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Bacterial Isolate MRL3-1 from Jeju Island Induced Systemic Resistance in Crop Plants against Plant Diseases Caused by *Colletotrichum orbiculare* and *Phytophthora infestans*

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제주에서 분리한 길항근권세균 MRL3-1의 *Colletotrichum orbiculare* 및 *Phytophthora infestans*에 의한 작물병에 대한 전신적 유조저항성

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ABSTRACT : Bacterial isolate MRL3-1 showing antifungal activity in vitro test against plant pathogens was tested the ability of resistance induction by bioassay. The pre-treatment with MRL3-1 at the concentration 1.0 x 108cfu/ml in the rhizosphere of cucumber plants could induce systemic resistance in the aerial part of cucumber plants against anthracnose caused by *Colletotrichum orbiculare*. The lower concentration of the bacterial isolate resulted in the decrease of the ability inducing systemic resistance after challenge inoculation with *C. orbiculare*. Similarly the pre-treatment with MRL3-1 could trigger the systemic resistance against late blight disease caused by *Phytophthora infestans* in tomato plants. As a positive control the treatment with DL-3 amino butyric acid caused a remarkable reduction of disease severity whereas the lesions on the leaves of untreated plants developed apparently after the fungal inoculation. From these results it was discussed that disease control using the bacterial isolate inducing systemic resistance in the field where chemical application is forbid.

Keywords: **Plant growth promoting rhizobacteria (PGPR), Cucumber, Antifungal activity, Induced systemic resistance, *Colletotrichum orbiculare***

INTRODUCTION

One of the strategy for plant protection is using crops expressing a systemic induced resistance, which can be triggered in the plant by pre-inoculation with plant growth promoting rhizobacteria (PGPR) (van Loon et al. 1998a). ThePGPR-mediated resistance has been defined as induced systemic resistance (ISR) (van Loon et al. 1998a). The treatment with PGPR may enhance the plant own defense mechanism, which result in expression of systemic resistance on the aerialpart of the plants (van Loon et al. 1998a). Moreover, for

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expression of resistance the PGPR need not be contact with plant pathogens and the PGPR can grow well in the rhizosphere. Therefore, using PGPR is one of the possible strategies for plant protection in the field (van Loon et al. 1998a).

PGPR enhance the growth of plant and most of them have a direct anti-fungal activity, whereas plant pathogens or chemical activators have not (Jeun et al. 2001). Furthermore, ISR can be triggered in the plants without an accumulation of salicylic acid (SA), which is obligatorily necessary for expression of systemic acquired resistance (SAR) by pathogen of some activators (Pieterse et al. 1996). Using transgenic plants, they have revealed that the signal mechanism of ISR is different to those of SAR. They were also shown that jasmonic acid and ethylene might involve to the resistance signal in arabidopsis after pre-inoculated with PGPR. The resistance expression may also be different between SAR and ISR (Sticher et al. 1997; van Loon et al. 1998a). For example, the pathogenesis related protein (PR-protein) have been known as one of the important resistance factors for expression of SAR in many plants (Sticher et al. 1997), whereas the PR-protein was not found in arabidopsis plants expressing ISR (van Loon et al. 1998a).

In the previous study bacterial strains were isolated from the rhizosphere of the plant growing in Jeju and the anti-fungal activities of the isolates against several plant pathogens were tested and selected (Lee et al. 2003). In this study aim for the selection of an effective ISR inducing agent, efficacy of the selected isolate MRL3-1 for induced systemic resistance was tested by inoculation with *C. orbiculare* in cucumber or *P. infestans* in tomato plants pre-treated with the suspension of MRL3-1.

MATERIALS AND METHODS

Plants

Cucumber seeds (Cucumis sativus L. cv. Eun Sung) were sown in a plastic sowing plate (72 holes, 4cm diameter each) filled with commercial soil (Choroc Nala®, Bokyung Nongsang, Korea) containing 10 % of perlite (Parat® Sam Son, Korea). Cucumber seedlings were grown in the greenhouse maintaining 28℃ at daytime and 25 °C at night. Plants were watered once every day about 30ml per plants.

Seedlings of tomato plants (Lycopersicum esculentum Mill cv. Super Dotaernag) were grown in plastic pots (Ø 8 cm) filled with a sterile soil - sand mixture (soil : sand, 3 : 1, v / v) in a greenhouse at 25 °C during the day and at 20 °C during the night. Plants of the 4-leaf stage were used for soil treatment with suspension of MRL3-1.

Treatment of bacterial isolates in the plants for triggering of ISR

The bacterial isolate MRL3-1 showing anti-fungal effect was selected for test triggering of ISR in plants. The strain was grown in tryptic soy agar at 28 °C for 24 h. The concentration of bacterial strain was adjusted to be1.0 x 10^6 colony forming unit (cfu) / mlaccording to the methods described by Park and Kloepper (2000). Thirty ml of the bacterial suspension was soil-drenched per cucumber and tomato plants 7 days before challenge inoculation with *C. orbiculare* and *P. infestans*, respectively. For negative control,

H2O was applied on the cucumber plants instead of the bacterial suspension.One mM of DL-3-amino butyric acid(BABA) wasdrenched in the soil for triggering SAR 3 days before the fungal inoculation. The BABA treated plants were used as a positive control.

Challenge inoculation with anthracnose pathogen

Anthracnose pathogen *C. 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 than the fungal conidia were harvested by using a brush. The conidial concentration were adjusted to be 2.5 x 10^5 conidia / ml. This conidial suspension with 100μl/L tween 20, which enhances the adhesion of conidia on leaf surface, was used as inoculum for challenge inoculation on cucumber leaves.

P. infestans (Mont.) de Bary was grown on V8 agar medium for 7 days at 15 °C to induce sporangium formation. For the initiation of zoospore release from sporangia 10 ml H2O bidest 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 and the concentration of the zoospores was adjusted to 1.5×10^5 zoospores /ml for the inoculation of tomato plants.

The conidial suspension of *C. orbiculare* or suspension containing zoospores of *P. infestans* was sprayed on the aerial cucumber leaves 5 days after the treatment with the suspension of MRL3-1. The plants inoculated with the fungal suspension 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.

Evaluation of resistance

Disease severity caused by *C. orbiculare* on the inoculated leaves was determined 7 days after challenge-inoculation. Late blight caused by *P. infestans* was determined at 5, 8 and 11 days after challenge inoculation. Both disease severities were established by visual estimating the leaf area occupied by lesion. 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. The data of disease severity caused by *C. orbiculare* and *P. infestans* were statistically analyzed using Duncan's multiple range test and a paired t-test, respectively, in the bacterial isolatepre-treated and the non-treated plants.

RESULTS

The bacterial isolate MRL3-1 showing antifunal activity in vitro test was selected in order to determine their resistance efficiencies against disease caused by *C. orbiculare* in cucumber plants and by *P. infestans* in tomato plants, respectively. The lesion caused by anthracnose was well developed in the leaves of cucumber plants untreated control after inoculation with *Colletotrichum orbiculare* and at 5 days the disease severity was about 70 % (Fig. 3). When the bacterial isolate MRL3-1 was pre-treated with the high concentration (1.0 x 10^8 cfu / ml), the lesion development was limited at theinfected sites on the leaves (Fig. 1 and 2). Also the disease was significantly reduced

compared to the control (Fig. 3) resulting in about 55 % of disease protection (Table 1). Similarly, the pre-treatment of MRL3-1 with both lower concentration at 1.0 x 10^7 cfu / ml and 1.0 x 10^6 cfu / ml could decrease the disease severity and resulted in protection rate of the disease at 47.6 and 36.6%, respectively (Table 1). The treatment with DL-3-amino butyric acid, which was used as a positive control, caused the remarkable reduction of disease severity and the high protection against anthracnose at the concentration of 1 mM (Fig. 3 and Table 1). The statistic analysis showed that the disease severity in the cucumber leaves MRL3-1 pre-treated was significantly different from that of untreated control (Table 1).

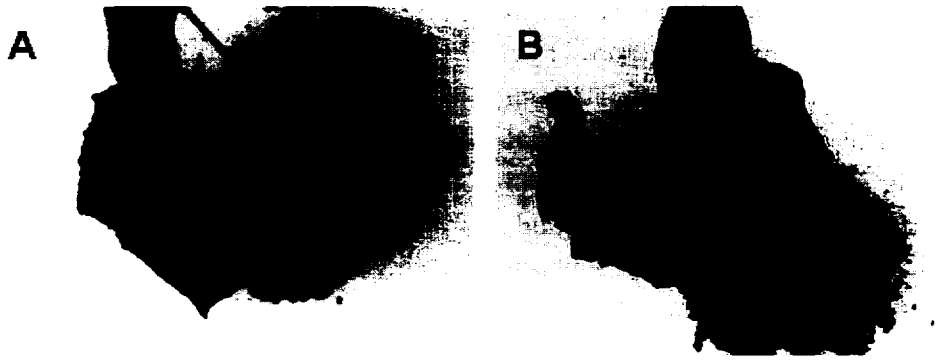

Fig. 1: Induction of systemically induced resistance in cucumber plants against anthracnose disease 7 days after inoculation with *Colletotrichum orbiculare* (1.0 x 10^5 conidia / ml). The presented plants were pre-treated with 30 ml of bacterial suspension of MRL3-1 (1.0 x 10^8 cfu/ml) (A) and untreated control (B) at 5 days before the challenge inoculation

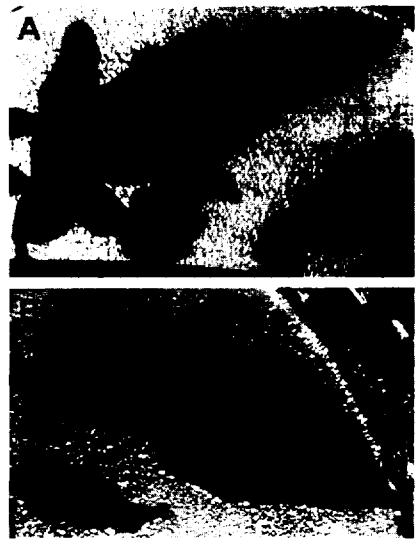

Fig. 2: Induction of systemically induced resistance in tomato plants against late blight disease 7 days after inoculation with *Phytophthora infestans* (1.5 x 10^5 zoospores / ml). The presented plants were pre-treated with 30 ml of bacterial suspension of MRL3-1 (1.0 x 10^8cfu/ml) (A) and untreated control (B) at 5 days before the challenge inoculation

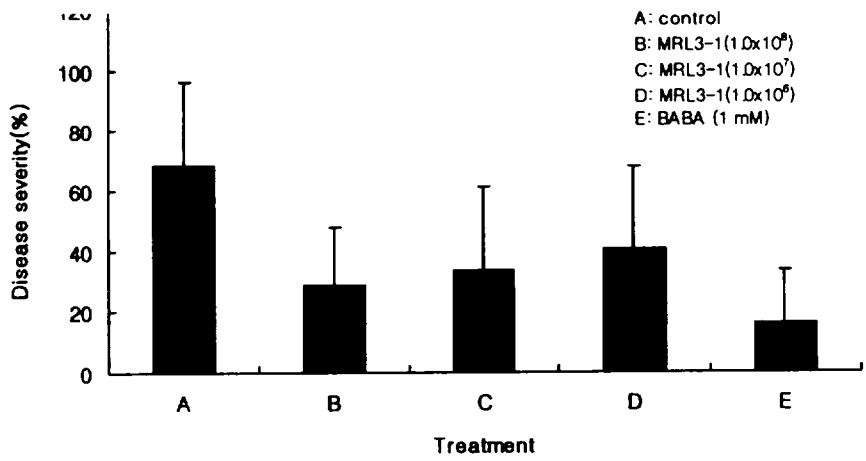

Fig. 3: Disease severity on the leaves of cucumber plants pre-treated with different concentration of bacterial isolate MRL3-1 and non-treated after inoculation with *C. orbiculare* (2.5 x 10^5 conidia/ml). The disease severities were measured at 7 days after challenge inoculation. The verticalbars indicate the standard deviation of the three separated experiments each containing 6 plants per treatment

Table 1. Protection rate and duncan's multiple range test of diseased cucumber leaves treated with different concentration of MRL3-1 or BABA

	Concentration of MRL3-1 (cfu/ml)				BABA
	0	1.0 x 10^8	1.0 x 10^7	1.0 x 10^6	1mM
Protection rate (%)[a]	-	55.1	47.9	36.6	74.8
DMRT[b]	a	bc	b	b	c

[a]Protection rate(%)=(1-(disease severity of treated/disease severity of control))×100[b]
Duncan's multiple range test

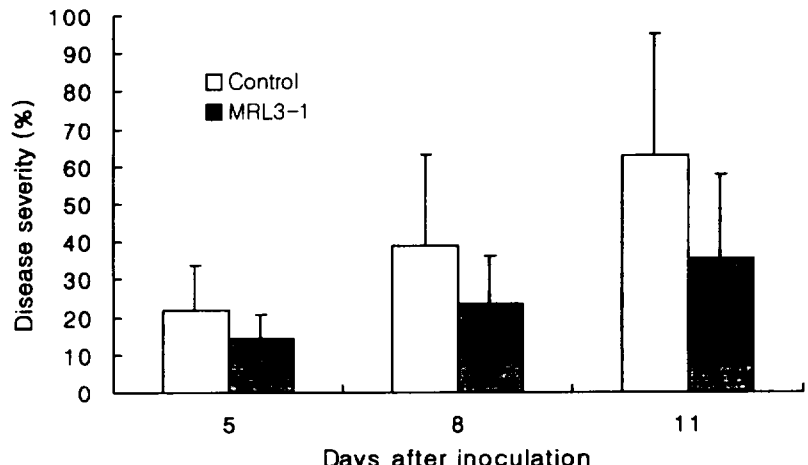

Fig. 4: Disease severity on the leaves of tomato plants pre-treated with MRL3-1 (1.0 x 10^8 cfu/ml) and non-treated at different days after inoculation with *P. infestans* (1.5 x 10^5 spores/ml). The verticalbars indicate the standard deviation of the three separated experiments each containing 6 plants per treatment

Table 2. Protection rate and t-test of diseased cucumber leaves treated with MRL3-1 at different days after fungal inoculation

	Days after inoculation		
	5	8	11
Protection rate (%)[a]	33.9	39.7	43.6
t-test[b]	*	*	**

[a]Protection rate(%) = (1-(disease severity of treated/disease severity of control)) x 100
[b]* = significant at the 5 % probability level; ** = significant at the 1 % probability level

The lesion caused by late blight was well developed in the untreated tomato plants, which reached about 70 % at 11 days after inoculation with *P. infestans* (Fig. 4). Similarly in the leaves of the tomato plants, which the bacterial isolate MRL3-1 was pre-treated, the lesion development was limited (Fig. 4). The protection rate was increased by the inoculation time, which was 33.9 % at 5 days and 43.6 % at 11 days after inoculation (Table 2). The disease severities determined were significantly reduced at 5, 8, 11 days after inoculation (Table 2).

DISCUSSION

Using microorganisms for disease control has been considered for many years because this strategy results in the reduction of chemical application. However, using the antagonistic microorganisms to control of plant diseases has not been always successful in the field, new strategy of the biological control such as using crop plants expressing an induced systemic resistance (ISR) has been looking for control plant diseases (van Loon et al. 1998a). It may be resulted in reduction of chemical application in the field. In this study to select an effective microorganism inducing ISR against plant diseases, the selected rhizobacteria showing antifungal activity weretested with cucumber-anthracnose and late blight-tomato plants interaction systems.

In our study ISR could not be effectively-triggered when the bacterial isolate was pre-treated with high concentration (Fig 2). Similarly the treatments with lower concentrations of the isolate were resulted in the significant reduction of disease severity (Fig. 3). However, it seems to be a certain concentrationof PGPR to begin triggering of ISR in plants. Some PGPR strains such as Serratia marcescens or Pseu-domonas 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. 2004), which has been studied in details (Sticher et al. 1997). In contrast to SAR, some PGPR strains mediating systemic resistancehave direct antifungal activity. In our previous study both bacterial isolates showed direct antifungal effect in vitro test (Lee et al. 2003). Another mechanism of expression of ISR 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, too. The other resistance mechanisms of ISR, however, 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). In this study BABA could effectively induce systemic resistance showing as a positive control (Fig. 1 and 2). Also untreated plants as a negative control showed a high severity compared with the treated plants (Fig. 1 and 2). These controls clearly revealed that the pre-treatments of both bacterial isolates with a certain concentration could induce systemic resistance.

In summary, the bacterial isolate MRL3-1 could trigger ISR in cucumber plants against anthracnose as well as in tomato plants against late blight.Although the ISR by the isolate was not higher compared to those of SAR by BABA, it is suggested that the protection by using microorganism may be usefulin the field or a certain condition, for example, where chemical application is forbid. For this purpose, more research concerning ISR should be carried out.

적 요

식물 병원균에 대한 in vitro 상에서 항균효과가 있는 길항근권 세균 MRL3-1에 대한 저항성 유도 능력을 생물 검정을 통하여 조사하였다. MRL3-1을 1.0×10^8cfu/ml 농도로 오이식물의 근권에 처리하였더니 *Colletotrichum orbiculare*에 의한 오이탄저병에 대해 지상부에서 전신적 저항성이 나타났다. 이 길항근권 세균을 저농도로 처리하면 병원균 접종 후 전신적 저항성 유도 능력이 다소 떨어졌다. 또한 MRL3-1은 토마토에서도 전처리를 하면 *Phytophthora infestans*에 의한 역병에 대해서도 전신적 저항성을 나타낼 수 있었다. 긍정적 비교구로 DL-3-amino butyric acid를 처리한 식물에서는 병 감염율이 확연히 감소하였는데 비하여 부정적 비교구인 무처리한 식물에서는 상당한 병반 진전이 관찰되었다. 이들 결과를 통하여 길항근권세균을 이용한 식물병 방제 수단이 농약사용이 제한된 포장에서 유용하리라 생각된다.

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