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Observations of Infection Structures on the Leaves of Cucumber Plants Pre-treated with Arbuscular Mycorrhiza *Glomus intraradices* after Challenge Inoculation with *Colletotrichum orbiculare*

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Resistance inductions on the leaves of cucumber plant by an arbuscular mycorrhiza *Glomus intraradices* were investigated. In addition, the infection structures were observed at the penetration sites on the leaves of plant inoculated with *Colletotrichum orbiculare* using a fluorescence microscope. The severity of anthracnose disease caused by *Colletotrichum orbiculare* was significantly decreased on the leaves of cucumber plant colonized with *G intraradices* compared with those of non-treated control plants. As a positive control, pre-treatment with DL-3-aminobutyric acid (BABA) caused a remarkable reduction of the disease severity on the pathogen-inoculated leaves. There were no significant differences in the frequency of either germination or appressorium formation of the plant pathogen between mycorrhiza colonized and non-treated plants. It was also the same on the BABA pre-treated plants. However, the frequency of callose formation was significantly high on the leaves of *G intraradices* colonized plants compared to those of non-treated control plants at 5 days after challenge inoculation. On the leaves of BABA treated plants callose formation was not significantly high than those of non-treated, although the disease severity was more strongly suppressed. It was suggested that the resistance induced by colonization with *G intraradices* might be related to the enhancement of callose formation at the penetrate sites on the leaves invaded by the pathogen, whereas resistance by BABA did not.

Keywords : arbuscular mycorrhiza, *Colletotrichum orbiculare*, cucumber plants, DL-3-Aminobutyric acid (BABA), *Glomus intraradices*, induced systemic resistance (ISR), systemic acquired resistance (SAR)

Plant can be resistant against plant disease when the plants exposed to the exogenous stimuli such as an invasion of plant pathogens, chemicals, or microorganisms (Sticher et al., 1997). Although the mechanisms of the resistance are not clearly explained yet, in some plant-pathogen interactions the signal pathways of the induced resistance were evidenced (Sticher et al., 1997). One of the mostly known mechanism is defined as systemic acquired resistance (SAR), which is mostly triggered by pre-inoculation with pathogen to certain parts of plants (Sticher et al., 1997). Usually, SAR is expressed after hypersensitive reaction (HR) (Siegrist et al., 2000) or by necrosis on the treated parts of the plants (van Loon, 1997).

Another form of resistance termed as induced systemic resistance (ISR) has been reported, in which the resistance mostly induced by pre-inoculation with plant growth promoting rhizobacteria (PGPR) (van Loon et al., 1998). ISR is distinguished from the classical SAR by different signal pathway and resistance expression (Knoester et al., 1999; Pieterse et al., 1996; Press et al., 1997; Van Wees et al., 1997). In some cases of plants expressing ISR, PR-proteins were not accumulated (Pieterse et al., 1996; van Wees et al., 1997). Although neither HR nor necrosis is formed in the roots inoculated with PGPR, the aerial parts of plants become resistant against plant pathogen (Kloepper et al., 1980). Furthermore, the signaling of ISR is usually independent on the accumulation of SA (van Loon et al., 1998). However, in the ethylene or jasmonic acid insensitive Arabidopsis plants, ISR was not triggered after pre-inoculation with PGPR (Pieterse et al., 1998), indicating an important role of ethylene or jasmonic acid for triggering of resistance by PGPR.

Some microorganisms maintain endosymbiotic interaction with plant, such as the mutualistic symbiosis between plant roots and arbuscular mycorrhizal fungi (AMF). The plants colonized with AMF benefit not only to improve plant health but also to be resistant against plant pathogens (Azcón-Aguilar and Barea, 1996). Like the case of PGPR, the AMF can be potential biocontrol agents for agricultural crop species (Pozo et al., 2002). Indeed, in the tomato plants colonized with *Glomus mosseae* in root system the

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disease severity caused by *Phytophthora parasitica* was reduced (Cordier et al., 1996, 1998; Pozo et al., 1996, 1999; Trotta et al., 1996; Vigo et al., 2000). In some of studies the resistance mechanisms of symbiotic associations between plants and AMF has been reported (Pozo et al., 2002). The pathogenesis-related proteins such as chitinases, chitosanases and β-1,3-glucanses in the mycorrhizal colonized roots of tomato plants were accumulated (Pozo et al., 1996, 1998, 1999) indicating the effective defense reaction by antifungal proteins. Furthermore, in bean and wheat plants colonized by *G. interaradices* the catalase and peroxidase were accumulated (Blee and Anderson. 2000). The increases of these enzymes are involved with defense mechanism against plant pathogen. However, the mechanisms of this type of resistance have not been clearly understood.

In the present study, protection effects by colonization with *G. intraradices* against cucumber anthracnose caused by *Colletotrichum orbiculare* were investigated on the leaves of cucumber plants colonized with *G. intraradices*. Furthermore, infection structures of the pathogen and defense responses of plant were cytologically examined on the leaf surfaces of the cucumber plants colonized with *G. intraradices*. Additionally, the resistance mechanism by chemical inducer BABA was also discussed.

Materials and Methods

Plant and pathogen. 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.

Anthracnose pathogen, *Colletotrichum orbiculare* was grown in green beans agar medium for 5 days. Ten ml distilled water was poured in the medium grown the anthracnose pathogen and then the fungal conidia were harvested by using a brush. 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.

Propagation of the mycorrhizal fungus. *Glomus intraradices* (BEG 110) were propagated several times on white clover grown on autoclaved substrate (sand : vermiculite = 1 : 1) at 121°C for 30 min. For propagation, the inoculum always comprised 10% (v/v) of the pot volume, where usually approximately 0.5 g seeds were used per 500 g of substrate. Pots were placed on a balance one or two times per day and watered with distilled water to maintain a soil water content equivalent to 65% of field capacity. After 6 weeks, a nutrient solution containing 2 mM Ca $(NO_3)_2$ was daily watered to compensate the nitrogen deficiency for the plants. Plants were grown in a greenhouse for ten weeks. Thereafter, the substrate was left to dry out (until plants wilted), re-irrigated and left to dry for a further two weeks, both drying periods serving to promote spore production. Roots and substrate were sieved (1 mm in diameter) and the percentage colonization of roots was determined (usually around 50%). The percentage of root length colonized by mycorrhizal fungi was determined on roots stained in trypan blue (Koske and Gemma 1989) using the gridline-intersect method (Giovannetti and Mosse 1980). The air-dried substrate with spores and colonized root pieces were stored at 4°C and used as inoculum.

Colonization with *G. intraradices* and treatment with BABA. Ten% (V/V) of inoculum of *G. intraradices* was mixed with the commercial soil, which was already sterilized at 100°C for 1 h. The cucumber seeds were sown in the soil mixture with inoculum of *G. intraradices*.

The plants pre-treated with DL-3-aminobutyric acid (BABA) were used as a positive control for induction of systemic resistance. Thirty ml of 10 mM BABA solution per plant was soil-drenched at 5 days before challenge inoculation with *C. orbiculare*. As a negative control, water treated plants were used.

Challenge inoculation and disease assessment. The conidial suspension of *C. orbiculare* (2.5×10^5 conidia/ml) was sprayed on the aerial leaves of cucumber plants. Five days after treatment with BABA the plants grown in the soil colonized with *G. intraradices*, BABA pre-treated plants, and non-treated plants were inoculated. The plants inoculated with the conidial suspension of the fungus were kept in a humid chamber maintaining 100% RH for 24 h and then transferred into a growth chamber with a day/night temperature of 28/25°C and a relative humidity of 60%.

The development of lesions on the inoculated leaves was determined at 5 days after challenge inoculation by visual estimation of the leaf area occupied by anthracnose lesion. Protection rate was calculated as described by Cohen (1994), that is the rate (%) = $100\ (1 - x/y)$ in which x and y are number of lesions on the leaves of treated and non-treated plants, respectively.

Observation of infection structures using light 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 (5-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 0.02% 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). Numbers of conidia, germinated conidia and appressoria of fungi and autofluorescent plant cells under the appressorium were counted on the leaf surfaces of the plants non-treated, colonized with mycorrhiza and treated with BABA, 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 lesion areas of the plant leaves, the germination rate and frequency of appressorium formation of the fungus, and fluorescent cells in the inoculated leaves were compared using a paired t-test (P=0.001) between BABA as well as mycorrhiza treated and the non-treated control plants, respectively.

Results

Effects of colonization with arbuscular mycorrhiza on anthracnose severity. There was no visible difference in phenotype between the mychorrhizal plants and the untreated control plants after colonization with *G. intraradices* in the root system of cucumber plants (Fig. 1). Five days after challenge-inoculation, visible lesion spots were formed on the leaves of the control as well as the mycorrhizal plants. However, lesion area was significantly reduced in the leaves of the mycorrhizal plants compared with the control plants (Figs. 1 and 2). Furthermore, the size of lesions on the leaves of untreated plants was rapidly increased at 7 days after challenge inoculation, indicating rapid development of fungal growth. In contrast to the control plants, the development of anthracnose fungus was restricted on the leaves of mycorrhizal plants (Fig. 1).

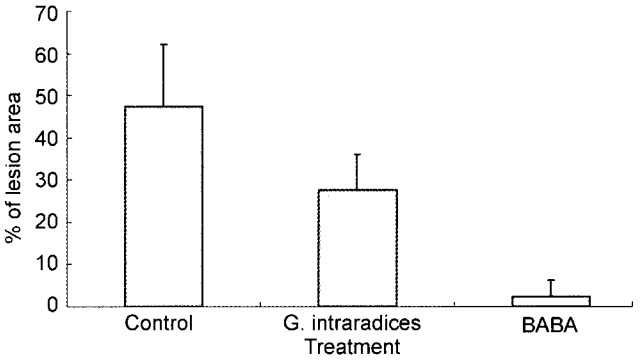

Fig. 2. Reduction of lesion area causing *C. orbiculare* in the leaves of cucumber plants by colonization with *G. intraradices* as well as by pre-treatment with BABA. The lesion areas were measured at 7 days after inoculation with *C. orbiculare* (1.0×10^8 conidia/ml). The vertical bars indicate the standard deviation of the 5 separated experiments each containing 12 plants per treatment.

The treatment with BABA had no difference in phenotype of cucumber plant compared to that of control plants (Fig. 1). BABA was effective on mediating resistance against cucumber anthracnose in the aerial parts of the plants (Fig. 1 and 2). The protection rate by BABA treatment against anthracnose reached to approximately 95% indicating that BABA could be an effective inducer of systemic acquired resistance (Fig. 1). In particular, the reduction of lesion area by BABA treatment was much higher than those by *G. intraradices* colonization (Fig. 1), indicating that defense reaction by chemicals may be much

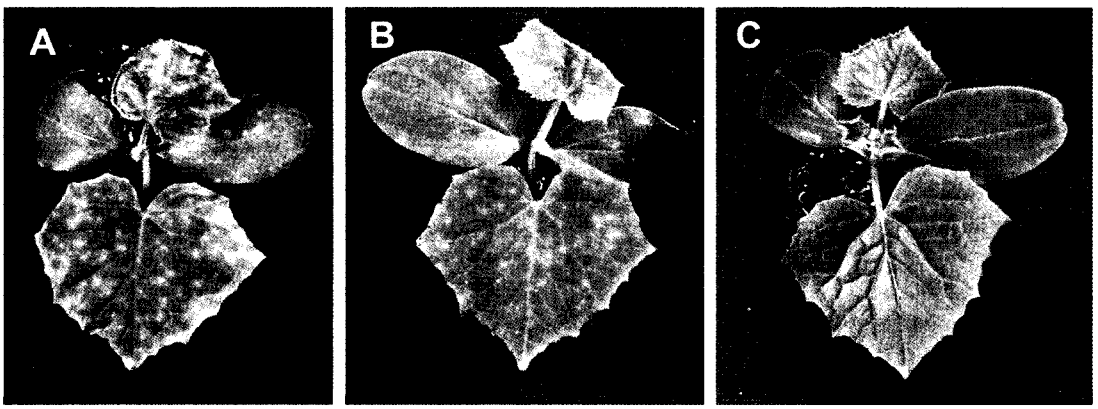

Fig. 1. Induction of systemically induced resistance in cucumber plants against anthracnose disease at 7 days after inoculation with *C. orbiculare* (1.0×10^8 conidia/ml). The presented plants were untreated control (A), colonized with *G. intraradices* (B) and drenched with 30 ml of 10 mM BABA 5 days before the challenge inoculation.

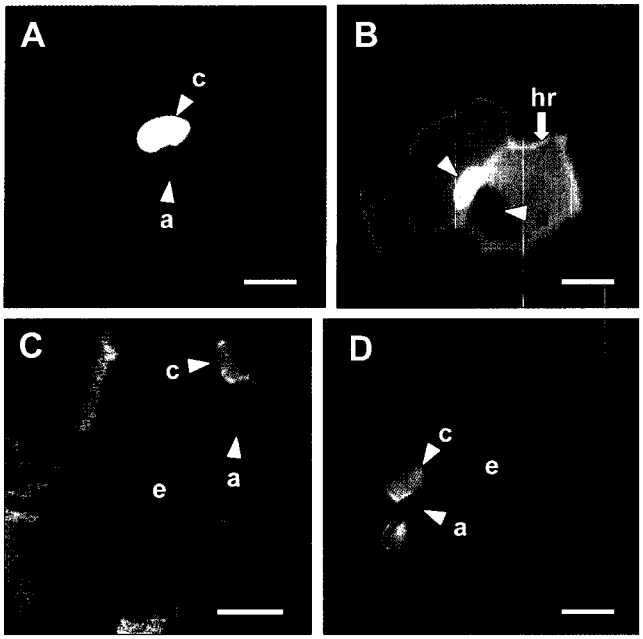

Fig. 3. Fluorescence microscopical observation of infection structures and resistance responses on the leaves of the cucumber plants untreated (A), pre-treated with BABA (B) and colonized with *G. intraradices* at 3 days (C) and 5 days (D) after challenge-inoculation with *C. orbiculare*, respectively. The arrow showed plant cell fluorescent by staining with aniline blue and indicated a reaction of plant cell hypersensitively. The bars = 20 μm. Abbreviations: a, appressorium; c, conidium; e, epidermal cell; hr, hypersensitive reaction

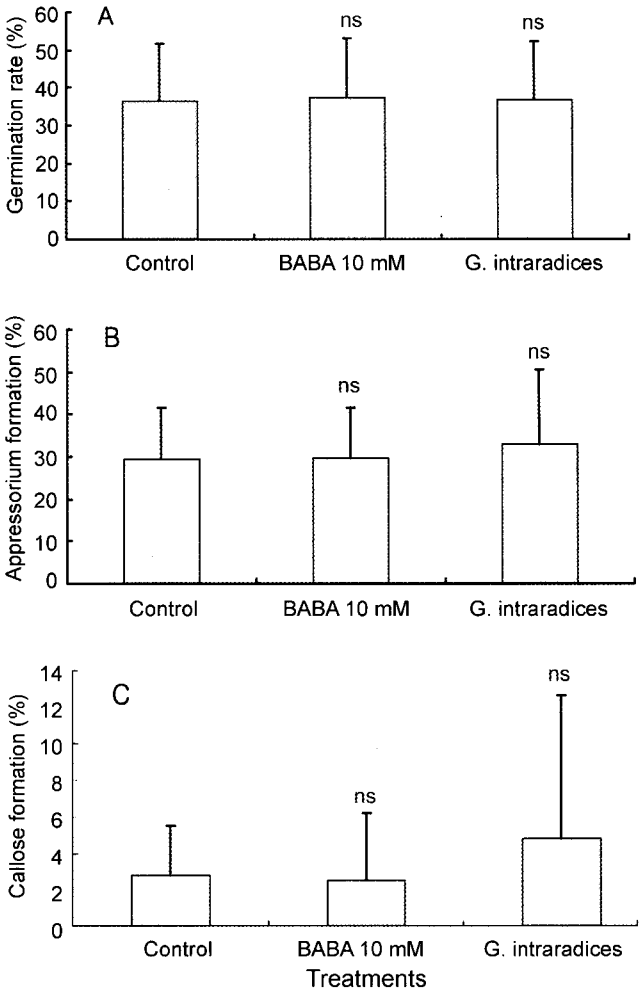

Fig. 4. Frequency of conidial germination, appressorium formation of *C. orbiculare* and callose formation of the plant cells on the leaves of cucumber plants untreated control, colonized with *G. intraradices* and pre-treated with BABA. The leaves were attached at 3 days after challenge inoculation with *C. orbiculare* (1.0×10^8 conidia/ml). The vertical bars indicate the standard deviation of the 3 separated experiments each containing 4 leaf discs from 6 plants per treatment. ns, non significant

more effective than those induced by mycorrhiza colonization.

Microscopical observation on the leaf surface. Using fluorescence microscope the resistance expression was examined both on the leaf surface and in the epidermal cell layer of cucumber plants colonized with *G. intraradices* as well as treated with BABA. On the leaf surfaces of non-treated plants about 36.4% of total conidia were germinated and only 29.3% of total conidia formed appressoria at 3 days after inoculation. Some conidia were germinated but failed to form appressoria. Most of appressoria formed melanin, which was identified as black spot under the microscope (Fig. 3). Most of the penetration sites were not intensively fluorescent, indicating no active defense reaction of the host cells (Fig. 3A).

On the leaf of plants colonized with *G. intraradices* some penetration sites became fluorescent at 3 days after challenge inoculation, indicating the plant response to the fungal invasion of pathogen (Fig. 3C). However, there was no significant difference in callose formation, although by mycorrhizal colonization was slightly increased callose formation compare to those of control one (Fig. 4C). Similarly, there were no differences in germination rate or in appressorium formation between non-treated and both BABA and mycorrhizal treated plants (Fig. 4A, 4B).

Two days later, the conidia germination on the leaf surface of non-treated plants increased slightly while the conidia germination of both mycorrhiza and BABA treated plants were not increased (Figs. 4A and 5A). There were no significant differences in germination rate and in appressorium formation between non-treated and both treated plants at 5 days after inoculation (Fig. 5A, 5B). However, callose was more frequently formed at the penetration sites on the BABA treated as well as the *G. intraradices* colonized plants at 5 days after challenge

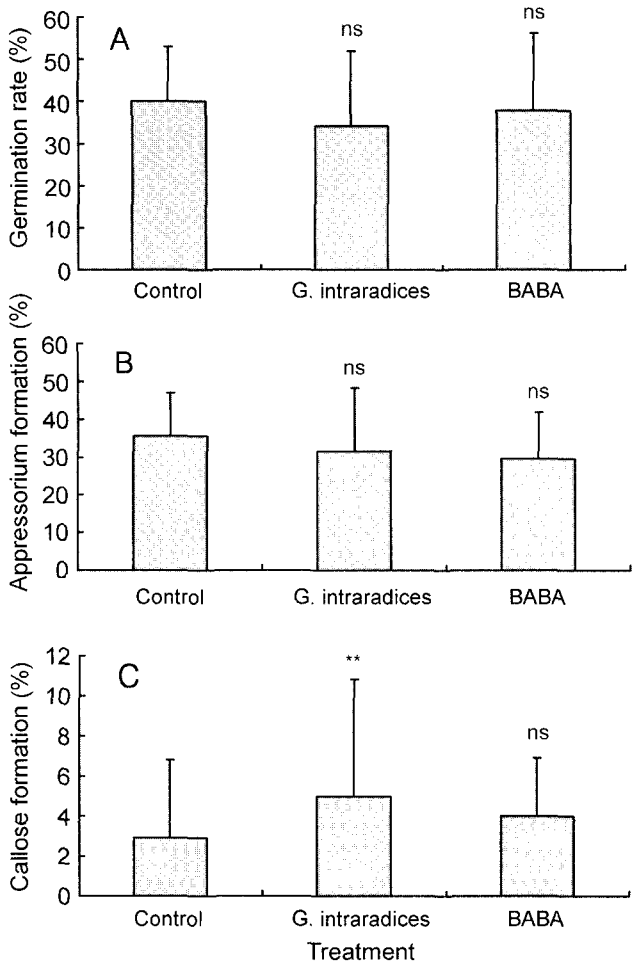

Fig. 5. Frequency of conidial germination, appressorium formation of *C. orbiculare* and callose formation of the plant cells on the leaves of cucumber plants untreated control, colonized with *G. intraradices* and pre-treated with BABA. The leaves were attached at 5 days after challenge inoculation with *C. orbiculare* (1.0×10^8 conidia/ml). The vertical bars indicate the standard deviation of the 3 separated experiments each containing 4 leaf discs from 6 plants per treatment. **, significant at the 0.1% probability level; ns, non significant

inoculation compare to those of 3 days, whereas no difference was found on the leaves of control leaves (Figs. 4C and 5C). In the BABA pre-treated plants the autofluorescence was very strong at the penetrated site (Fig. 3B). Remarkably, the callose formation on the mycorrhiza-colonized plants was significantly increased compared to those of control plants (Fig. 5C), indicating an enhancement of defense reaction of the plants. The autofluorescence intensity was not stronger than that of at 3 days (Fig. 3C, D).

In contrast to the case of mycorrhizal plants, the BABA pre-treated plants had no significant difference in the frequency of the fluorescent cells compared with that of untreated plants, although fluorescent cells were slightly increased (Fig. 5C).

Discussion

The AMF can not only improve plant health but also enhance resistance against an invasion of plant pathogens. The plant reaches an optimal level of nutrition by the fungal symbionts (Burleigh and Bechmann, 2002) and the AMF play a role in the regulation of plant nutrient transporter genes, which regulated by a feed-back mechanism (Burleigh and Bechmann, 2002). On the other hand, the plant resistance/tolerance against biotic stress including pathogenic bacteria and fungi was enhanced in the AMF colonized plants (Azcón-Aguilar and Barea, 1996; van Driesche and Bellows, 1996). As one of the effective AMF *Glomus intraradices* as well as *G. mosseae* have been reported, which enhance plant resistance against Phytophthora disease in tomato plants (Pozo et al., 2002). In the present study the colonization with *G. intraradices* in cucumber roots triggered resistance against anthracnose disease caused by *C. orbiculare* (Figs. 1 and 2), while *G. mosseae* did not (data not shown).

It has been reported that treatment with BABA showed no direct antimicrobial activity against fungal plant pathogen *Phytophthora infestans* (Cohen, 1994) as well as bacterial pathogen *Pseudomonas parasitica* (Zimmerli et al., 2000). Our results revealed also no direct antifungal effect of BABA on the anthracnose pathogen, *C. orbiculare in vitro* test (data not shown). Nevertheless, the treatment with BABA caused the disease reduction of anthracnose in cucumber indicating an effective resistance induction by BABA (Figs. 1 and 2).

The results in this study showed that the lesion area was decreased by approximately 42% in the plants colonized with *G. intraradices*, whereas about 95% of lesion area was decreased by the application of BABA (Fig. 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 mycorrhiza. Similar results have been observed in our previous study e.g. the pre-treated with plant with promoting rhizobacteria (PGPR). 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 this cytological study we attempted to illustrate the resistance mechanism mediated by colonization with arbuscular mycorrhiza such as *G. intraradices*, and to compare to those of resistance by chemical BABA. Generally, the conidia of *C. orbiculare* germinate on the surface of leaves of cucumber plants under optimal condition of humidity and temperature. The rate of conidial 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 between both resistance induced and non-treated susceptible plants (Figs. 4A and 5A), indicating no role of conidial germination in expressing resistance.

Like some other fungi, the anthracnose fungus forms an appressorium, which is structurally differentiated from a conidium, in order to penetrate the host cell walls. In some plant-pathogen interactions, the formation of appressorium may be enhanced by a certain compound excreted from the host (Hwang and Kolattukudy, 1995; Lee and Dean, 1993). Because anthracnose fungi cannot penetrate into the host cells without formation of appressorium, the plant may acquire resistance against anthracnose 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 colonized with *G. intraradices* (Figs. 4B and 5B). Nevertheless, resistance against cucumber anthracnose was triggered by the colonization with the mycorrhiza (Fig. 2). This result indicates that some resistance mechanisms, other than the suppression of appressorium formation, may be involved in the expression of resistance induced by *G. intraradices*.

Numerous autofluorescent cells were detected at the penetration sites on the leaves of the plants colonized with *G. intraradices* compared to those of untreated control plants at 5 days after challenge inoculation (Fig. 5C). Although it was not significant, the callose formation of *G. intraradices* colonized plants was higher than that of control plants at 3 days after challenge inoculation (Fig. 4C). The autofluorescent 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 (Strömberg and Brishammar, 1993; Kovats et al., 1991b). Similar results were observed in our previous study, in which the callose formation was enhanced on the leaves of cucumber plants pre-inoculated with PGPR (Jeun et al., 2004). In contrast to the mycorrhiza, there was no difference in callose formation between the BABA treated and non-treated plants (Figs. 4C and 5C), indicating the resistance expressing by BABA without the thickening of cell walls forming callose deposit.

On the bases of the results of cytological observations, it is suggested that the callose deposits at the penetration site, one of the defense response of the plant cells against pathogen, may be play an important role for expressing a resistance against anthracnose pathogen in the cucumber plants colonized with *G. intraradices*. In BABA treated plants, however, callose deposits was not important for expressing resistance although the disease severity was more effectively suppressed by BABA. These different resistance expressions may be caused the different protection values by *G. intraradices* colonized plant and BABA treated plant. It could be also involved in 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). To confirm this hypothesis, further investigations are required at the biochemical level.

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