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Observations of Infection Structures after Inoculation with *Colletotrichum orbiculare* on the Leaves of Cucumber Plants Pre-inoculated with Two Bacterial Strains *Pseudomonas putida* or *Micrococcus luteus*

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Infection structures were observed at the penetration sites on the leaves of cucumber plants inoculated with *Colletotrichum orbiculare* using a fluorescence microscope. The cucumber plants were previously drenched with suspension of bacterial strains *Pseudomonas putida* or *Micrococcus luteus*. The plants pre-inoculated with both bacterial strains were resistant against anthracnose after inoculation with *C. orbiculare*. To investigate the resistance mechanism by both bacterial strains, the surface of infected leaves was observed at the different time after challenge inoculation. At 3 days after inoculation there were no differences in the germination and appressorium formation of conidia of *C. orbiculare* as well as in the callose formation of the plants between both bacteria pre-inoculated and non-treated. At 5 days, the germination and appressorium formation of the fungal conidia were, however, significantly decreased on the leaves of plants pre-inoculated with *M. luteus* at the concentration with 1.0×10^7 cfu/*ml*. Furthermore, callose formation of plants cells at the penetration sites was apparently increased. In contrast, there were no defense reactions of the plants at the concentration with 1.0×10^6 cfu/*ml* of *M. luteus*. Similarly, inoculation *P. putida* caused no plant resistance at the low concentration, whereas increase of callose formation was observed at the higher concentration. The results of this study suggest that the resistant mechanisms might be differently expressed by the concentration of pre-treatment with bacterial suspension.

KEYWORDS: ***Colletotrichum orbiculare*, Defense response, Induced systemic resistance (ISR), Infection structure, *Micrococcus luteus*, *Pseudomonas putida*, Systemic acquired resistance (SAR)**

Since many years ago, the increased amounts of chemicals for plant protection were resulting in a destruction of agricultural ecosystem, the occurrence of resistance of plant pathogens or insect against chemicals, and harmful to human health by the residue of chemicals. Recently, a lot of attention has been focused on environment for the last decade. In the case of plant protection a new strategy has been looked for, by which the amount of chemical usage could be decreased.

One of the environment-favorable strategies for plant protection is use of plants expressing the induced systemic resistance (ISR). ISR is a resistance mechanism, which is mostly operated by pre-inoculation with plant growth promoting rhizobacteria (PGPR) (Van Loon *et al.*, 1998). PGPR enhances the plant growth, when they are colonized in the rhizosphere of the plants (Kleopper *et al.*, 1980). Also, it has been found that the plants become resistant against various plants diseases including anthracnose by the colonization of the PGPR (Van Loon *et al.*, 1998).

ISR expressing plants have been introduced as an alterative strategy because of some benefits for environment by using ISR plants (Van Loon *et al.*, 1998). First, ISR is effective to a broad spectrum of diseases caused by fungi, bacteria, and virus. Especially, because there are no commercial chemicals against disease caused by virus yet, protection strategy by using ISR should be very useful to control the virus diseases. Second, there is no induction of resistance of plant pathogens or insect against chemicals. Because ISR is mostly expressed by enhancement of plant's own defense reaction, there would be no possibility for adaptation of pathogen or insect to this phenomenon. Third, the products from the plants expressing ISR are not harmful to human health. Although some metabolites are produced in the ISR expressing plants, which may play a role for plant resistance, such as phenolic compounds, but not affect to human.

For an optimal application of the ISR expressing plants to the field, more research concerning ISR should be performed, such as signal transfer for ISR, mechanism of ISR expression, or environment of crops, which may effect to ISR expression.

Until now, however, the mechanism of ISR has not been clearly illustrated. In our previous study the disease severity in the cucumber plants pre-inoculated with both bacterial strains *P. putida* and *M. luteus* were suppressed after challenge inoculation with *C. orbiculare* (Jeun *et al.*, 2004a). In the present study, infection structures of the

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pathogen and defense responses of its host plants were cytologically examined on the leaf surfaces of the cucumber plants pre-inoculated with *P. putida* or *M. luteus*.

Materials and Methods

Strains and culture conditions. Anthracnose pathogen, *Colletotrichum orbiculare*, which was obtained from Plant Pathology Div., National Institute of Agricultural Science and Technology, RDA, Suwon, was grown in green beans agar medium for 5 days. Ten ml of distilled water was poured in the medium grown the anthracnose pathogen and then the fungal conidia were harvested by using a loop. This conidial suspension (2.5×10^5 conidia/*ml*) with 100 μl l^{-1} of Tween 20, which enhances the adhesion of conidia on leaf surface, was used as inoculum for challenge inoculation on cucumber leaves. *Pseudomonas putida* or *Micrococcus luteus*, which were isolated form Jeju Island (Lee *et al.*, 2003), were grown in tryptic soy agar at 28°C for 48 h.

Triggering of ISR. Cucumber seeds (*Cucumis sativus* L. cv. Eun Sung) were sown in plastic pots (10-cm in diameter) filled with commercial soil (Choroc Nala®, Bokyung Nongsang, Korea) containing 10% of Perlite (Parat®, Sam Son, Korea). Cucumber seedlings were grown in a growth chamber with a day/night temperature of 28/25°C. The concentration of each bacterial strain was adjusted to 1.0×10^7 and 1.0×10^6 colony forming unit (cfu)/*ml* according to the methods described by Park and Kloepper (2000). Thirty ml of each bacterial suspension with different concentration was soil-drenched around each cucumber plant 7 days before challenge of *C. orbiculare*. Distilled water as negative control was drenched around the cucumber plants instead of the bacterial suspension.

Challenge inoculation with anthracnose pathogen. The conidial suspension of *C. orbiculare* (2.5×10^5 conidia/*ml*) was sprayed on the aerial cucumber leaves 7 days after treatment of the bacterial suspension. The plants inoculated with the conidial suspension of the pathogen were kept in a humid chamber maintaining 100% RH for 24 h and then transferred to the greenhouse at 28°C during the day and 25°C at night with 60% humidity.

Observation of infection structures using fluorescence microscope. Leaves of the inoculated cucumber plants were detached at 3 and 5 days after the challenge-inoculation. The leaf tissues were stained according to the method described by Jeun *et al.* (2000). The leaves were cut with a cork borer (9-mm in diameter) and fixed with 2% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2) for 2 h. After washing three times in the phosphate buffer three times, for 10 min each, the leaf disks were stained with 0.005% (w/v) aniline blue (Serva, Heidelberg) containing the fluorochrome 'Sirofluor' in phosphate buffer (Kauss, 1989) and then with 0.02% Uvitex 2B (w/v) (Diethanol) for 20 min, in order to observe fungal structure. After washing in the phosphate buffer, the leaf disks were mounted on glass slides in 50% glycerin. The infection structures of the anthracnose fungus at the penetration sites were observed using fluorescent microscopy (Olympus) equipped with filter set 05 (BP 400-440, FT 460, LP 470). Number of germinated conidia and appressoria of *C. orbiculare* and autofluorescent plant cells under the appressorium were counted on the leaf surfaces of the plants non-treated, pre-inoculated with both bacterial strains, respectively. The rate of appressorium formation and autofluorescent cells at the penetration sites were calculated from the data counted on the 4 leaf discs detached from each 4 plants in the 3 separated experiments.

Data analysis. The germination rate and frequency of appressorium formation of the fungus, and fluorescent cells in the inoculated leaves were compared using a *t*-test (P=0.01 and 0.005) between non-treated and bacteria pre-inoculated plants, respectively.

Results

Disease severity. Disease severity was determined on the leaves of cucumber plants non-treated and both bacteria pre-inoculated after challenge inoculation with anthracnose pathogen *Colletotrichum orbiculare*. The growth of cucumber plants were enhanced after inoculation with bacterial strains *Pseudomonas putida* as well as *Micrococcus luteus* (data not shown). The visible lesion spots were formed on the leaves of the non-treated as well as the bacteria pre-inoculated plants 5 days after challenge-inoculation. However, the number of lesions was significantly reduced on the leaves of the bacteria pre-inoculated plants compared with the non-treated plants. Furthermore, the size of lesions on the leaves of untreated plants was bigger than those of non-treated plants (Fig. 1). Generally, the disease severity of the both bacteria pre-inoculated plants was decreased compared to that of non-treated plants, indicating a resistance induction in the plants by pre-inoculation with both bacterial strains.

Observation of infection structures. The resistance expression was examined using a fluorescence microscope on the leaves of surface and in the epidermal cell layer of cucumber plants pre-inoculated with both bacterial strains. A conidium of the pathogen germinated on the leaf surface of the inoculated plants at 1 day later and then began to form an appressorium at 2 days after inoculation (Fig. 2). At 3 days after challenge inoculation about

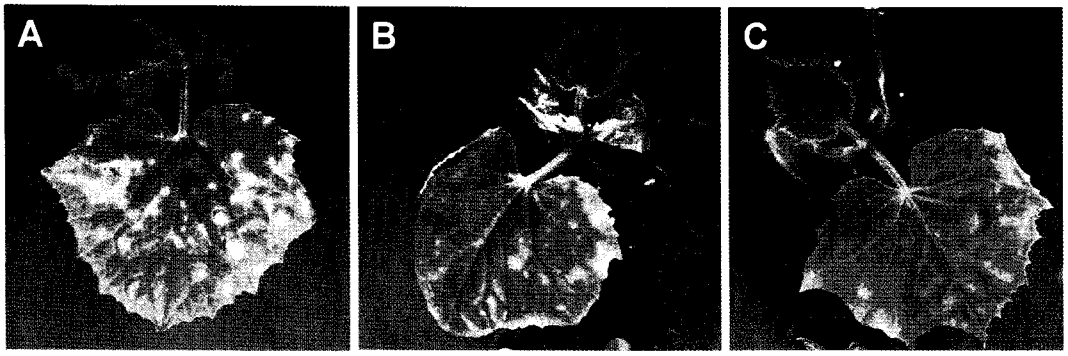

Fig. 1. Induction of systemically induced resistance in cucumber plants against anthracnose disease 7 days after inoculation with *Colletotrichum orbiculare* (2.5×10^5 conidia/*ml*). The presented plants were non-treated control (A), pre-inoculated with *Pseudomonas putida* (B) and *Micrococcus luteus* (C) 7 days before the challenge inoculation. The present pictures showed the developed lesions by anthracnose pathogen on the non-treated control plants (A) and the suppression of lesions on the leaves of *P. putida* (B) or *M. luteus* (C) pre-inoculated plants.

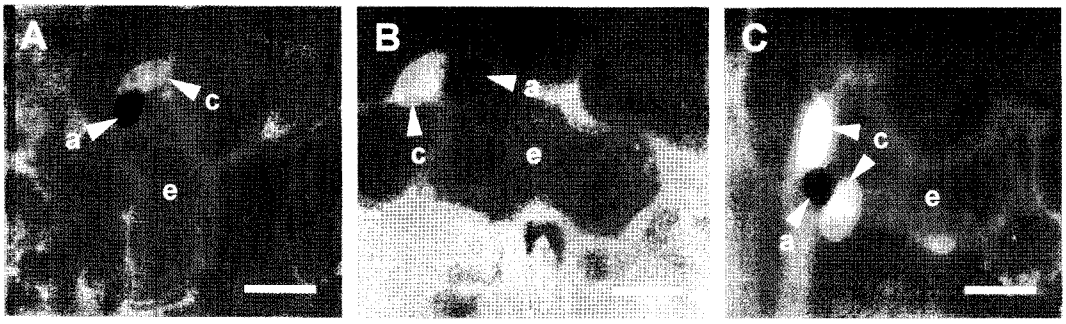

Fig. 2. Fluorescence microscopical observation of infection structures and resistance responses on the leaves of the cucumber plants non-treated (A), pre-inoculated with *Pseudomonas putida* (B) and *Micrococcus luteus* (C) at 5 days after challenge-inoculation with *Colletotrichum orbiculare*. Pre-inoculation with both bacterial strains (1.0×10^7 cfu/*ml*) were carried out 7 days before the challenge inoculation. The bars = 20 μm. Abbreviations: a, appressorium; c, conidium; e, epidermal cell.

35% of total conidia were germinated on the leaf surfaces of non-treated plants and about 30% of total conidia formed appressoria (Fig 3). There were some germinated conidia without appressoria formation. Most of appressoria colored as black, which contained melanin (Fig. 2), whereas a few appressoria lacking of melanin had no color. The fluorescence cells regarded as the cells, which form callose in the cell wall, because aniline blue dyes β-glucans (Kauss, 1989). The callose formation from plant epidermal cells, however, was not often detected on the leaf tissues of the non-treated plants (Fig. 2).

There were no differences in infection structure and no significant difference between non-treated and both bacteria pre-inoculated plants in the germination rate and the appressorium formation of the conidia and in the callose formation of the plant cells at 3 days after challenge inoculation (Fig. 3).

At 5 days after challenge inoculation, the germination rate and appressorium formation were slightly decreased on the leaves of *M. luteus* pre-inoculated plants at 1.0×10^7 cfu/*ml* (Fig. 3). However, the pre-inoculation with *M. luteus* at lower concentration could affect neither to the germination rate nor appressorium of the conidia (Fig. 3). Similarly, on the leaves of *P. putida* pre-inoculated plants, there were no differences in the germination rate and appressorium formation compared to those of non-treated plants (Fig. 3). In the case of epidermal cells of plants, however, the pre-inoculation with both bacteria at higher concentration caused the enhancement of callose formation, whereas at lower concentration did not (Fig. 3). It indicated that the active defense response of the plant cells against attack of *C. orbiculare* was triggered by pre-inoculation with high concentration of both bacteria.

Discussion

The resistance mechanism triggered by plant growth promoting rhizobacteria (PGPR) has been studied in many laboratories (Park and Kleopper, 2000; Jeun *et al.*, 2004b). The mechanism of ISR could be classified mostly into two parts; one is signal transfer of ISR and the other is resistance expression in plants pre-inoculated with PGPR.

The signaling of ISR is usually independent on the

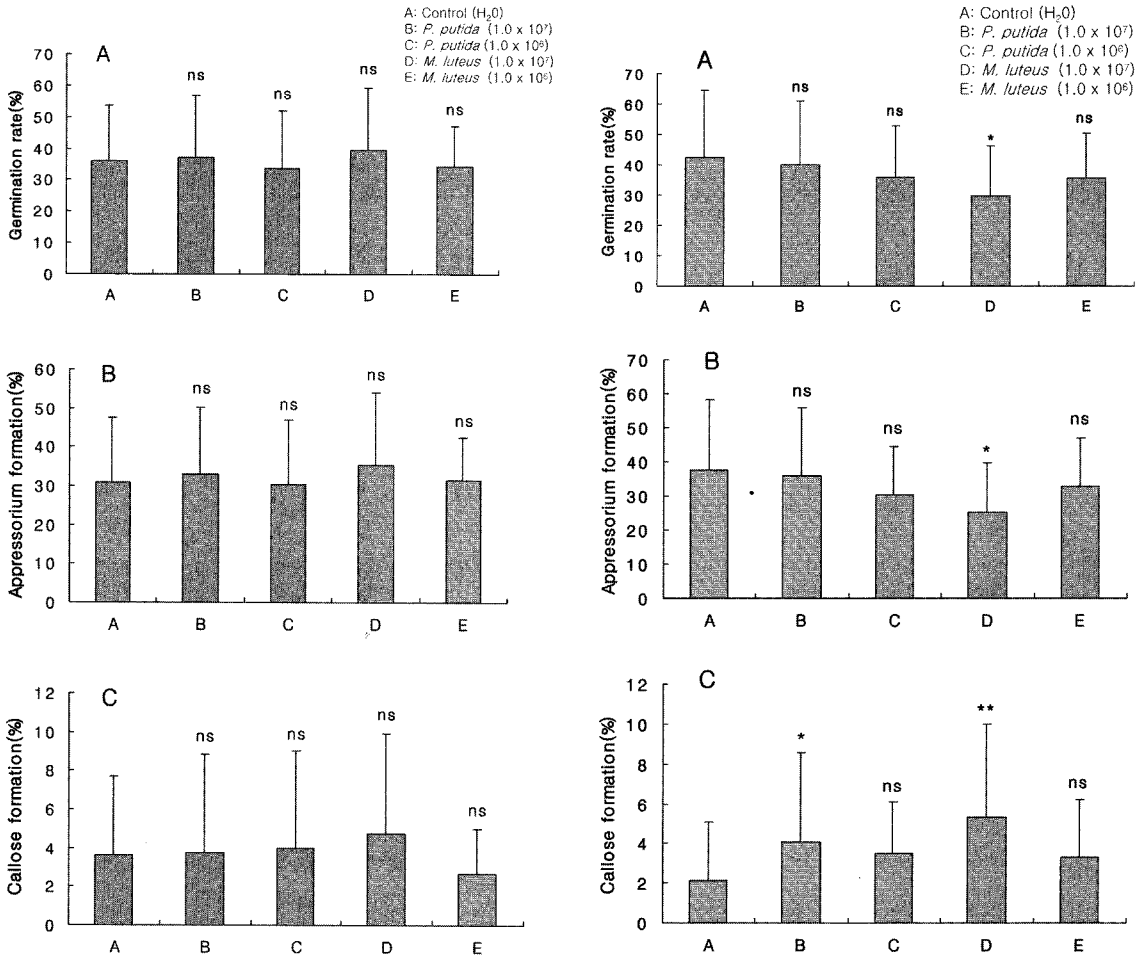

Fig. 3. Frequency of conidial germination (A) and appressorium formation (B) of *Colletotrichum orbiculare* and callose formation of plant cells (C) at the penetration sites on the leaves of cucumber plants non-treated, pre-inoculated with both bacterial strains *Pseudomonas putida* and *Micrococcus luteus* at 3 days (left) or 5days (right) after challenge inoculation with the fungal pathogen. The pre-inoculation with both bacterial strains (1.0×10^7 cfu/*ml*) were carried out 7 before the challenge inoculation, respectively. The vertical bars indicate the standard deviation of the 3 separated experiments each containing 4 leaf discs from 4 plants per treatment. * = significant at the 1% probability level; ** = significant at the 0.5% probability level; ns = non-significant.

accumulation of salicylic acid (Van Loon *et al.*, 1998), which play an important role for the signal transfer in the plants expressing acquired systemic resistance (SAR) by stimulation with plant pathogen or chemicals (Malamy *et al.*, 1990). Pieterse *et al.* (1998) has reported that in the ethylene or jasmonic acid insensitive arabidopsis plants, ISR was not triggered after pre-inoculation with PGPR. It indicated that the role of ethylene or jasmonic acid, rather than SA, might be important for triggering of resistance by PGPR.

The resistance expression by PGPR seems to be different to that triggered by plant pathogen or chemicals. In some cases of plants expressing ISR, PR-proteins were not accumulated (Pieterse *et al.*, 1996; Van Wees *et al.*, 1997), whereas PR-proteins always accumulated in the plants expressing SAR (Sticher *et al.*, 1997). Furthermore, some PGPR strains mediating systemic resistance have direct antifungal activity. In our previous study both bacterial isolates TRL2-3 (*Pseudomonas putida*) as well as TRK2-2 (*Micrococcus luteus*) showed direct antifungal effect *in vitro* test (Lee *et al.*, 2003). Another mechanism of expression of ISR is competition for mineral elements such as ion (Fe), which is easily captured by siderophores produced in PGPR (Maurhofer *et al.*, 1994; Van Loon *et al.*, 1997; 1998).

In the cucumber plants pre-inoculated with PGPR *Pseudomonas putida* as well as *Micrococcus luteus*, disease severity was decreased after inoculation with anthracnose pathogen *Colletotrichum orbiculare*. Similarly, in our previous study, the efficacy of both strains for triggering of ISR induction was already revealed (Jeun *et al.*, 2004a). However, it has not clearly explained how the resistance response was expressed in the cucumber plants pre-inoculated with both bacteria.

In present study, using fluorescent microscope the resistance mechanism was investigated on the leaf surface of the cucumber plants expressing ISR by both bacteria. The germination rate and appressorium formation of *C. orbiculare* conidia were slightly decreased on the leaves of *M. luteus* pre-inoculated plants at 1.0×10^7 cfu/*ml* at 5 days after challenge inoculation. However, it seems to be not main point of defense reaction in this system, because the suppression was not dramatically enough to express the resistance. Contrast to that, callose formation, one of the defense reactions of plants against attack of pathogen (Strömberg and Brishammar, 1993; Kovats *et al.*, 1991), was significantly increased at the penetration site on the leaves of *M. luteus* pre-inoculated plants at 1.0×10^7 cfu/*ml*. Although it was not significantly increased in the plants pre-inoculated with *P. putida*, the callose was more formed (Fig. 3). These results indicated that both bacterial strains induced active callose formation against fungal attack resulting in decrease of disease development. Similar results were shown in the pre-inoculated cucumber plants with PGPR strains such as *Serratia marcescens* or *Pseudomonas fluorescens* after challenge inoculation with *Colletotrichum orbiculare* (Jeun *et al.*, 2004b). Also, the callose deposits at the penetration site were increased in the cucumber plants expressing ISR by pre-inoculation with an arbuscular mycorriza *G. intraradices* (in our unpublished data). However, the cucumber plants expressing SAR by DL-3-aminobutyric acid (BABA) were different, where the germination rate as well as appressorium formation of the anthracnose pathogen were dramatically decreased but not increase of callose formation (Jeun *et al.*, 2004b). Strangely, there were no different in germination rate, appressorium formation, and callose formation compared to non-treated plants, when both bacteria were pre-inoculated with the low concentration 1.0×10^6 cfu/*ml* (Fig. 3). There is no clear explaining why the bacteria cannot affect to the fungal infection structure or plant response at different concentration.

In summary, from these results it was discussed that the defense response of plants, such as callose formation, may be play an important role for expressing ISR against anthracnose pathogen. However, in order to illustrate the mechanism of ISR, the other defense responses, such as the production of anti-fungal substance phytoalexin (Somssich and Hahlbrok, 1998), the accumulation of PR-proteins (Hwang *et al.*, 1997; Jeun, 2000), and encoding of enzymes involved in the metabolism of reactive oxygen species (Lamb and Dixon, 1997), should be investigated.

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