서도 이전에 수행된 실험과 마찬가지로 토마토 역병에 대하여 전신적 저항성을 유도하는 것을 확인하였다. 병 발생 감소의 원인을 알기 위해서 *Phytophthora infestans* 에 대한 항균효과를 실험한 결과 모든 선발 균이 직접적인 항균효과를 나타내지는 않아 이로 인한 직접적인 상관관계를 확인할 수 없었다. 한편, 이러한 분리 균의 생장촉진효과에 대한 실험을 수행한 결과 유의차는 크지 않지만, 생장을 촉진시키는 경향이 확인되었다. 또한 *Phytophthora infestans* 를 접종한 토마토 식물체 잎의 관통조직에서의 병원균의 침입기작을 형광현미경을 통하여 관찰한 결과 선발된 균을 처리한 식물과 BABA, 그리고 무처리 사이에서의 역병균의 발아율과 부착기 형성율은 유의적인 차이가 관찰되지 않았다. 하지만, 식물 방어기작 중의 하나인 callose 형성율에 있어서는 선발 균을 처리한 식물과 BABA를 처리한 식물에 있어서 무처리에 비하여 높은 빈도와 유의차를 확인할 수 있었다. 또한, BABA를 처리한 식물은 선발 균을 처리한 식물에 비해 저항성 유도효과가 매우 뛰어남에도 불구하고 거의 비슷한 callose 형성율을 나타냈으며, 병원균 침입부위에 있어서 선발균을 처리한 식물에 비해 강한 형광반응을 나타내는 것을 확인할 수 있었다. 따라서, 이와 같은 결과를 통하여 종합하여 볼 때 본 실험에서 분리한 선발균은 토마토 역병에 대한 저항성 유

도효과를 나타냈으며, 이들의 저항성 발현은 BABA에 의해 매개되는 저항성 기작과는 다른 어떤 것이라는 것을 보여준다.

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감사의 글

먼저 이 논문이 완성되기까지 많은 관심과 격려로 늘 변함없이 따뜻하게 지도해 주신 전용철 교수님께 진심으로 감사드립니다. 항상 누구보다 긍정적이고 적극적인 자세로 연구에 임하시고 학생들의 교육에 열의를 다하시는 교수님께 존경의 말씀을 전합니다. 또한 지난 2년간 교수님과 함께 하며 자연스레 배운 합리적인 태도와 부지런한 모습이 저에게는 살아가는데 귀중한 지표이자 재산이 될 것입니다.

제주대학교 대학원에 입학한 것이 정말 엊그제 같은데 벌써 졸업을 준비하게 되었습니다. 이렇게 빠르게 느껴지는 것은 학교생활이 저에게는 무척 소중하고 값진 것이었기 때문일 것입니다. 지난 2년간 구석구석 손때 묻은 병리학 실험실을 떠나려고 하니 차마 발이 떨어질 것 같지 않을 것 같습니다. 부족한 저에게 이렇게 논문을 마치고 졸업하는 것이 결코 쉽지는 않았지만, 뒤쳐지지 않고 무사히 졸업을 할 수 있게 된 것은 뒤돌아보면 그동안 저를 도와준 많은 고마운 분들이 계셨다는 것을 다시금 생각하게 합니다.

바쁘신 와중에도 열과 성의를 다하여 저의 미흡한 논문 심사를 맡아주신 현해남 교수님, 김동순 교수님에게 진심으로 감사드립니다. 또한 대학에 입학해서부터 오늘의 제가 있기까지 지도해주신 고영우 교수님, 조남기 교수님, 강영길 교수님, 송창길 교수님께도 진심으로 머리 숙여 감사의 말씀을 전합니다.

또한, 대학원 과정을 마치기까지 정말 많은 관심과 도움을 베풀어 주셨던 미라누나, 충선 형님, 성준 형님, 상현 형님, 형철 형님, 진영 형님, 정환 형님, 성배, 태근, 연동, 민웅이를 비롯한 많은 대학 및 대학원 선후배님들 정말 감사드립니다. 특히, 지난 2년간 같은 식물병리학실험실에서 연구에 대한 열의를 갖고 동고동락하며, 때로는 다투고 얼굴을 붉히기도 하였지만 평생에 잊지 못할 좋은 추억을 함께 만들었던 경후 형님, 또한 이번에 같이 졸업하는 효정이, 혜영이, 그리고 우리를 뒤이어 앞으로 남게 되는 명희 누님과 소영 누나, 그리고 학부생 진영이, 소연이 에게도 감사의 마음을 전합니다.

또한, 제 중요한 인생의 기로에서 항상 따뜻한 말과 격려로 든든한 후원자가 되어주신 태일이 삼촌과 우리 양희 누나, 경희 누나, 동생 소희, 그리고 매형들에게도 정말 감사하다는 말을 전하고 싶습니다. 이외에도 좁은 지면에 그분들을 일일이 열거하면서 감사의 마음을 전하지는 못하지만 이 모든 것이 결코 저 혼자의 힘만으로 된 것이 아니었음을 고백하지 않을 수 없습니다.

끝으로 제가 학업을 무사히 마치기까지 아무 말 없이 저를 믿고 기다려주신 사랑하는 어머니에게 이 논문을 바칩니다.

2006 년 12 월 29일

안 용 준

너무 정들었던 5347호 연구실에서...