Thesis for the Degree of Master of Agriculture

Resistance Induction and Enhanced Tuber Production by Pre-inoculation with Bacterial Strains in Potato Plants against *Phytophthora infestans*

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ABSTRACT

Efficacy of resistance induction by the bacterial strains *Pseudomonas putida* (TRL2-3), *Micrococcus luteus* (TRK2-2) and *Flexibacteraceae bacterium* (MRL412), which were isolated from the rhizosphere of plants growing in Jeju mountain, were tested in a green house. The disease severity caused by *Phytophthora infestans* was effectively reduced in the potato plants pre-inoculated with the three bacterial strains compared with those of the untreated control plants. In order to estimate the level of protection by the three bacterial strains, Mancozeb WP (Diesen M^{TM}, Kyong nong) and DL-3-amino butyric acid (BABA) were treated before the challenge inoculation with the pathogen, whereas Dimethomorph WP (Forum^{TM}, Kyong nong) and potassium phosphonate (KH_2PO_3 or K_2HPO_3) were treated after the challenge inoculation. Disease severities of chemical pre-treated as well as post-treated plants were reduced compare to those of the untreated. The disease reduction in the plants pre-treated with Mancozeb WP was the highest, whereas that of post-treated with Dimethomorph WP was the lowest. The yields of plants pre-inoculated with three bacterial isolates were greatly increased than those of control plants. To clarify the cause of the disease severity reduction, the infection structure were observed at the penetration sites on the leaves of plant inoculated with *P. infestans* using a fluorescence microscope. In this study the rates of germination and appressorium formation of the cysts of *P. infestans* were observed on the leaf surfaces of the potato plants expressing systemic resistance mediated by the three bacterial strains as well as by BABA. Furthermore, the frequencies of callose formation of the epidermal cells at the penetration sites were observed after the fungal inoculation. There were no differences on the germination rates among the untreated, the three bacterial strains pre-inoculated and BABA pre-treated

plants. However decrease of appressorium formation was observed in BABA pre-treated but not in the bacterial strains pre-inoculated compared to untreated plants. Also, the frequencies of callose formation of the three bacterial strains pre-inoculated were higher than that of untreated plants, indicating an active defense reaction of the pre-inoculated host cells against fungal attack. However, the frequency of callose formation on BABA pre-treated plants were not high as those of bacterial strains pre-inoculated plants. Contrastively, the stronger fluorescent of epidermal cells were observed at the penetration sites compared to those of bacterial strains. Based on these results it is suggested that the defense reduction were differently expressed by the pre-inoculation with the bacterial strains or by the pre-treatment with BABA.

I . INTRODUCTION

The oomycetes *Phytophthora infestans*, the cause of potato (*Solanum tuberosum*) and tomato (*Lycopersicon essulentum*) late blight, is an extremely destructive disease which can destroy potato fields in just a few days. The late blight disease has been often occurred in potato cultivating area under cool and rainy weather conditions and can result in a severe damage (Erwin and Ribeiro 1996). The pathogen survives in volunteer potato plant material in the fields which becomes the primary source of inoculum in the next year. Under favorable conditions, potato plants are infected by the overwintering inoculum (Jones et al. 1991). In the infected plant tissues, the fungus sporulates and forms sporangia on typical sporangiophores. The sporangia drift off by wind or are dispersed by rain and cause infections by releasing zoospores which can again rapidly infect wet leaves, stems and fruits under optimal temperature and humidity conditions (Erwin and Ribeiro 1996). Beside asexual sporulation, the late blight fungus is able to perform sexual recombination resulting in oospore production. The sexual life cycle may affect the epidemiology and increase of selection of fungicide resistant strains in the population of the late blight fungus in potato growing regions (Fry and Goodwin 1995).

Late blight is one of the diseases which are difficult to control in the field. Generally, varieties which are resistant to only one or a few races of the late blight fungus, can become susceptible when they are attacked by new virulent races of the fungus. Beside the vertical resistance, some varieties possess horizontal resistance of varying degree, which is effective against all races of the blight fungus. Cultivating resistant varieties is not sufficient to control the late blight disease, since under favorable conditions *P. infestans* can severely infect even resistant varieties. The control of late blight

fungicide was register 18 types. These kinds of fungicide such as metalaxyl, mancozeb, dimethomorph, fosetyl-Al, ethaboxan, oxadixyl and propamocarb widespread of use in potato field (Korea Crop Protection Association, 2006).

Consequently, farmers have relied heavily on periodic application of fungicides. Phenylamide fungicides including metalaxyl have a biochemical action site to pathogens belong to the order *Peronosporales* (Bruck et al., 1980) However, their high efficacy and specificity lead consequently to widespread of their use in agriculture and resulted in potential problems such as development of fungicide resistance (Davidse et al., 1981). For example, chemical control strategies for potato late blight changed with the migration of phenylamide resistant isolates of *P. infestans* into the United States (Johnson et al., 2000). Similarly in potato fields in Korea occurrence of resistant strains of *P. infestans* to metalaxyl was reported by Choi et al. (1992). The resistance of pathogen continuously requires a development of new fungicides with different modes of action.

In recent years, research on the biological control of Phytophthora blight of potato has been expanded because of the fungicide resistance of pathogens. Moreover, potato cultivars grown commercially in field, do not have high levels of general resistance to late blight. One of the strategies for plant protection against late blight disease may be using crops expressing a induced systemic resistance, which can be triggered in the plant by pre-inoculation with plant growth promoting rhizobacteria (PGPR) (van Loon et al. 1998a). Free-living root colonizing bacteria (rhizobacteria) have been studied for the past decade as possible inoculants for increasing plant productivity and controlling microbial pathogens (Kloepper, 1992). Soil or seed applications with PGPR have been used to enhance the growth of several crops (Glick, 1995) as well as to suppress the growth of plant pathogens (Kloepper et al., 2004). PGPR colonize in root systems through seed applications and protect plants from foliar diseases including *Pseudomonas*

spp. *Bacillus* spp. *Paebacillus* spp., and *Serratia* sp. (Kloepper et al., 2004). Some strains of PGPR are known to survive both in the rhizosphere and phyllosphere (Krishnamurthy and Gnanamanickam, 1998). The PGPR-mediated resistance has been defined as induced systemic resistance (ISR) (van Loon et al. 1998a). ISR is occurred when the plant's defense mechanisms are stimulated and triggered resistance to attack of pathogens (Conrath et al., 2002). The treatment with PGPR in the rhizosphere may result in expression of systemic resistance on the aerial part of the plants (van Loon et al. 1998a). Moreover, for expression of resistance the PGPR need not be contact with plant pathogens and the PGPR can grow well in the rhizosphere.

On the other hand, signaling of ISR is different from that of systemic acquired resistance (SAR). Normally, SAR is triggered by treatment with a chemical or biological agent and have a broad spectrum of pathogens. SAR is expressed by plant defense response following hypersensitive response after the inoculation of plant pathogens (Durrant and Dong, 2004; Loon et al., 1998) which is the ability of plants to defend themselves against pathogens through a distinct signal transduction pathway (Ryals et al, 1996).

In recent years, the use of PGPR as an inducer of systemic resistance in crop plants against different pathogens has been demonstrated under field conditions (Bharathi et al., 2004). It is considered natural, eco-friendly and safe, and provides resistance against a broad spectrum of pathogens (Radjacommare et al., 2002; saravanakumar, 2006).

For this study three bacterial strains were obtained from plant pathology lab Cheju university which isolated from the rhizosphere of the plant growing in Jeju and showed the anti-fungal activities against several plant pathogens (Lee et al. 2003). Aiming for the selection of an effective ISR inducing agent, efficacy of the selected bacterial strains *Pseudomonas putida* (TRL2-3), *Micrococcus luteus* (TRK2-2) and *Flexibacteraceae bacterium* (MRL412) against late blight caused by *P. infestans* was tested in potato plants.

Furthermore, to estimate the control efficacy of the bacterial strains, disease severities of plants treated with two resistance activators and two commercial fungicides were compared after inoculation with the late blight fungus. Also, microscopical observation of infection process of the pathogen and defense response of potato plant were cytologically examined on the leaf surfaces after fungal inoculation. Additionally, the total weights of tubers per potato plants were compared among the bacterial isolates pre-inoculated, resistance activators treated, and commercial fungicides treated plants.

Ⅱ. MATERIALS AND METHODS

1. Plants

Seed tuber of potatoes (*Solanum tuberosum* L. cv. Deajima) were grown in a styrofoam bed (volume 0.032 ㎥, W x L x D = 31 x 51 x 20 cm in size) filled with perlite (Parat®, Sam son, Korea) and peatmoss (Tuksimi®, Nongwoogreentec, Korea) mixture (1 : 2, v/v). Each bed was fertilized with 80g of the essentiality microelement (Osmocote® Scotts Korea™) and covered commercial soil. Potato plug seedling were planted at intervals of twelve centimeters eight seed tuber of potato in one bed. Plants were in a glass greenhouse at 25℃ during the day and at 20℃ during the night for about 90 days.

2. Fungal pathogen

Phytophthora infestans (Mont.) de Bary KACC 40718 was obtained from Korean Agricultual Culture Collection (KACC) and grown on oatmeal agar medium for 14 days at 15℃ until induction of sporangium formation. For the initiation of zoospore release from the sporangia, 20 ㎖ distilled water were added to the agar plate grown with the fungal mycelium. A spatula was used to remove air between hyphae so that sporangia were submerged in water. Then the plates were immediately placed in a refrigerator at 4℃ until zoospores were released. The suspension containing zoospores was filtered through three times folded cheesecloth. The number of zoospores at each concentration was determined to 1.0 x 10^4 zoospores/㎖ using a haemocytometer under a light microscope. To induce encystment, zoospore suspensions were intensively shaken using a vortex mixer for 2-3 min and used as an inoculum.

3. Treatment with three bacterial isolates, two resistance activators and two commercial fungicides in the potato plants

The bacterial isolates TRL2-3, TRK2-2, MRL412 showing antifungal effect were obtained from plant pathology lab Cheju university and were tested for triggering of ISR in potato plants. TRL2-3, TRK2-2 were identified as *Pseudomonas putida* and *Micrococcus luteus*, respectively, by fatty acid analysis (Sherlock microbial identification system, MIDI, Inc.) and Biolog MicroLog3 system (Biolog, Inc.)(Jeun et al., 2004a). The bacterial isolate MRL412 was identified as *Flexibacteraceae bacterium* by analysis of 16S rRNA sequence in plant pathology lab Chonnam university.

The *Pseudomonas putida* (TRL2-3), *Micrococcus luteus* (TRK2-2) and *Flexibacteraceae bacterium* (MRL412) were grown in TSA medium at 28℃ for 24h. The concentration of bacterial strains was adjusted to be 1.0 x 10^7 colony forming unit (cfu)/㎖. Thirty ㎖ of the bacterial suspension was soil-drenched per potato plants 7 days before challenge inoculation with *P. infestans*. For negative control, H_2O was applied on the potato plants instead of the bacterial suspension.

In order to estimate the level of protection by the bacterial strains, Mancozeb wettable powder (WP) (Diesen M^{TM}, Kyong nong 2,000 ppm) and DL-3-amino butyric acid (BABA 10mM) were pre-treated 7 days before challenge inoculation, whereas Dimethomorph WP (Forum^{TM}, Kyong nong 1,000 ppm) and potassium phosphonate (KH_2PO_3 or K_2HPO_3 2,000 ppm) were post-treated 7 days after the challenge inoculation with pathogen. The potassium phosphonate was prepared a rate of phosphonic acid (H_3PO_3 : 97%) 100 : potassium hydroxide (KOH : 85%) 83 (Hong et al., 2003).

All trials were designed in randomized complete block with three experiments, replicated with time intervals and every experiment carried out with 3 replications containing 8 plants each.

4. Challenge inoculation and evaluation of resistance

The cysts suspension with 100$\mu\ell L^{-1}$ of Tween 20, for enhances the adhesion of *P. infestans*, was sprayed on the aerial potato leaves at 7 days after the treatment with the suspension of bacterial suspension or chemicals. The potato plants inoculated with suspension of *P. infestans* were covered with black polyethylene vinyl for 24h and for keeping the relative humid to 100 %. After eliminating the polyethylene vinyl the inoculated plants were kept in the greenhouse until evaluating the disease severity.

Disease severity caused by *P. infestans* was determined at 14 days after the challenge inoculation. The disease severities were established using the following scale: 0, no lesion; 1, up to ~20% of the leaf area blighted; 2, 20~40%; 3, 40~70%; 4, 70~90%; 5, plant entirely blighted. The disease severities of every branch were determined and presented the mean of the total scale numbers. Percentage protection against the disease was calculated as 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.

5. Determination of increase of yield by the bacterial isolates

To determine the increase of yield by the pre-inoculation with the bacterial strains the seed tuber of potatoes were sown in plastic pots (∅ 25cm). Thirty ㎖ of the suspension of bacterial strains *Pseudomonas putida* (TRL2-3), *Micrococcus luteus* (TRK2-2) and *Flexibacteraceae bacterium* (MRL412) was soil-drenched per potato plants at four weeks before harvest. For control, BABA as well as Mancozeb WP were also treated at the same time in stead of the bacterial inoculation. The total weight of potato tubers harvested per plant was measured using a balance. The experiment was carried out with 3 replications containing 3 plants each.

6. Microscopical observation of infection process of fungal pathogen

The leaves of potato plants inoculation with bacterial strains, treatment with BABA and fungicide of 8-9 branch leaves growth stadium were attached and inoculated with 20㎕ droplets of cyst suspension of *P. infestans* (1.0x 10^4 cysts/㎖) at six positions. The inoculated leaves were kept at 100% RH in a petri-dish (∅ 14cm) at 15℃. Observation of infection process on the leaves of the inoculated potato plants were performed at 3 days after the challenge inoculation.

Staining of leaf tissues was carried out according to the method of Jeun et al.(2000). The infected leaf tissues were cut out with a corkborer (11-mm in diameter) and fixed for at least 2h in 2% glutaraldehyde in 0.05M phosphate buffer, pH 7.2. After washing in the phosphate buffer three times, for 10 min each. Sections were stained for 20 min with 0.5% (w/v) aniline blue. Papillae were detected by staining with aniline blue fluorochrome specific for callose. After washing with phosphate buffer for 10 min 3 times. The leaf disks were stained with 0.02% Uvitex 2B (w/v) (Diethanol) for 20 min. Diethanol is a fluorochrome for β-glucans. Papillae also in potato tuber tissue have been shown to contain mainly callose (β-1, 3-glucan) and cellulose (β-1, 4-glucan) as structural glucans (Hachler and Hohl 1984). After washing in the phosphate buffer, the leaf disks were mounted on glass slides in 50% glycerin and then covered with cover glass.

Fungal structures were observed using a fluorescence microscope (Olympus, Japan) equipped with a 'U' exciter cube-filter was used to observe the infected leaf cells by ultraviolet epifluorescence (BP 400-440, FT 460, LP 470). Photomicrographs were taken with a fluorescence microscope of four hundred magnifications (X400).

Total number of germinated cysts, appressorium formations, and callose depositions at the penetration sites were counted on the leaf surfaces and in the epidermal cells of the potato plants untreated, pre-inoculated with the

bacterial strains, and pre-treated with BABA or Mancozeb (WP). The rate of appressorium formation fluorescent cells at the penetration sites were calculated from the data counted on the 5 leaf discs detached from each 5 plants in the 3 separated experiments.

7. Statistical analyses

The data of disease severity caused by *P. infestans*, the germination rate, frequency of appressorium formation of the fungus, fluorescent cells in the inoculated leaves and total tuber weight were statistically analyzed using Duncan's multiple range tests (DMRT). Statistical analysis of the experimental data were conducted using the Statistical Analysis System (SAS institute, version 8.02)

Ⅲ. RESULTS

1. Induced resistance against *Phytophthora infestans* in potato plants

The bacterial strains *Pseudomonas putida* (TRL2-3), *Micrococcus luteus* (TRK2-2) and *Flexibacteraceae bacterium* (MRL412) showing antifungal activity in vitro test (Lee et al. 2003) was pre-inoculated and determined their resistance efficiencies against late blight disease caused by *P. infestans* in potato plants. The symptom of late blight disease was visually identified at 14 days after the challenge inoculation. The infected lesions were visually observed at 10 days after challenge inoculation on the leaves of untreated. The lesion area of untreated plants was rapidly spread out at 14 days after fungal inoculation the disease severity was over 80% (Fig. 1 and 2).

In contrast to the untreated plant, disease severity of late blight was significantly suppressed by the pre-inoculation with all three bacterial strains (Fig. 2). Although in the first experiment the protection rate of the *Micrococcus luteus* (TRK2-2) pre-inoculated plants was a little lower than those of other bacterial isolate pre-inoculated plants, similarly high protection rate of the disease severities were shown both in second and third experiments (Fig. 2).

The disease severity of pre-treated with BABA was significantly reduced compared to those of untreated plants (Fig. 2), showed lower disease severity compare to those of the bacterial strains pre-inoculated at the first stage of infection. Also the disease control efficacy by BABA was higher than those of bacterial strains in the first experiment but similar in both second and third experiments (Fig. 2).

The protection of late blight by pre-treated with chemical Mancozeb WP was remarkably hight in all experiments (Fig. 2). Similarly, the post-treatment with potassium phosphonate (KH_2PO_3 or K_2HPO_3) could reduce

the disease severity as case of BABA pre-treated (Fig. 2). Interestingly, protection rate by the post-treatment with Dimethomorph WP was the lower than those by three bacterial strains (Fig. 2).

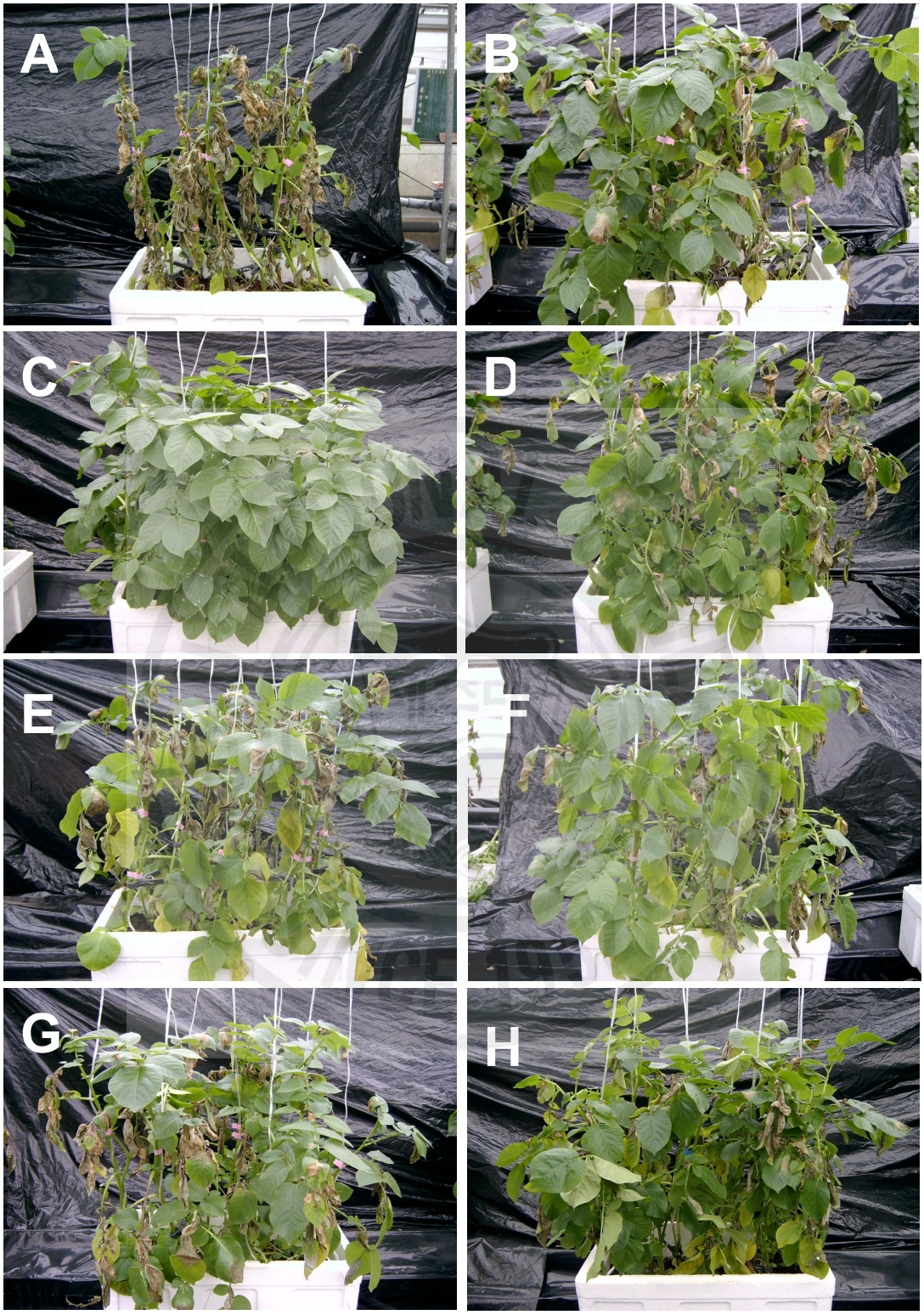
A
B
C
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Fig. 1: Induction of systemically induced resistance in potato plants against late blight disease at 14 days after challenge inoculation with *P. infestans* ($1.0x10^4$ cysts/㎖). The presented plants were (A) untreated control, pre-treated 7 days before challenge inoculation with (B) DL-3-amino butyric acid (BABA; 10 mM), (C) Mancozeb WP (Diesen^{TM} M, 2,000 ppm), pre-inoculation 7 days before challenge inoculation with bacterial suspension of (D) *Pseudomonas putida* (TRL2-3), (E) *Micrococcus luteus* (TRK2-2), (F) *Flexibacteraceae bacterium* (MRL412) with the concentration of $1.0x10^7$ cfu/㎖ each, post-treated 7 days after challenge inoculation with (G) Dimethomorph WP (Forum^{TM} 1,000 ppm), and (H) potassium phosphonate (KH_2PO_3 or K_2HPO_3 2,000 ppm).

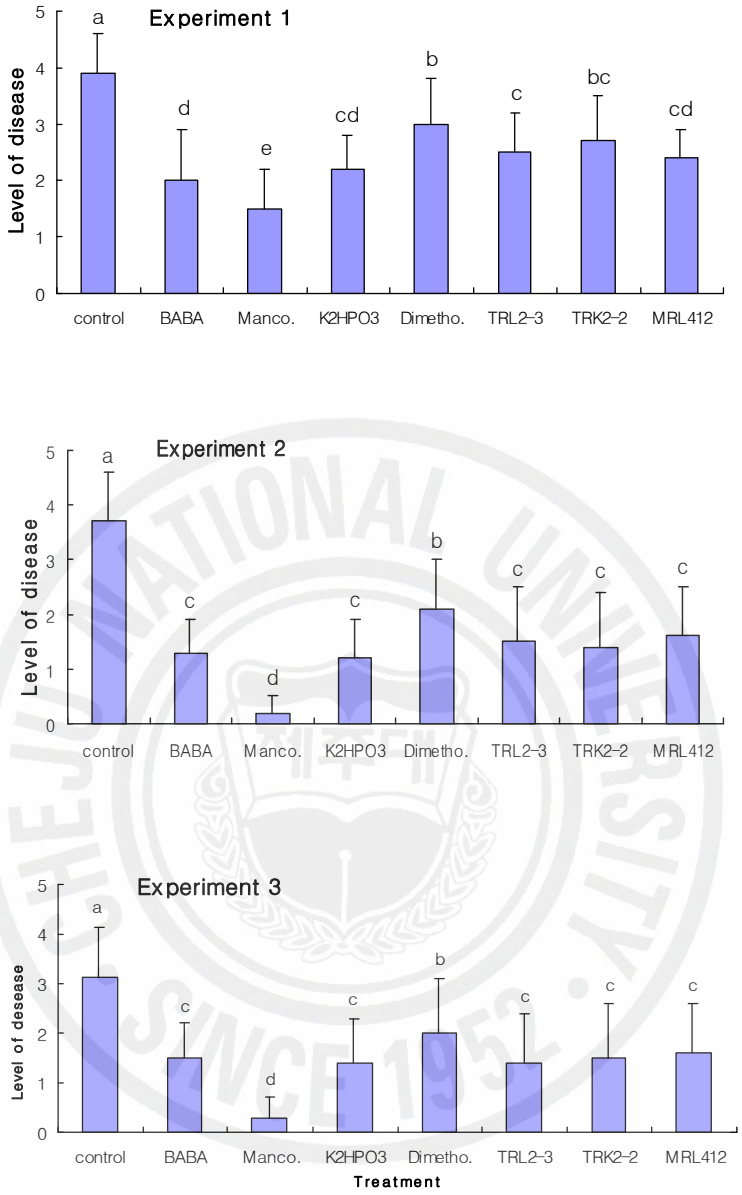

Fig. 2: Comparison of reduction of disease severities and duncan's multiple range test in the leaves of potato plants by three selected bacterial isolates, by two resistance activators and by two commercial fungicides after challenge inoculation with *P. infestans* ($1.0x10^4$ cysts/㎖). Disease severity were estimated with the scale of infected leaves as following: 0, no lesion; 1, up to ~20% of the leaf area blighted; 2, 20~40%; 3, 40~70%; 4, 70~90%; 5, plant entirely blighted. The vertical bars indicate the standard deviation of the 3 replications each containing 8 plants per treatment. Different letters on the columns are significantly ($P < 0.001$) different according to Duncan's multiple test.

2. Observation of infection behavior on the leaf surface using a fluorescence microscope

The rates of germination and appressorium formation of the cysts of *P. infestans* were observed on the leaf surfaces of the potato plants expressing systemic resistance mediated by the three bacterial strains as well as by BABA. Furthermore, the frequencies of callose formation of the epidermal cells at the penetration sites were observed after the fungal inoculation. On the leaves of untreated plants most of the cysts were germinated and formed appressoria at 24h after the inoculation. In most cases, germination rate and appressorium formation on the leaf surface were over the 90% after 48h (Fig. 4 and Fig 5). At 72h after fungal inoculation there were no significant increase in germination rate and appressorium formation compare to those at 48h (data not shown).

In the plants of pre-inoculated bacterial strains there were no different percentage of germination rate and appressorium formation compare to those of untreated plants (Fig. 4 and Fig. 5). Staining with the fluorochrome aniline blue, indicative of callose, revealed an accumulation of yellow-fluorescing material located beneath some of the appressorium (Fig. 3). The frequencies of callose formation on the bacterial strains pre-inoculated plants were higher than that of untreated plants, indicating an active defense reaction of the pre-inoculated host cells against fungal attack (Fig. 6).

Similar to the case of bacterial strains, there were no differences on the rates of germination of the fungal cysts between the BABA pre-treated and untreated plants (Fig. 4). However, in contrast to the bacterial strains slightly decrease of appressorium formation were observed on the leaves of BABA pre-treated in second and third experiments (Fig. 5), and no significant increasement in the frequency of fluorescent cells compared with that of untreated plants (Fig. 6). Particularly, the highly intensive yellow fluorescence were showed by the pre-treatment with BABA at the penetration sites

compared to those of bacterial strains (Fig. 3). On the other hand, on the leaves of pre-treated with Mancozeb WP the cyst did not observed after the challenge inoculation (data not shown).

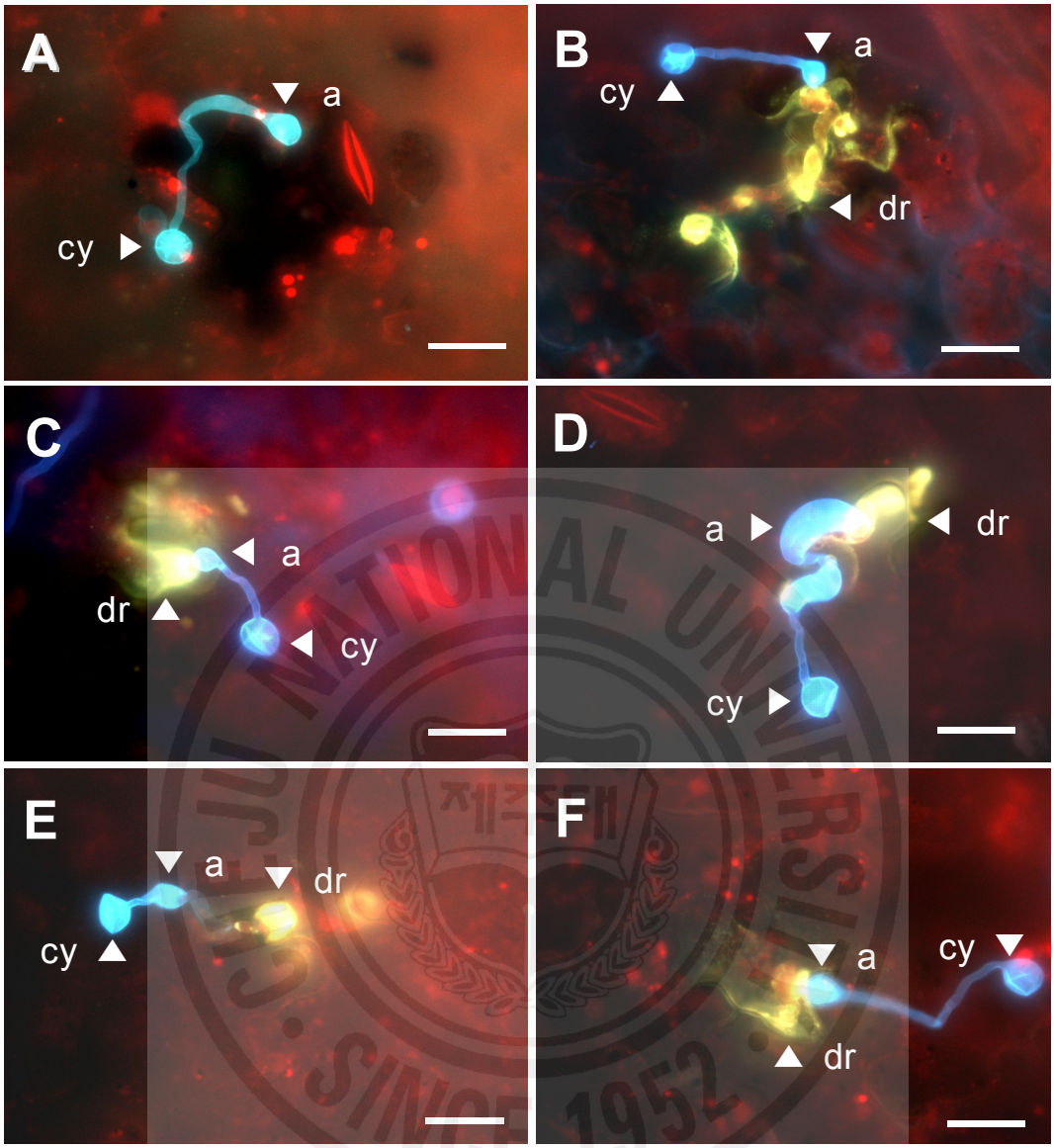

Fig. 3. Fluorescence micrographs of the penetration sites of *P. infestans* on leaf surface and epidermal layer of potato leaves of (A) untreated plants, pre-treated 7 days before challenge inoculation with (B and C) DL-3-amino butyric acid (BABA; 10 mM), pre-inoculated with bacterial suspension (D) TRL2-3, (E) TRK2-2 and (F) MRL412 72h after inoculation with *P. infestans*. The bacterial strains were pre-inoculated at the concentrate with 1.0×10^7 cfu/㎖. All bars= 20㎛. *Abbreviations*: a, appressorium; cy, cyst; dr, defense reaction.

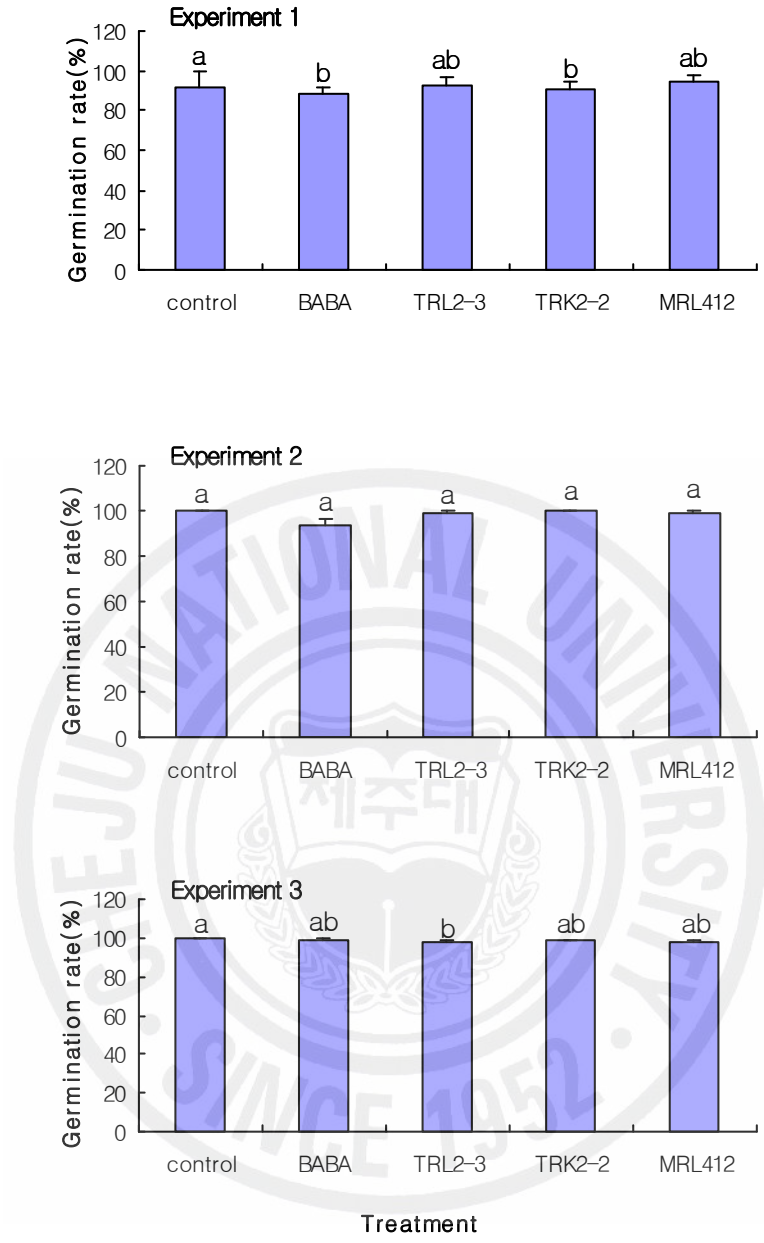

Fig. 4. Germination rate of cyst of *P. infestans* on the leaves of potato plants untreated control, pre-inoculated/-treated with bacterial strains or BABA. The leaves were attached at 72h after challenge inoculation with *P. infestans* (1.0 X 10^4 cysts/㎖). The vertical bars indicated the standard deviation of the 3 separated experiments each containing six plants. Different letters on the columns are significantly ($P < 0.001$) different according to Duncan's multiple test.

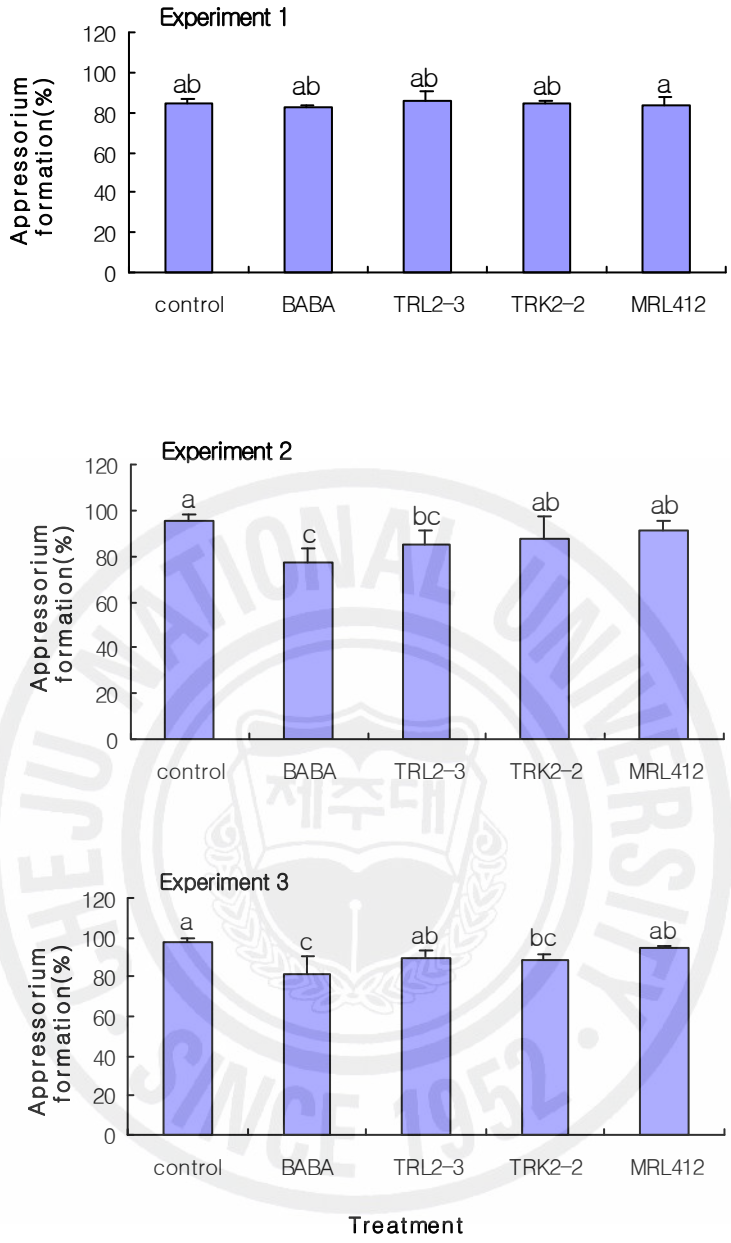

Fig. 5. Appressorium formation rate of cyst of *P. infestans* on the leaves of potato plants untreated control, pre-inoculated/-treated with bacterial strains or BABA. The leaves were attached at 72h after challenge inoculation with *P. infestans* (1.0 X 10^4 cysts/㎖). The vertical bars indicated the standard deviation of the 3 separated experiments each containing six plants. Different letters on the columns are significantly ($P < 0.001$) different according to Duncan's multiple test.

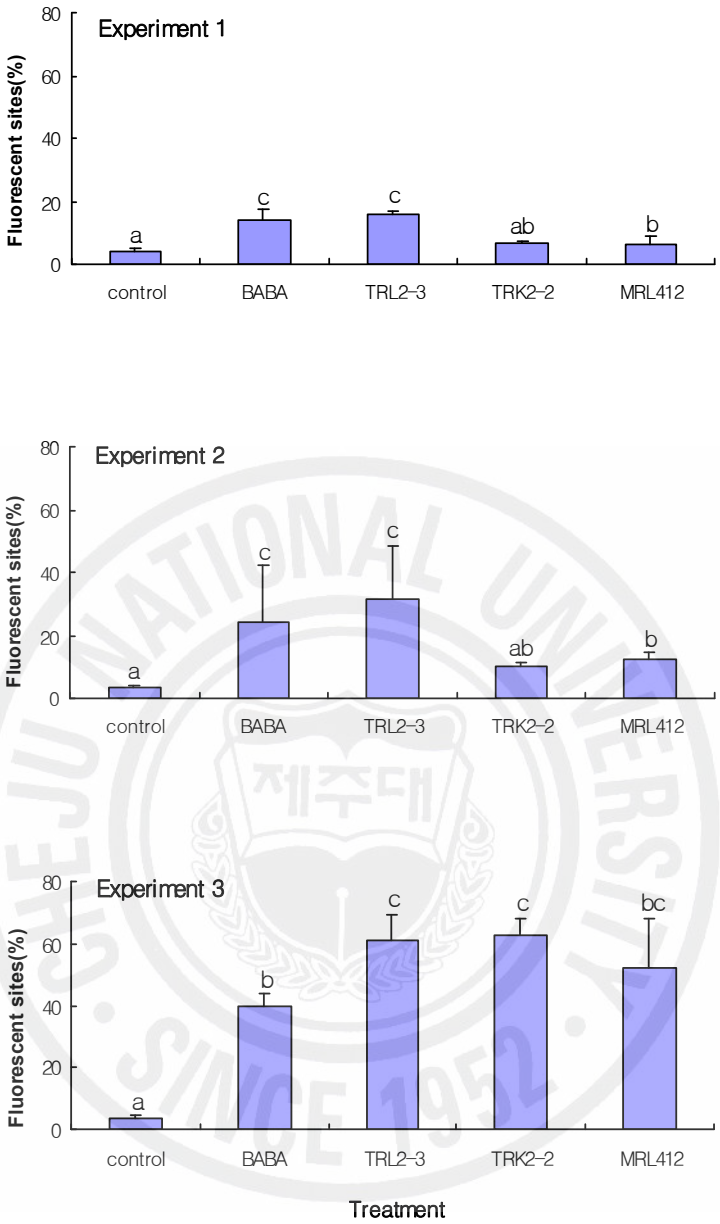

Fig. 6. Frequency of fluorescent cells at the penetration sites on the leaves of potato plants pre-inoculated with *Pseudomonas putida* (TRL2-3), *Micrococcus luteus* (TRK2-2) and *Flexibacteraceae bacterium* (MRL412) and pre-treated with DL-3 amino butyric acid (BABA) at 72h after challenge inoculation with *P. infestans* (1.0 X 10^4 cysts/㎖). The vertical bars indicated the standard deviation of the 3 separated experiments each containing six plants. Different letters on the columns are significantly ($P < 0.001$) different according to Duncan's multiple test.

3. Enhancement of tuber production by pre-inoculation with bacterial strains

To investigate the enhancement of growth by the bacterial strains, a total weight of tubers as measured from the pre-inoculated with the bacterial strains, pre-treated with BABA, commercial fungicides and untreated plants. The pre-inoculation with all three bacterial isolates caused an increase of yields of the potato tubers. Both bacterial isolates *Pseudomonas putida* (TRL2-3) and *Micrococcus luteus* (TRK2-2) could remarkably increase the total weight of tubers per plants compared to those of control plants (Table 1 and Fig. 7). Also, the pre-inoculation with *Flexibacteraceae bacterium* (MRL412) could cause the significant increase of tuber production per plants (Table 1 and Fig. 7). Although the yields were increased in the plants pre-treated with both chemical BABA and fungicide Mancozeb WP, compared to that of untreated control they were not as high as the cases of *Pseudomonas putida* (TRL2-3) or *Micrococcus luteus* (TRK2-2) (Table 1 and Fig. 7).

Table 1: Comparison of total tuber weight of potato plants pre-inoculated with three bacterial strains, pre-treated with Mancozeb.

Treatment[a]	Total weight of tubers per plant (g)	Duncan's test
TRL2-3	269.3 ± 66.7[b]	c[c]
TRK2-2	313.9 ± 52.3	c
MRL412	174.3 ± 42.8	b
Mancozeb	124.5 ± 20.1	ab
BABA	103.6 ± 16.5	ab
Control	76.4 ± 6.7	a

[a]The bacterial isolates *Pseudomonas putida* (TRL2-3), *Micrococcus luteus* (TRK2-2) and *Flexibacteraceae bacterium* (MRL412) were pre-inoculated and Mancozeb WP (Diesen M^{TM}, Kyong-nong) and DL-3-amino butyric acid (BABA) were pre-treated at 4 weeks before harvest.

[b]Mean of values standard deviation of the three replications.

[c]Significant difference, $P < 0.001$ by Duncan's multiple test.

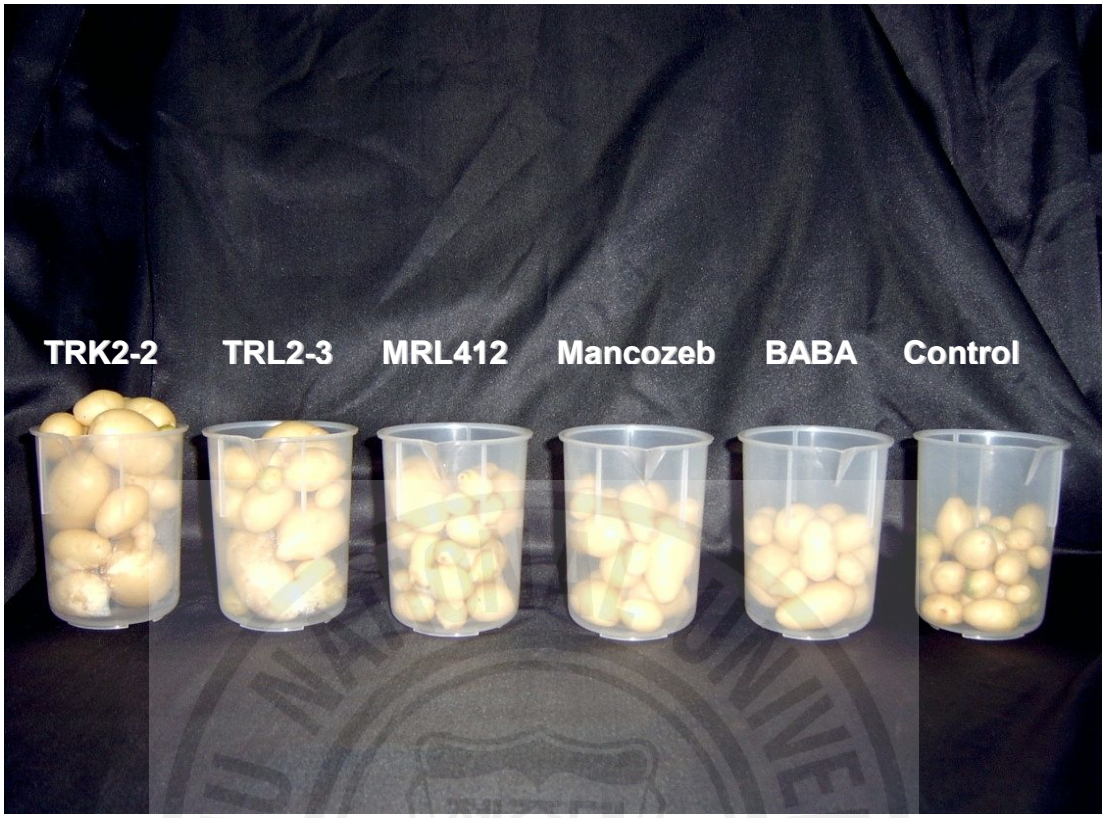

Fig. 7. Effect of increase tuber production by pre-inoculation with three bacterial strains. The presented tubers were harvested from the plants pre-inoculated with bacterial suspension of *Pseudomonas putida* (TRL2-3), *Micrococcus luteus* (TRK2-2) and *Flexibacteraceae bacterium* (MRL412) at every concentration with ($1.0x10^7$ cfu/㎖) and pre-treated with DL-3-amino butyric acid (BABA; 10 mM), and Mancozeb WP (Diesen M™, 2,000 ppm) and untreated plants.

Ⅳ. DISCUSSION

Using microorganisms for disease control has been considered for many years because this strategy may result in the reduction of chemical application. However, using the antagonistic microorganisms to control of plant diseases has not been always successful in the field. Therefore, new strategy of the biological control such as using crop plants expressing an induced systemic resistance (ISR) has been looking for controling plant diseases (van Loon et al. 1998b). In this study to find an effective microorganism inducing ISR against late blight diseases in potato, some rhizobacteria showing antifungal activity were tested their's efficacy of inducing systemic resistance.

In the potato plants ISR could be effectively triggered when all three bacterial strains were pre-inoculated in the green house (Fig 2). In other study the efficacies of resistance induction of *Pseudomonas putida* (TRL2-3) and *Micrococcus luteus* (TRK2-2) were also shown in cucumber plants after challenge inoculation with anthracnose pathogen *Colletotrichum orbiculare* (Jeun et al. 2004a). Similarly some PGPR strains such as *Serratia marcescens* or *Pseudomonas fluorescens* could effectively induce systemic resistance in cucumber plants against anthracnose disease at certain concentration (Liu et al. 1995).

The mechanisms of ISR have been compared with those of systemic acquired resistance (SAR) (Jeun et al. 2004b; Sticher et al. 1997). Different expression of ISR compare to SAR is competition mineral element such as ion (Fe), which is easily captured by siderophores produced in PGPR (Maurhofer et al., 1994; van Loon et al., 1997; 1998b). The resistance expression by competition of nutrient has not been reported in the plants expressing SAR. In contrast to SAR, some PGPR strains mediating systemic

resistance have direct antifungal activity. It has been reported that both bacterial isolates TRL2-3 and TRK2-2 showed direct antifungal effect *in vitro* test (Lee et al. 2003). The other resistance mechanisms of ISR, seem to be similar with those of SAR, which is involved in the resistant gene *npr1* (Pieterse and van Loon 1999).

DL-3 amino butyric acid (BABA) is well known as an activator in many plants (Cohen 2002; Jeun and Park 2003; Zimmerli et al. 2000). It has been previously shown that pre-treatment with BABA in the cucumber plants caused the decrease of disease severity after inoculation with anthracnose pathogen *Colletotrichum orbiculare* (Jeun et al., 2001). Also, the increase of salicylic acid (SA) level in the leaves of BABA-treated tomato or tobacco plants was reported (Jeun et al., 2000a, siegrist et al., 2000). However, the signal pathway of resistance mediated by BABA is not yet clearly illustrated.

In this study to biological control, as a protection method of Phytophthora blight in potato plants, researches have been conducted for the effective control of Phytophthora blight by treatment with a non-fungicidal synthetic chemical BABA to potato plants. Pre-treatment with the BABA showed a synergistic effect on the protection against *P. infestans* (Fig. 2). As a positive control, BABA pre-treated plants showed highly significant differences compare to those of pre-inoculated the bacterial strains as well as untreated plants (Fig. 1 and Fig. 2).

Similarly, the disease severity was reduced in the plants post-treated with potassium phosphonate (KH_2PO_3 or K_2HPO_3)(Fig. 2). Disease control with phosphorous acid (H_3PO_3) has been already reported in some studies especially against the disease caused by oomycetes (Grant et al. 1990; Guest et al. 1995). The chemical is primarily responsible for the fungicidal activity of fosetyl-Al as a breakdown product in plant tissues and is the only available phloem-translocated fungicide that moves upward and downward through the xylem and phloem (Guest et al., 1995; Ouimette and Coffey,

1989). Phosphonate refers to compounds containing a phosphorous-hydrogen (P-H) bond that confers biological activity against oomycetes and host plants (Guest et al., 1995). In these all three experiments the disease control efficacy of the bacterial strains were comparable to those of both ISR inducers BABA and potassium phosphonate (Fig. 2).

The pre-treatment of commercial fungicide Mancozeb WP was the best control strategy for protection of late blight in potato plants. Interestingly, the post-treatment of another fungicide Dimethomorph WP could not decrease the disease severity as the case of pre-inoculated with three bacterial isolates (Fig. 2). Based on these results late blight in potato plants may be effectively controlled by spraying a protective fungicide before break out of the disease. Furthermore, the disease control efficacy by the bacterial strains was higher than those of fungicide Dimethomorph WP (Fig. 2). These results indicated the possibility of alternative disease control strategy using bacterial strains triggering ISR in the potato in a green house.

In this study, the cytological study has been carried out on the leaves of epidermal cells at the penetration sites using a fluorescence microscope. There was no different on germination rate among the bacterial strains pre-inoculated, BABA pre-treated, and untreated plants (Fig. 4). These observations indicated no suppression of germination neither by pre-inoculated with the bacterial strains nor pre-treated with BABA. However it was found a slight reduction of appressorium formation on leaves of BABA pre-treated (Fig 5). Similar results was reported in cucumber plants pre-treated with BABA after inoculation with *C. orbiculare* (Jeun et al., 2004b).

Callose formation is well-known as a resistance mechanism in many host-parasite interactions (Kovats et al., 1991; Strömberg and Brishammar, 1993). In this study the frequency of callose formation on bacterial strains pre-inoculated or BABA pre-treated plants were higher than that of untreated plant. (Fig 3 and 6). The significantly increase of callose formation indicated

the thickening of cell walls forming callose deposit as resistance expressing by the bacterial strains. In the BABA pre-treated plants the autofluorescence was very strong at the penetrated site (Fig 3). Although, on the bacterial strains pre-inoculated plants the frequency of fluorescent cells was generally higher compared with that of BABA pre-treated plants, the protection rate of BABA were higher than those of bacterial strains. Similar results were observed in the other study, in which the calllose formation was enhanced on the leaves of cucumber plants pre-inoculated with plant growth promoting rhizobacteria (PGPR) comparing to BABA pre-treated (Jeun et al., 2004b ; Lee et al., 2005). Based on these results it is suggested how many defence at the penetration sites may not play an important role for expression of systemic resistance in potato plants against late blight. It could be also involved in the other defense responses such as the accumulation PR-proteins (Jeun, 2000b) and production of anti-fungal substance phytoalexins (Maurhofer et al., 1994; Bowles, 1990).

In addition to disease management, rhizobacterial strains could increase the plant growth after their inoculation in rice, potato, radish, mango and sugar beet (Kloepper et al., 1980b; Kloepper et al., 1997; Vivekananthan et al., 2004). In the present study, bacterial strains treated plants had higher yields than those of the chemical and untreated plants. A total weight of tubers was greatly increased in plants pre-inoculated with bacterial isolates TRL2-3 or TRK2-2 compared to those of untreated control plants (Table 1). Although the fungicide Mancozeb WP and resistance activator BABA caused increase of yield, the total weight of tubers of both chemical treated plants was not significantly different to that of untreated control plants (Table 1). These results suggest that both the protection efficacy against late blight and yield increase of tubers might be expressed in potato plants cultivating with the green house by the pre-inoculation with the bacterial strains. The enhancement of plant growth and resistance indcution mediated by

rhizobacteria have been reported in many host-pathogen interactions (Gamo and Ahn 1991; Kloepper et al. 1980a).

Summarily our results suggested that the bacterial strains TRL2-3 TRK2-2 and MRL412 could trigger ISR in potato plants against late blight. Although the ISR by the strains was not higher compared to those of commercial fungicide Mancozeb, it is suggested that the protection by using the microorganism may be useful in a green house, where chemical application is forbid.

Ⅴ. 適要

제주도에서 자생하는 식물의 근권에서 순수 분리한 길항 근권 세균인 *Pseudomonas putida* (TRL2-3), *Micrococcus luteus* (TRK2-2) and *Flexibacteraceae bacterium* (MRL412)을 이용하여 감자 역병균에 대한 저항성 발현 여부를 조사하였다. *Phytophthora infestans*에 의한 병 발생 정도는 근권 세균을 처리한 식물이 무처리 식물보다 병 발생정도가 효과적으로 감소되는 것을 확인하였다. 근권 세균과 살균제인 Mancozeb 수화제와 DL-3-amino butyric acid (BABA)는 역병 접종 전 처리로 이용하여 실험을 하였으며, 반면에 살균제인 Dimethomorph 수화제와 potassium phosphonate (KH_2PO_3 or K_2HPO_3)는 역병 접종 후 처리로 이용하여 병 발생 정도를 조사하였다. 화학 살균제의 전 처리와 후 처리 식물의 병 발생 정도는 무처리 식물 보다 병 발생 정도가 감소되는 것을 확인하였다. 그중에서 유기합성 농약인 Mancozeb 수화제를 전 처리한 식물이 병 발생 정도가 가장 효과적으로 억제 되었으며, Dimethomorph 수화제를 후 처리한 식물의 역병 방제 효과는 다른 처리구에 비해 효과가 다소 낮게 나타났음을 실험을 통해 확인 하였다. 역병 발생의 감소의 이유를 설명하기 위해 *P. infestans*를 접종한 감자 잎의 관통조직에서 병원균의 침입 기작을 형광 현미경을 통하여 관찰하였다. 역병균의 cyst의 발아율과 부착기 형성율은 근권 세균 뿐만 아니라 전신적 저항성 기작을 나타내는 BABA를 이용하여 감자 잎 표면에서 병원균의 침입 기작을 관찰했다. 또한, 표피 세포에서의 callose 형성율의 빈도는 역병균 접종 후 관찰했다. 무처리 식물과 세 가지 근권 세균을 전 처리한 식물, BABA를 전 처리한 식물 사이에서 cyst의 발아율은 차이가 거의 없었다. 그러나 BABA를 전 처리한 식물은 근권 세균을 전 처리한 식물과 무처리 식물에 비해 부착기 형성율의 감소와 유의차를 확인할 수 있었다. 또한 근권 세균을 전 처리한 식물의 callose 형성율은 무처리 식물 보다 높은 빈도를 나타냈으며, 이는 근권 세균을 전 처리한 식물에서 병원균의 침입에 대한 기주 세포의 강한 방어 반응을 나타내는 것임을 확인할 수 있었다. 그러나 BABA를 전 처리한 식물에서는 근권 세균을 전 처리한 식물 보다 callose 형성율의 빈도가 높지 않았다. 반면에 병원균 침입 부위에

있어서 BABA를 처리한 식물은 근권 세균을 처리한 식물에 비해 강한 형광 반응을 나타내는 것을 확인할 수 있었다. 이와 같은 결과를 기초로, 병원균 침입에 의한 방어 반응은 근권 세균을 처리한 식물과 BABA를 처리한 식물에서 저항성 유도의 기작이 다르게 나타나는 것으로 사료 된다.

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이 한권의 논문이 완성되기 까지 도움을 주신 많은 분들과 울고 웃었던 시간들이 이젠 제가 간직할 소중한 추억이 되었습니다. 다시금 한분, 한분 떠올리며 감사의 마음을 전합니다.

바쁘신 중에도 제 논문의 심사를 맡아서 많은 조언과 격려를 아끼지 않으신 강영길 교수님과 홍순영 박사님께 깊은 감사의 마음을 전합니다. 또한 존경하는 조남기 명예교수님, 고영우 명예 교수님, 송창길 교수님, 현해남 교수님, 김동순 교수님께도 진심으로 감사드립니다.

언제나 많은 관심을 가져 주셨던 고미라, 이충선, 박성준 조교 선생님과 늘 친절하게 제 질문에 좋은 답변 해주시던 정말 고마우신 김찬우 박사님과 박정식 선배님께 감사드리며, 따뜻한 마음으로 후배를 챙겨 주시던 송진영, 강정환, 우성배, 서연동 선배님들을 비롯한 많은 선후배님들께 마음을 다해 감사드립니다.

이제는 가족같이 느껴지는 우리 병리학 실험실 식구들에게 좀더 잘해주지 못했던 죄송한 마음과 더불어 고마운 마음을 전합니다.

그리고 말없이 묵묵히 응원해주던 친구같이 편한 상현오빠에게 감사드립니다.

가끔 나태해지고 용기를 잃었을 때 마다 곁에서 따끔한 충고 아끼지 않고 진심으로 걱정해주고 챙겨주시던 친오빠 같은 경후오빠...정말 고맙습니다.

항상 곁에 있는 것만으로도 든든한 소중한 벗 혜영이... 비록 지금은 멀리 떨어져 있지만 함께 쌓았던 소중한 우정 평생 잊지 못할 것입니다.

함께 병리학 학문을 연구하면서 도움을 주신 같이 졸업하는 용준오빠에게도 감사드립니다.

아마도 매주 월요일 오전 10시 lab meeting 시간이 가슴 짠하게 그리운 날이 올 것이란 생각이 듭니다. 힘들었지만 결코 힘들지만은 않았던 시간들...

소소한 실험실 일상생활에서 얻은 큰 교훈들...

그 소중한 시간들을 가슴 깊이 새기고 석사 과정의 마침표를 찍으려 합니다.

논문을 보시면 우리 딸 대견스럽다고 어깨를 두들겨 주실 것 같은 부모님...

그리고 언제나 언니를 믿고 응원해주는 은정이....

제게 이런 가족이란 소중한 사랑을 주신 부처님께 감사드립니다.

마지막으로, 저의 큰 버팀목이자 제게 살아가는 힘이 되어주시는 너무나 소중한 아버지, 어머니, 그리고 동생 은정이에게 이 논문을 바칩니다.

감사합니다.