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CUCUMBER LEAF EXTRACT INHIBITS DEVELOPMENT OF CUCUMBER ANTHRACNOSE

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ABSTRACT

The cucumber plant sprayed with the supernatant of cucumber leaf extract both of healthy and diseased plants became to be resistant against *Colletotrichum orbiculare*, the causal agent of cucumber (*Cucumis sativus*) anthracnose. The upper leaves of pre-treated plant showed mild symptom suggesting that the systemic resistance was induced by the treatment of diseased and even healthy leaf extracts. Furthermore, the leaf treated with healthy leaf extract also showed relatively mild symptom suggesting that the leaf extract directly inhibited the fungal infection before the induction of the systemic resistance. The appressorium formation was inhibited in the healthy leaf extract. The healthy cucumber leaf extract may affect doubly on the infection of *C. orbiculare* and be used as a biological control agent against the cucumber anthracnose and/or other diseases.

Key words: Appresorrium formation, biological control, *Colletotricum orbiculare*, *Cucumis sativus*, systemic resistance induction

INTRODUCTION

The biological control (biocontrol) of plant diseases has become popular measure in the control of plant diseases worldwide. Japanese government also has recommended the registration of biocontrol agents as official agrichemicals under the Agricultural Chemicals Control Low and the number of bio-pesticides has been increased gradually in Japan (Anonymous, 2011).

The main mechanisms by which the biocontrol agents work against plant diseases are categorized into three mechanisms namely antimicrobial activity, pre-inhabitance and induced-resistance (Arie, 2007). The antimicrobial activity can be detected relatively easy *in vitro* by culturing the antagonistic microorganism and the target pathogenic organism on opposite side of the medium plate surface to observe the occurrence of growth hindered area between the colonies. As the effect of antimicrobial organism basically depended on the concentration of antimicrobial substances produced by the organism, it is very important to keep high population density. However, it is very difficult to keep a high density of a certain microorganism in the field because of the existence of so many competitive microbes. The pre-inhabitant microbes of the pathogen are the microbes that are treated in relatively large amount and covered the surface of the crops to prevent from the infection by the pathogen. Some strains of *Bacillus subtilis* and *Trichodema* spp. have been registered and used for the control of some diseases of vegetables and rice (Anonymous, 2011; Taguchi *et al*., 2003).

The third is the induced-resistance. The effect of the induced-resistance usually can be detected and/or estimated under field experiment. It is needed large amount of test plants and also long times to find useful isolate that can induce the disease resistance in plant. Kuc *et al*. (1975) succeeded in inducing the systemic resistance in cucumber pre-inoculated with *C.lagenarium* against *Colletotrichum lagenarium* (syn. *C. orbiculare*), the causal agent of cucumber anthracnose. Other workers successfully tested the same *C. lagenarium* strain as a biocontrol agent to protect the plants from bean anthracnose (*C. lindemuthianum*) (Elliston *et al*, 1976a; Elliston *et al*, 1976b) and similar trial had also been conducted by Vakalounakis and Williams (1991). However, the use of such pathogenic isolate is not practical because of its strong pathogenicity against other important crops.

Chai and Doke (1987) succeeded to induce the systemic resistance against *Phytophthora infestans*, the causal agent of potato late blight, by treating the wall component of the pathogen itself suggesting that a part of the hyphae of the pathogen that was not alive could induce the systemic resistance in the host plant. It is very convenient to use the pathogenic strain as a biological control agent rather than the nonpathogenic isolate because it can be found easily.

In this study, the effects of leaf extracts of the healthy and diseased cucumber plants were evaluated in relation with the systemic resistance induction against the infection by *C. orbiculare.*

MATERIALS AND METHODS

Inoculum and Seedling Preparation

Colletotrichum orbiculare isolate CL3, provided by Meiji University, was used in this experiment. The fungus was grown on potato dextrose agar (PDA) slant medium at 25^{o}C for 10 days. The conidia were harvested in 10 ml sterilized distilled water. The concentration of conidia was adjusted to 1.0 x 10^{6} conidia ml^{-1}. The seeds of cucumber (*Cucumis sativus* cv. Sagami-Hanjiro-Fushinari) produced by Takii & Co. Ltd., Japan were sawn individually in a plastic pot (4.5cm in diameter) filled with sterilized soil and grown in the air conditioned greenhouse at 25^{o}C before the experiment. In each experiment, at least 3 seedlings were used for each treatment.

Inoculation Procedure

Conidia suspension (25 - 30 ml per seedling) was sprayed on the whole of 2.5 and 5 leaf stage cucumber seedlings. The inoculated seedlings were kept in humid chamber (almost 100% relative humidity) at 25^{o}C for 1 day and incubated in the air conditioned greenhouse at 25^{o}C. The symptom occurring on the seedling was observed for 5 days after inoculation. If the growth stage affects anthracnose sensitivity strongly, the growth stage of cucumber plant must be strictly uniform in further experiments.

Induction of Systemic Resistance

The first and the second leaves of 2.5 leaf stage cucumber seedlings were pre-inoculated with conidia suspension of isolate CL3 (1.0 x 10^{6} conidia ml^{-1}) and kept in humid chamber for 1 day at 25^{o}C followed by the incubation in the air-conditioned greenhouse at 25^{o}C. Five days after the pre-inoculation, the third, the fourth and the fifth leaves of the seedlings (almost at 5 leaf stage) were inoculated with conidia suspension of isolate CL3 (challenge inoculation). About 5 ml of conidia suspension (1 x 10^{6} conidia ml^{-1}) was sprayed on each leaf. After the challenge inoculation, the plants were kept in the air-conditioned greenhouse at 25^{o}C for 5 days to observe the occurrence of the symptom. The seedlings for controls were sprayed with sterile distilled water.

The second and the third leaves of 4.3 leaf stage cucumber seedlings were pre-inoculated

by spraying the conidia suspension (1.0 x 10^6 conidia ml^{-1}) of CL3. Incubated for 1, 2 or 3 days in the air-conditioned greenhouse at 25°C, challenge inoculation was conducted on the fourth leaves. The controls were pre-treated by spraying with distilled water followed by the challenge inoculation as same as the pre-inoculated plants. The plants tested were kept at 25°C for 1 day in the humid chamber and incubated in air-conditioned greenhouse at 25°C following 4 days to observe the occurrence of the symptom.

Extract Preparation and Application

Ten gram of diseased leaves bearing anthracnose lesions were frozen in liquid nitrogen or deep freezer (-80°C) and macerated with 10 ml of distilled water. The suspension was centrifuged at 8,000 rpm for 10 minutes and the supernatant was collected and used for experiment. As a control, the supernatant of the healthy leaf extract was also prepared by the same manner as the diseased leaves. The first and the second leaves of 2.5 leaf stage cucumber seedlings were sprayed with 1 ml of healthy or diseased leaves supernatants followed by the challenge inoculation with conidia suspension of isolate CL3 (1.0 x 10^6 conidia ml^{-1}) on the third and the fourth leaves 5 days after extracts treatments. The seedlings sprayed with sterile distilled water and challenge-inoculated with isolate CL3 were used as controls. Five days after the challenge inoculation, the number of anthracnose lesions occurring on the challenge-inoculated leaves was counted.

The 2.5 leaf stage cucumber seedlings were pre-treated with the leaf extract of the healthy cucumber plant. The challenge inoculation was conducted 1 or 2 days after the pre-treatment on the first, the second and the third leaves of the pre-treated cucumber seedling to confirm the direct effect of the leaf extract on the pathogen infection. The plants were incubated in the air-conditioned greenhouse at 25°C to count the anthracnose lesions on the pre-treated leaves themselves up to 7 days after the challenge inoculation. The controls were pre-treated with sterile distilled water.

Application of Leaf Extract

The supernatant of the healthy leaf extract prepared by the manner mentioned above was diluted with distilled water to make 1/5 and 1/10 concentrated solutions. Eighty microliter of conidia suspension (more than 5.0 x 10^6 conidia ml^{-1}) was mixed with the same amount of undiluted, 1/5 and 1/10 concentrated solutions to make 1/2, 1/10 and 1/20 concentrated samples containing conidia of CL3, respectively. Each 40 microliter of samples were dropped on the slide glass and incubated at 23°C for 6 hours in a Petri dish and observed under the microscope to count the conidia germinated and the germ tubes with appressoria.

RESULTS

Susceptibility of Cucumber Plant

There was almost no difference in the appearance and the development of anthracnose symptoms occurred on 2.5 leaf stage seedlings and on 5 leaf stage seedlings. In general, the first anthracnose lesion appeared on the inoculated leaf 3 days after inoculation. The brown lesions enlarged along with the time passing up to 5 days after the symptom appearance followed by fusing with each other to make large diseased area on the leaves. As the growth stage did not affect anthracnose sensitivity, the cucumber seedlings at 2.5 to 5 leaf stages were used in this experiment.

Induction of Systemic Resistance

The first and the second leaves of the pre-inoculated seedlings showed severe anthracnose symptom including lesion formation while their upper leaves and the control plants did not show any

disorder 5 days after the pre-inoculation. The pre-inoculated leaves showed the first anthracnose lesion 3 days after inoculation. The lesion number and the size increased along with the time passing by 5 days after pre-inoculation. Some enlarged lesions fused with each other to make large diseased areas on the leaves thereafter.

The challenge inoculation done 1 and 2 days after the pre-inoculation caused very severe symptom on the third, the fourth and the fifth leaves of the control plants and also of the pre-inoculated plants. Those leaves showed almost the same severe symptom as appeared in the pre-inoculated plants. However, the challenge inoculation done 3 days after the pre-inoculation caused very mild symptom. The number of lesions seen on the third, the fourth and the fifth leaves of the pre-inoculated plant was apparently smaller than the controls and the lesion size did not enlarge. The induced resistance could be seen clearly 3 days after pre-inoculation (Fig. 1).

Fig. 1. The symptom appeared on the third leaves of cucumber plant that were taken under challenge inoculation with *Colletotricum orbiculare* 3 days after the pre-inoculation with the same fungus on the first and the second leaves. Left is the leaf of pre-inoculated plant and right is the leaf of control plant that had not been pre-inoculated but sprayed with distilled water. The leaf of pre-inoculated plant showed mild symptom than of control.

Effects of the Leaf Extract on the Anthracnose Development

The third and the fourth leaves of non-sprayed controls began to show symptom from 3 days after the challenge inoculation. The number of lesions 5 days after inoculation on control was 377 lesions while on plants treated with healthy and diseased leaves extract was 134 and 25 lesions respectively (Table 1). In general, the size of lesions appeared on the control plant were larger than those of treated plants. In addition, the leaves of control plants did not fully expand (Fig. 2). The treatments with healthy and diseased leaves extracts obviously induced the systemic resistance on the third and the fourth leaves. Disease development on the upper leaves was inhibited by the leaf extract treatment.

Disease development was also inhibited by the leaf extract treatment directly. The number of lesions on the first and the second leaves reduced considerably irrespective of the duration between the extract treatment and the challenge inoculation. The disease development on the treated leaves was not only inhibited by the induced resistance but also by other components contained in the leaf extract. The effect on the third leaf was relatively unstable compared with the ones on the first and the second leaves where the lesion number on the treated leaves reduced less than 10% of the control (Table 2).

Table 1. Number of anthracnose lesions occurring on the upper leaves of the cucumber plant sprayed with the leaf extract of diseased and healthy cucumber plants [a)]

Pre-treatment	Number of lesions [b)]
Diseased cucumber leaf extract	25
Healthy cucumber leaf extract	134
Control	377

a) The conidia suspension (1.0×10^6 conidia/ml) of cucumber anthracnose fungus (*Colletotrichum orbiculare*) was inoculated on the upper leaves of the plant that was sprayed with different materials including the centrifugal supernatant of macerated diseased or healthy leaf extract 5 days prior to the inoculation. The control was sprayed with distilled water.

b) The number of the lesions was counted at 5 days after the inoculation.

Table 2. Number of anthracnose lesions occurring on the leaf pre-treated with the extract of healthy cucumber leaf followed by challenge inoculation [a)]

Pre-treatment	Days after pre-treatment	Number of lesions [b)]		
		First leaf	Second leaf	Third leaf
Healthy leaf Extract	1	15	3	7
	2	7	6	12
Control	1	166	122	108
	2	171	174	40

a) One or two days after the spraying with healthy cucumber leaf extract, the conidia suspension (2.5×10^5 conidia/ml) of cucumber anthracnose fungus (*Colletotrichum orbiculare*) was inoculated. The control was pre-treated with distilled water.

b) The lesion number was counted 7 days after the inoculation.

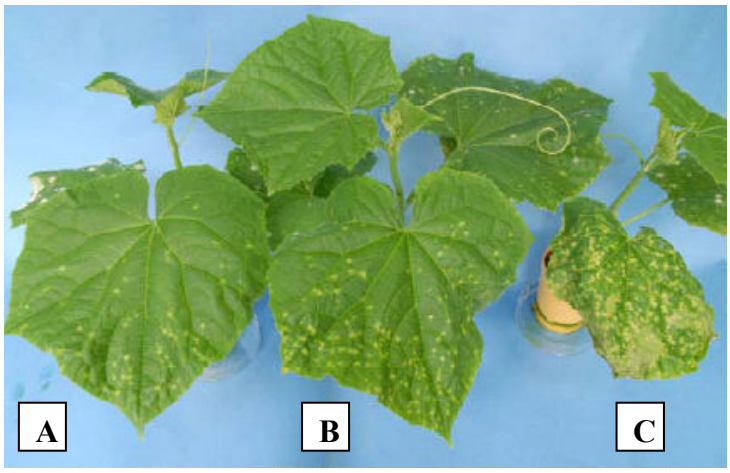

Fig. 2. The symptom appeared on the challenge inoculated third leaves of cucumber seedlings taken under the different pre-treatment on their first and the second leaves. From the left, (A) diseased leaf extract, (B) healthy leaf extract or (C) sterile distilled water was pre-treated 3 days before the challenge inoculation with *Colletotrichum orbiculare*. The symptoms appeared on the pre-treated plants were milder than on the control.

The Effect on Conidia Germination and Appressorium Formation

The germination rate of the conidia was not obviously affected by the extract treatment. Of about 300 conidia observed in each treatment, almost all of the conidia developed the germ tubes normally in every treatment. However, no appressorium was formed in the treatment with 1/2 strength of leaf extract. About 10% and 40% of the germinated conidia formed appressoria in 1/10 and 1/20 strength leaf extract, respectively (Table 3). The appressorium forming ratio was strongly affected with the concentration of the leaf extract. Such inhibition effect on the appressorium formation was also detected on the cucumber cotyledon and true leaf that were treated with the extract of the healthy leaf (Arimoto, unpublished).

Table 3. The inhibitory effect of the extract of healthy cucumber leaf on the conidia germination and the appressorium formation of *Colletotrichum orbiculare* [a)]

	Number of conidia [b)]					
Diluted level	1/2		1/10		1/20	
Observed	300	-	339	-	313	-
Germinated	300	(100)	333	(98)	313	(100)
Forming appressoria	0	(0)	35	(10)	125	(40)

a) The healthy cucumber leaves were frozen by liquid nitrogen and macerated with the same weight of distilled water to make crude sap solutions followed by the centrifugation to make the supernatant. The supernatant was diluted with distilled water to make 1/2, 1/10 and 1/20 concentration samples.

b) Suspended in the samples, the conidia of *Collototrichum orbiculare* CL3 were incubated at 23°C for 6 hours followed by counting the number of conidia germinated and germ tubes with appressoria. The figures within the parentheses are the proportions (%) to the total numbers of conidia observed. All the conidia suspended in distilled water germinated and formed the appressorium in the same condition.

DISCUSSION

We had expected the induction of systemic resistance only through the pre-treatment with the diseased leaf extract that contained some components of plant pathogen. Chai and Doke (1987) already succeeded to elicit the resistant response in the potato plant against *Phytophthora infestans* through the pre-treatment with the components of the pathogen. Our results showed that the treatments with both the healthy and diseased leaves extracts obviously induced the systemic resistance (Table 1). Kubota and Nishi (2006) reported that salicylic acid accumulation could be detected only in the resistance induced plant that was pre-treated with the pathogenic fungus. The contents of free salicylic acid in the fresh healthy leaf and the diseased leaf were 13.3 ng g^{-1} and 188.6 ng g^{-1}, respectively, and of total salicylate compounds were 27.2 ng g^{-1} and 1,850.9 ng g^{-1} as well (Arimoto, unpublished). The resistance induced by the healthy leaf extract in our experiment may not be depended on some unknown substances other than salicylic acid. The phenomenon of the resistance induction seen on cucumber cv. Sagami-Hanjiro-Fushinari was also confirmed on the other 5 cultivars including Sharp One, Tokiwa, Chikanari-Yotsuba, Yotsuba-Kyuuri and Tsuyu-Shirazu that are popular in Japan (Arimoto, unpublished). The resistance induced through by the healthy leaf extract may be common in almost all the cucumber cultivars.

Induced systemic resistance has been applied on the disease control in the commercial field both as biological and chemical measures. The chemicals named probenazole and acibenzolar-S-methyl are used for the control of rice diseases including blast and bacterial blight and of some vegetable diseases, respectively. The induced resistance is generally effective against more than one disease (Arie, 2007). Those chemicals are thought to have no potential chemical resistance on pathogens (Anonymous, 2011). Nonpathogenic isolate of *Fusarium oxysporum* had been registered as a pesticide named “Marukaraito” that protect sweet potato (*Ipomoea batatas*) Fusarium

wilt caused by *F. oxysporum* f. sp. *batatas* through seedling immersion into the bud cell suspension (Ogawa and Komada, 1984). The mechanism of the non-pathogenic isolate was systemic resistance induction (Ogawa and Komada, 1986) and has contributed to develop several other biocontrol agents (Ichikawa, 2002; Katsube and Akasaka, 1997; Tezuka and Makino, 1991). However, nonpathogenic isolate cannot be found easily. There is no valid procedure to select the useful nonpathogenic isolate except to carry out the field experiment or at least the pot experiment. On the other hand, it is very easy to prepare the supernatant of the healthy leaf extract that can induce the systemic resistance only by spraying. According to our results, it is not necessary to find any particular isolate or material.

Although we succeeded in inducing the systemic resistance by treatment with the healthy leaf extract, how the resistance was induced was unclear. P robenazole, a synthetic resistance-inducing chemical, was known its mechanism to induce resistance precisely (Arie, 2007). So we must investigate the mechanism of the resistance induction by the leaf extract. The mechanism of the resistance induction shown in this study may not be the same as the procedure related with salicylic acid that is accumulated only in the diseased or inoculated leaf (Kubota and Nishi, 2006). It is suggested that such resistance inducing substances in our experiments are originally contained in the plant or originated through the maceration that may be only one physical procedure to create the new substances.

On the other hand, the leaves pre-treated with the leaf extract showed the protective response against the inoculation of *C. orbiculare* almost just after the pre-treatment (Table 2). Very small numbers of anthracnose lesions were seen on the first and the second leaves irrespective of the duration between the pre-treatment and the challenge inoculation. The third leaf showed also the protective but unstable response suggesting the effect of insufficient leaf expanding conditions at the pre-treatment and/or the challenge inoculation. This protective response seemed to be derived from the direct effect by the leaf extract because the systemic resistance induction could be seen at least three days after the pre-treatment (Kuc *et al*, 1975; Elliston *et al*, 1976a and 1976b). There must be some components that affect the infection procedure of *C. orbiculare*. Although the appresorrium formation was inhibited in the solution of the healthy leaf extract in spite of that no inhibition effect could be seen on the conidia germination (Table 3). T he inhibition effect was also detected on the cucumber cotyledon and true leaf that were treated with the healthy leaf extract (Arimoto, unpublished). So the leaf extract may operate doubly in resistance induction on the plant and in direct effect on the pathogen.

Under Agricultural Chemicals Control Act in Japan at present, we cannot use any substances that are not registered as pesticides except the specific protective materials including vinegar (acetic acid), baking soda (sodium bicarbonate) and domestic natural enemy to protect the crop diseases. If we intend to use leaf extract for disease control in the field commonly in Japan, the extract should be permitted as a specific protective material legally. The healthy leaf extract is hopeful to be prevailed as a specific protective material because of its disease protecting effect and also of the safety from the plant pathogenicity and for the environment.

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REFERENCES

Anonymous 2011. Guidebook of Registered Pesticides in Japan. Japan Plant Protection Association, Tokyo, Japan. 689p.

Arie, T. 2007. Induced systemic resistance in plants. Brain Techno News. 121: 10-13.

Chai, H. B. and Doke, N. 1987. Systemic activation of O-2 generating reaction, superoxide dismutase, and peroxidase in potato plants in relation to induction of systemic resistance to *Phytophthora infestans*. Ann. Phytopathol. Soc. Jpn. 53:585-590.

Elliston, J., Kuc, J. and Williams, E. B. 1976a. Protection of *Phaseolus vulgaris* against anthracnose by *Colletotrichum* species nonpathogenic to bean. Phytopath. Z. 86:117-126.

Elliston, J., Kuc, J. and Williams, E. B. 1976b. A comparative study of the development of compatible, incompatible and induced incompatible interaction between *Colletotrichum* spp. and *Phaseolus vulgaris*. Phytopath. Z. 87:289-303.

Ichikawa, K. 2002. Biological control of cymbidium yellow spot by a weakly virulent *Fusarium* sp. strain HPF-1. Ann. Phytopathol. Soc. Jpn. 68:21-27.

Katsube, K. and Akasaka, Y. 1997. Control of *Fusarium* wilt of spinach by transplanting seedlings pretreated with non-pathogenic *Fusarium oxysporum*. Ann. Phytopathol. Soc. Jpn. 63:389-394. (Japanese)

Kubota, M. and Nishi, K. 2006. Salicylic acid accumulation in the roots and hypocotyls after inoculation of cucumber leaves with *Colletotrichum lagenarium*. Jour. Pl. Physiol. 163: 1111-1117.

Kuc, J., Shockley, G. and Kearney, K. 1975. Protection of cucumber against *Colletotrichum lagenarium* by *Colletotrichum lagenarium*. Physiol. Pl. Pathol. 7: 191-199.

Ogawa, K. and Komada, H. 1984. Biological control of Fusarium wilt of sweet potato by non-pathogenic *Fusarium oxysporum*. Ann. Phytopathol. Soc. Jpn. 50:1-9.

Ogawa, K. and Komada, H. 1986. Induction of systemic resistance against Fusarium wilt of sweet potato by non-pathogenic *Fusarium oxysporum*. Ann. Phytopathol. Soc. Jpn. 52:15-21.

Taguchi, Y., Hyakumachi, M., Horinouchi, Y. and Kawane, F. 2003. Biocontrol of rice blast by *Bacillus subtilis* strain IK-1080. Ann. Phytopathol. Soc. Jpn. 69: 85-93.

Tezuka, N. and Makino, T. 1991. Biological control of Fusarium wilt of strawberry by nonpathogenic *Fusarium oxysporum* isolated from strawberry. Ann. Phytopathol. Soc. Jpn. 57:506-511.

Vakalounakis, D. J. and Williams, P. H. 1991. A cotyledon double inoculation technique for evaluating resistance to anthracnose (*Colletotrichum orbiculare*) and scab (*Cladosporium cucumerinum*) in cucumber. Ann. Appl. Biol. 118: 273-282.