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L-Alanine Augments Rhizobacteria-Induced Systemic Resistance in Cucumber

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ABSTRACT. *Bacillus vallismortis* strain EXTN-1 is a proven biotic elicitor of systemic resistance in many crops against various pathogens. L-Alanine (Ala) was tested in cucumber as a chemical elicitor of induced systemic resistance (ISR) against *Colletotrichum orbiculare*. In the greenhouse, both Ala and EXTN-1 induced significant levels of disease suppression in cucumber against anthracnose. When cucumber plants were treated with EXTN-1 and Ala together, augmentative disease suppression was observed. Experiments with transgenic tobacco plants carrying pathogenesis-related genes fused with the β-glucuronidase (GUS) reported gene (PR-1a::GUS & PDF 1.2::GUS) showed an enhanced activation of both PR-1a and PDF 1.2 genes upon combined treatment with Ala and EXTN-1. RT-PCR analysis with transgenic (PR-1a or PDF 1.2 over expressing) *Arabidopsis* plant showed more enhanced expression of resistance genes PR-1a and PDF 1.2 upon combined treatment with Ala and EXTN-1 than either alone. An augmentative ISR effect, when the bacterial elicitor and chemical elicitor were combined together, was confirmed.

Abbreviations

Ala	L-alanine	Jas	jasmonic acid (2-(*cis*-2-pentenyl)-3-carboxymethylcyclopentanone)
BTH	2,1,3-benzothiadiazole	RT-PCR	reverse transcriptase polymerase chain reaction
GUS	β-glucuronidase	Sal	salicylic acid (2-hydroxybenzoic acid)
ISR	induced systemic resistance		

Plants develop an enhanced defensive capacity against a broad spectrum of plant pathogens after colonization of the roots by certain strains of microorganisms. Research on these lines has led to the identification of several species of microorganisms with strong activity, microbial components and also several chemical elicitors with similar effects. Many of these have been already commercialized and are extensively in use. The widely investigated chemical compounds are 2,6-dichloroisonicotinic acid, BTH, methyl jasmonate and probenazole (Staub *et al*. 1993; Lawton *et al*. 1996; Epple *et al*. 1997; Yoshioka *et al*. 2001). Chen *et al*. (2006) observed that Ala activated a programmed cell death response in *Vitis labrusca* cells, in contrast to other amino acids. The programmed cell death was reported to be accompanied by coordinated activation of phenylalanine–ammonia lyase, cinnamic-acid 4-hydroxylase, and stilbene synthase gene expression, as well as by the accumulation of stilbenes and other phenolic compounds (Chen *et al*. 2006). Monselise *et al*. (2003) proposed that Ala is a universal first-stress signal expressed by cells. It would be advantageous if the systemic resistance offered by a bacterial strain to the host plant could be enhanced by a chemical elicitor.

A strain of plant-growth-promoting rhizobacterium, *Bacillus vallismortis* EXTN-1, has been reported to induce multispectral wide-range systemic resistance and growth promotion in several crops (Park *et al*. 2000, 2001, 2006*a*,*b*). EXTN-1-mediated hypersensitive response, oxidative burst and enhanced vascular lignification have been documented (Ahn *et al*. 2002; Jeun *et al*. 2001). Furthermore, strain EXTN-1 was shown to perform a broad spectrum of disease-controlling activity against viral, bacterial, and fungal plant pathogens comprising cucumber mosaic virus, tobacco mosaic virus, potato virus Y, *Pseudomonas syringae* pv. *lacrymans*, *Ralstonia solanacearum*, *Colletotrichum orbiculare*, *Magnaporthe grisea*, and *Fusarium oxysporum* (Park *et al*. 2001, 2006*a*).

An effort was made to enhance the EXTN-1-mediated induced systemic resistance with a chemical elicitor, Ala, in greenhouse studies with cucumber–*C. orbiculare* pathosystem. In parallel, molecular tools were employed to follow the activation of defense genes in transgenic tobacco and *Arabidopsis* plants.

MATERIALS AND METHODS

Microorganisms and plants. Bacillus vallismortis strain EXTN-1 was originally isolated from rhizosphere of red-pepper plant. The long-term preservation of the strain was done in glycerol at –80 °C. The anthracnose pathogen, *C. orbiculare*, was used for challenge inoculation of cucumber plant cv. Eunsung. EXTN-1 was multiplied on tryptic soy agar plates; *C. orbiculare* was grown on green bean agar at 25 °C. Ala was obtained from *Sigma* (USA). Transgenic (PR-1a::GUS, PDF 1.2::GUS) tobacco plants and transgenic (PR-1a/PDF 1.2 over expressing) *Arabidopsis* plants (Mitter *et al.* 1998) were obtained from the *Ohio State University Stock Center, Ohio State University* (Columbus, USA) and cultivated in a growth chamber at set temperature (20–24 °C) and relative humidity 65 %. All experiments were done as triplicates, data being statistically analyzed with ANOVA. Means were compared with LSD at $p = 0.05$.

Greenhouse experiments. Cucumber seeds were sown in 100-mm diameter plastic pots filled with a commercial soilless mix (TKS 2; *Floragard*) containing 10 % of perlite. At the two-leaf stage, the seedlings (one each) were transplanted to fresh cups, watered daily and fertilized weekly with 1 % Wuxal Super (6 : 2 : 3; *Aglukon*; Germany). Appropriate temperature and humidity were maintained in the green house during the experiment.

Cotyledons of 2-week-old cucumber plants were infiltrated with a cell suspension (10^7 CFU/mL) of EXTN-1 using a needle-less syringe. Ala was infiltrated at 100 ppm concentration in a similar way. The positive control was treated with 0.1 mmol/L BTH. One week later, the elicitor-treated plants were challenge-inoculated with *C. orbiculare* (the anthracnose fungus). A spore suspension (2×10^5 spores per mL) of the plate culture was prepared in sterile water. Spore suspension was sprayed onto the 3-week-old plants till runoff with 200 ppm Silwett L-77 (*van Meeuwen Chemicals BV*; The Netherlands) and kept in wet chamber for 1 d at 25 °C. One week later, lesion count per plant was recorded.

Defense gene (PR-1a and PDF 1.2) expression experiments in transgenic tobacco. Leaf infiltration of indicator tobacco plants were carried out with Ala concentration of 1, 10, 100 and 250 ppm using the same technique as *above*. The plants were also infiltrated with EXTN-1 (10^7 CFU/mL) and a combined application of EXTN-1 (10^7 CFU/mL) and Ala (100 ppm). Defense gene (PR-1a and PDF 1.2) expression was measured in terms of GUS activity (Table I); it was determined in the individual leaflets 7 d after infiltration by using a fluorometric assay (Jefferson 1987; Park and Kloepper 2000). GUS activity was determined from the rate of 4-methylumbelliferone produced from 4-methylumbelliferyl glucuronoside as substrate. Four replications of each treatment were maintained, the means being compared.

Table I. The GUS activity[a] (µmol 4-methylumbelliferone per 10 mg fresh mass per h) indicating the activation of PR-1a-GUS promoter by the inducing agents in PR-1a::GUS transgenic tobacco

Treatment		PR-1a-GUS	PDF 1.2-GUS
Control[b]		0.461	2.17
Ala	250 ppm	32.2	2.33
	100 ppm	27.2	2.55
	10 ppm	19.2	2.48
	1 ppm	0.528	2.37
EXTN-1	10^7 CFU	63.8	5.25
EXTN-1 +Ala	10^7 CFU 100 ppm	121.3	18.1
LSD[c]		7.67	2.48

[a]Determined from the rate of 4-methylumbelliferone produced from 4-methylumbelliferyl glucuronoside as substrate; a randomized complete block as quadruplicates per treatment.
[b]Sterile water. [c]$p = 0.05$.

RT-PCR. The seeds of *Arabidopsis thaliana* wild type (Col-0) obtained from the *Ohio State University Stock Center* were surface-sterilized (2 min, 70 % ethanol soaking followed by a 20-min 1 % sodium hypochlorite soaking), rinsed 4× in sterile, distilled water, placed on Petri dishes containing half-strength Murashige and Skoog salt medium (*Gibco–BRL*) containing 0.8 % agar and 1.5 % sucrose, adjusted to pH 5.7, and vernalized for 2 d at 4 °C in the absence of light. Seedlings then were placed in growth cabinets set to a 12-h-light/12-h-dark cycle under 40-W fluorescent lights; the temperature was maintained at 22 ± 1 °C with a relative humidity of 50–60 %. Two-week-old seedlings were transferred to 60 mL pots containing a potting soil mixture that had been autoclaved 2× for 1 h with a 1-d interval. Plants were cultivated in a growth chamber with a 9-h-day (200 $\mu E\ m^{-2}\ s^{-1}$ at 24 °C) and a 15-h-night (20 °C) cycle at 70 % relative humidity. Plants were watered on alternate days and once a week supplied with a modified half-strength Hoagland's nutrient solution (Pieterse *et al.* 1996).

After two weeks, the seedlings were infiltrated with cell suspension of EXTN-1, Ala and a suspension of EXTN-1 and Ala together as separate treatments. Infiltration of 0.1 mmol/L of BTH and sterile water served as a positive and negative control, respectively. Total RNA was isolated from leaf tissues after 12-h infiltration using TRIzol reagent (*Invitrogen*, USA) according to the manufacturer's instructions. RNA sam-

ples (1 μg per reaction) were reverse-transcribed into cDNAs by AMV reverse transcriptase according to the manufacturer's instructions (*Promega*, USA). RT-PCR was performed according to Kishimoto *et al*. (2005) with Ex *Taq* polymerase (*Takara Biomedicals*, Japan). The reaction mixture contained 0.1 μg of cDNA, 10 pmol each of forward and reverse primers, 250 nmol dNTPs, and 0.5 U of Ex *Taq* polymerse in 20 μL of buffer solution. The PCR was carried out in a *MJ Research* (USA) PTC-100 Thermal Cycler: 94 °C for 5 min followed by 94 °C for 1 min, 57 °C for 1 min for 25 cycles, and final 72 °C extension for 10 min. Primers for the defense genes were

PDF 1.2 (plant defensin, antifungal activity)
F – 5′-TGC GGT AAC ACC GAA CCA TAC-3′
R – 5′-CGA CAG TTG CAT TGG TCC TCT-3′

PR-1a (pathogenesis-related protein)
F – 5′-AAC CGC CAA AAG CAA ACG CA-3′
R – 5′-TCA CGG AGG CAC AAC CAA GTC-3′

Amplified PCR products were analyzed with 1.2 % agarose gel and gels were documented using LAS-3000 (*Fuji Photo Film*, Japan). In order to ascertain that RT-PCR was carried out with the same quality of template RNA in all samples, the level of the house-keeping gene (β-actin) was checked.

RESULTS

Green-house experiments. Treatment with Ala or EXTN-1 alone brought about considerable disease suppression in the challenge-inoculated cucumber plants. Percentage of the disease severity (expressed as the number of lesions) after challenge inoculation was 31 % for Ala-infiltrated plants and 17 % with EXTN-1 treatment. The simultaneous infiltration of Ala and EXTN-1 suppressed significantly more the disease development (only 7 % disease severity) in comparison with either of the treatments alone (Table II). The difference showed an augmentative effect on ISR when treatment with EXTN-1 was combined with Ala.

Defence gene (PR-1a and PDF 1.2) expression in transgenic tobacco. In order to understand the molecular ISR effect, Ala and EXTN-1 were infiltrated in transgenic (PR-1a::GUS & PDF 1.2::GUS) tobacco plants for induction of the PR-1a and PDF 1.2 genes. The result revealed that as little as 10 ppm of Ala could enhance the PR-1a gene expression to a significant level (Table I). Increase in the GUS activity was observed for increase in Ala concentration of 1–100 ppm but the difference between 100 and 250 ppm was not significant. Infiltration with EXTN-1 resulted in significant induction of both defense genes. However, the induction was much higher with PR-1a than with PDF 1.2. No significant over-expression of PDF 1.2 with application of Ala alone was observed, but it was elicited with EXTN-1 alone. Even though Ala did not induce a significant PDF 1.2 expression, there was a significant augmentative expression of the gene when the plants received a combined infiltration of both the inducers together. On the other hand, the augmentative effect on GUS activity upon combined application was not additive.

Table II. Disease severity (%) after various elicitor treatments ($p = 0.05$)

Elicitor	L-Ala	EXTN-1	L-Ala+EXTN-1	BTH
%	31	17	7	2

RT-PCR analysis for the induced expression of defence gene transcripts in Arabidopsis was carried out to confirm the activation of the defense genes, *viz*. PR-1a and PDF 1.2 with the above elicitors. Both Ala and EXTN-1 induced the PR-1a gene transcripts in transgenic *Arabidopsis* (Fig. 1). BTH in the positive con trol activated the PR-1a gene but not the PDF 1.2 one. Ala activated the defence gene expression for PR-1a and there was no significant activation of PDF 1.2. The activation of PR-1a was higher with EXTN-1 than with Ala but it was even higher with combined application of EXTN-1 and Ala. In case of PDF 1.2, there was

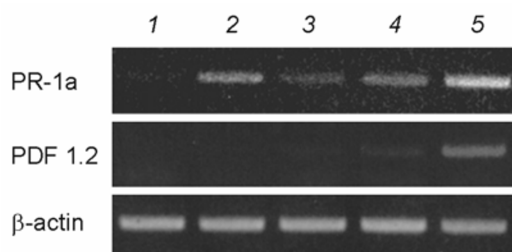

Fig. 1. RT-PCR analysis for the defense gene transcripts induced by the elicitors; *1* – control, *2* – BTH, *3* – L-Ala, *4* – EXTN-1, *5* – 100 ppm of L-Ala + EXTN-1); actin (control) was at nearly the same level in all samples.

significant induction only with EXTN-1, but not with Ala. Even with PDF 1.2, there was an augmentative effect with combined application of both the elicitors. It confirmed the enhanced bacterial ISR, with the chemical elicitor, Ala.

DISCUSSION

Induction of disease resistance following treatment with active microbial and chemical inducers has been reported to provide protection against invasion of pathogens in several plant species (van Loon *et al*. 1998). ISR by rhizobacteria has been proved against several bacterial, fungal and viral plant diseases (Leeman *et al*. 1995). The enhancement of bacterial (EXTN-1) ISR in cucumber with Ala is discussed.

B. vallismortis strain EXTN-1 is a member of efficient plant-growth-promoting rhizobacteria that improved growth and vigor of cucumber and tobacco plants as well as resistance against various pathogens (Park *et al*. 2001, 2006*a*,*b*). One of the mechanisms by which EXTN-1 brings about disease suppression was found to be by the induction of systemic resistance to the host plant (Ahn *et al*. 2002). This notion is supported by the findings that treatment of wild-type *A*. *thaliana* Col-0 with EXTN-1 resulted in simultaneous activation of PR-1 and PDF 1.2 genes (Park *et al*. 2001). The induced resistance mechanism has been proved to be effective against bacterial, fungal and viral pathogens of different crops (Ahn *et al*. 2002; Park *et al*. 2006*a*,*b*). As our experiments were aimed to enhance the EXTN-1 mediated ISR by a chemical elicitor (Ala), both agents were tested in greenhouse both individually and in combination. The *in planta* experiments showed that both agents alone induced systemic resistance in cucumber plants bringing about a significant level of anthracnose disease suppression. However, the potential of EXTN-1 as an elicitor of ISR to bring down disease severity was found to be by 20 % higher than that of Ala. When the disease severity in the EXTN-1-infiltrated plants was 31 %, it was only 7 % when the treatment was combined with Ala (100 ppm). It demonstrates an augmentative role of Ala toward disease-suppressive ability of EXTN-1.

This phenomenon shows that both Sal-mediated and Jas-mediated defense mechanisms are activated in the plant with both agents as the PR-1 gene is commonly used as an indicator of Sal-signaling and PDF 1.2 of Jas signaling (Reymond and Framer 1998). With application of Ala alone, the expression of PR-1a was more prominent than PDF 1.2, suggesting that Ala-mediated ISR works mainly through a Sal-dependant pathway. von Rad *et al*. (2004) have reported similar findings with other chemical elicitors.

Simultaneous application of Ala on EXTN-1-treated plants showed augmentative ISR against cucumber anthracnose. This throws light on the fact that the Sal-mediated and Jas-mediated systemic resistance mechanisms act in an additive mode to bring about a cumulative effect. This cumulative ISR confers higher disease suppression in plants. This is the first report of use of Ala as an ISR-elicitor; it could be an effective strategy on a practical scale to boost rhizobacteria-mediated ISR in crops.

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