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Induced Resistance to Rice Blast by Antagonistic Bacterium, *Serratia marcescens* Strain B2

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ABSTRACT

An antagonistic bacterium, *Serratia marcescens* strain B2, controlled rice blast after being sprayed onto rice phylloplane, as did the bacterial suspension when poured into rhizosphere soil of rice plants. Three days after root treatment, rice blast conidia were sprayed onto rice foliage. A week after pathogen inoculation, rice blast was suppressed and lesions caused by the pathogen decreased in size. Brown deposits were observed around sites of pathogen infection after root treatment. Induced resistance was not associated with an increase in the activitiy of peroxidase, phenylalanine ammonia lyase, tyrosine ammonia lyase, β-1,3-glucanase, β-1,4-glycosidase, *N*-acetylhexosaminidase or chitinase. However, lipoxygenase levels were elevated after the root treatment with strain B2 following inoculation with the pathogen. Strain B2 was not detected in rice foliage after root treatment. These data suggest that strain B2 induced resistance against rice blast caused by *Pyricularia oryzae*.

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Key words : biological control, antagonistic bacterium, rice blast, induced resistance, lipoxygenase activity.

INTRODUCTION

Plants utilize various chemical and physical barriers for protection against pathogens. These defense mechanisms are not restricted to the pathogen-attacked tissues, but are also extended to distal tissues in an attempt to increase resistance to a second challenge by the same or another pathogen. This phenomenon was first described by Ross, who termed it systemic acquired resistance (SAR)[24]. SAR was reported in various plant species including rice plant. Recently, nonpathogenic microbes, such as plant growth-promoting rhizobacteria (PGPR), have also been reported to induce local and systemic resistance. It was reported that an avirulent microbe, *Pseudomonas syringae* pv. *syringae* induces systemic resistance to *Pyricularia oryzae* in rice[27]. However, there are no reports on beneficial microbes used as biocontrol agents that induce systemic resistance to *P. oryzae* in rice.

Serratia marcescens strain B2, isolated from the phylloplane of tomato plants, effectively inhibits the *in vitro* growth of several phytopathogenic fungi including *P. oryzae* and controls cyclamen gray mold caused by *Botrytis cinerea* and cyclamen soil borne diseases caused by *Rhizoctonia solani* AG-4 and *Fusarium oxysporum* f. sp. *cyclaminis* in greenhouses[1,9,28-30]. Strain B2 produces lytic enzymes, antibiotics and siderophores as antifungal factors[30]. However, some pathogens, including *F. oxysporum*, were not inhibited by *S. marcescens* strain B2 in a dual culture assay[28,29]. Owing to these results, we presumed that *S. marcescens* strain B2 induces resistance in plants. In the present paper we demonstrate that an antagonistic bacterium, *S. marcescens* strain B2, may be used to induce resistance in rice plants against the fungal pathogen *Pyricularia oryzae*.

MATERIALS AND METHODS

Culture of plants Rice (*Oryza sativa* L. cv. Koshihikari) seeds were soaked in distilled water at 4°C for 48 hr and then incubated at 32°C for 48 hr. Germinated seeds were sown on 400 cm^3 soil (Shinano-Baiyodo Co., Kanuma, Japan) in plastic pots (10 cm × 20 cm, 5 cm tall) and kept in the dark for 3 days at 25°C. Plants were then grown for approximately 2 weeks under greenhouse conditions.

Microbial strains and culture conditions Freeze-dried samples of *Serratia marcescens* strain B2, isolated

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from the phylloplane of tomato plants growing in a field near Kyusyu University, were stored at Ibaraki University, Ibaraki, Japan. For bacterial growth, liquid or solid (1.5% [wt/vol] agar) Luria broth (10 g tryptone, 5 g NaCl, and 5 g yeast extract, in 1 liter of distilled water, pH 6.8) was used.

Rice blast pathogen, *Pyricularia oryzae* Cavara NAGA-68-138 (from the Ibaraki Agricultural Center, Agricultural Research Institute, Ibaraki, Japan) was grown on oatmeal agar medium at 22°C in the dark for 14 days, and incubated under black light blue (BLB) for 3 days. Conidia were suspended in 0.1% Tween 20 solution and adjusted to approximately 2×10^5 conidia ml^{-1}.

Plant inoculations The bacterial mass containing *S. marcescens*, incubated in liquid LB medium at 28°C on a reciprocal shaker (125 strokes/min) for 48 hr, was collected by centrifugation at 10,000 rpm for 10 min and diluted with sterile water to *ca.* 1×10^9 cfu/ml. Two-week-old rice seedlings were treated with the bacterium by adding 100 ml of the bacterial suspension to 400 cm^3 soil growing *ca.* 350 rice seedlings 3 days prior to the challenge inoculation with *P. oryzae*. Comparison treatments included bacterial suspension sprayed onto the phylloplane at 20 ml/pot, tricyclazole (Takeda Chemical Industries Ltd., Osaka, Japan) applied in a 200-ppm suspension at 20 ml/pot, probenazole (Meiji Seika Kaisha Ltd., Tokyo, Japan) applied at 1.6 g/pot, and control plants treated with sterile water. The phylloplane of rice plants were sprayed with the bacterial suspension, tricyclazole, and sterile water 1 hour prior to the challenge inoculation with *P. oryzae*. Rice plants were treated with probenazole 3 days prior to the challenge inoculation with *P. oryzae*.

Challenge inoculation Seedlings were sprayed with a 2×10^5 spores ml^{-1} suspension of *P. oryzae* at 20 ml/pot. After challenge inoculation, all experimental seedlings were maintained in a greenhouse, covered with vinyl bags, and kept at 100% RH for 16 hr. Plants were then removed from vinyl bag and grown in the greenhouse. Disease development was measured 7 days after inoculation.

Disease evaluation Each leaf was scored for number of lesions. Disease incidence was evaluated according to a lesion index: small brown spots (R; lesion diameter $<$ 2 mm), small brown lesion encircled by a whitish zone (RS; 2 mm $\leqq$ lesion diameter $<$ 5 mm), small whitish lesion encircled by a brown zone (MS; 2 mm $\leqq$ lesion diameter $<$ 5 mm) and large lesion (S; 5 mm $\leqq$ lesion diameter), and disease incidence (%) was calculated by the following formula: disease incidence (%) $=\{1\times n_R+2\times n_{RS}+3\times n_{MS}+4\times n_S\}$/seedlings$\}\times100$ (n_{R-S}: number of R-S index lesions). Approximately 1000 seedlings (three replicates) were tested each treatment. Each experiment was replicated three times. The mean disease incidence for each experiment were statistically analyzed using Tukey's method[31] (at $p=0.05$).

Each leaf sample was cut into approximately 1-cm^2 pieces and fixed in FAA (formalin : acetic acid : 50% ethanol=5 : 5 : 90) for 48 hr. The samples were then rinsed twice in sterile water and observed with a light microscope.

Localization of *S. marcescens* strain B2 in pot Disease incidence was evaluated, then the plant leaves, roots and soil were removed from pots, the soil carefully separated from the roots, and the roots were gently shaken. A 1 g sample of rhizosphere soil was transferred to 9 ml of washing buffer (0.1 M phosphate, 0.1% peptone, pH 7.0) in a 25-ml vial (rhizosphere soil), respectively. Likewise, 1-cm^2 pieces of leaves or 1 g of root were transferred to 9 ml of washing buffer, respectively. The vials were shaken and sonicated for 60 sec. The number of viable bacterial cells released into the washing buffer was determined, and the resulting data were used to represent rhizoplane-colonizing bacteria (rhizoplane). The washed root was then transferred to 70% ethanol for 10 sec, followed by 10% antiformin (WAKO Co., Ltd., Osaka, Japan) for 30 sec, and washed in sterile water three times. A 1-g sample of the root was homogenized in 9 ml of washing buffer and serially diluted with washing buffer (Endorhizosphere). Each dilution was cultured on LBCA plates containing 50-ppm ampicillin for 72 hr in the dark. Colonies exhibiting reddish pigment and chitinolytic halos were counted to estimate the strain B2 population.

Enzyme assays Bacterial suspension (100 ml, *ca.* 1×10^9 cfu/ml) was added to 400-cm^3 soil in which grew *ca.* 350 rice seedlings. Three days after bacterial inoculation, leaf samples were collected. Leaf tissue was frozen in liquid nitrogen and ground to a powder using a cold mortar and pestle. Ground leaves were resuspended in 0.1 M Tris-HCl, pH 8.0. Protein content in the suspension was determined according to Bradford[2]. Enzyme activities were measured as described previously for peroxidase (POX), phenylalanine ammonia lyase (PAL), tyrosine ammonia lyase (TAL), lipoxygenase (LOX), β-1, 3-glucanase, β-1,4-glycosidase, *N*-acetylhexosaminidase and chitinase[6,14,16,17,26,30,32]. Each treatment was replicated three times. The mean enzyme activities from three replicates for each experiment were statistically analyzed using Tukey's method[31] (at $p=0.05$).

Extraction of antifungal compounds from rice leaves Bacterial suspension (100 ml, *ca.* 1×10^9 cfu/ml) was added to 400-cm^3 soil containing *ca.* 350 rice seedlings. Three days after bacterial inoculation, leaf samples were collected. A 30-g sample of leaves was homogenized in 90 ml of 80% methanol. The slurry was centrifuged for 10 min at $5000\times g$ at 4°C and the supernatant was concentrated *in vacuo*. The aqueous residue

was resuspended in 10 ml sterile water, and 10 ml diethyl ether added. The mixture was shaken vigorously, and the ether phase was collected. The ether phase was dried, and the residue was resuspended in sterile water. Equal volumes of ethyl acetate was added, and the mixture was shaken vigorously. The ethyl layer was dried, and the residue was resuspended in 1 ml methanol. A paper disc (8 mm in diameter) was then treated with a 20-ml methanol phase, and dried *in vacuo*. The dried paper disc was then placed on oatmeal media. A mycelial disc (5 mm in diameter) of 7-day-old *P. oryzae* colonies on oatmeal media was placed on media containing the test paper disc. Three replicate plates were used in assay. Fungal growth was measured after inoculation at 22°C in the dark for 14 days, and mycelial growth was observed daily with a light microscope.

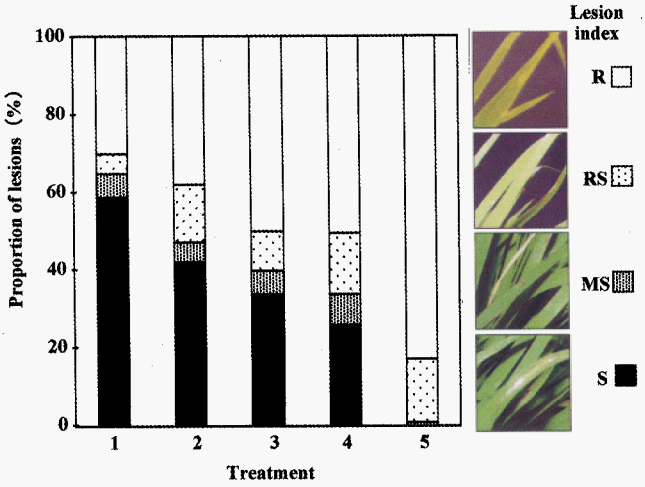

Fig. 1. Effect of *S. marcescens* strain B2 on rice blast lesion caused by *P. oryzae*. The treatments were as follows: 20 ml sterile water sprayed onto foliage of seedlings 1 hour prior to challenge inoculation with the pathogen (control, column 1); 20 ml bacterial suspension sprayed onto the foliage 1 hour prior to challenge inoculation (B2 on foliage, column 2); 100 ml bacterial suspension added to 400 cm^3 soil three days prior to challenge inoculation (B2 in root, column 3); probenazole applied at 1.6 g/pot 3 days prior to challenge inoculation (probenazole, column 4); or 20 ml tricyclazole solution (200-ppm) sprayed onto the foliage 1 hour prior to challenge inoculation (tricyclazole, column 5). Proportion of lesions was determined at 7 days after challenge inoculation using the following disease index: small brown spots (R; lesion diameter < 2 mm), small brown lesion encircled by a whitish zone (RS; 2 mm ≦ lesion diameter < 5 mm), small whitish lesion encircled by a brown zone (MS; 2 mm ≦ lesion diameter < 5 mm) and large lesion (S; 5 mm ≦ lesion diameter).

RESULTS

Suppressive effect of *S. marcescens* strain B2 against rice blast

Seven days after challenge-inoculation, control plants had a high incidence of rice blast. Plants inoculated with *S. marcescens* strain B2 on their leaves had decreased number and size of lesions (Fig. 1, 2). Likewise, plants with *S. marcescens* strain B2 added to rhizosphere 3 days prior to a challenge inoculation also had a markedly decreased size of lesion (Fig. 1). Disease incidence was reduced to 59% on rice seedlings inoculated with the bacterial suspension (Fig. 2). Few lesions were observed on rice leaves treated with tricyclazole (Fig. 2). We observed deposition of a brown material around sites of pathogen infection in *ca.* 20% of total lesions (Fig. 3). Hyphal growth was inhibited at the infection site in plants treated with strain B2 at the roots and with probenazole. *S. marcescens* strain B2 was not detected in the foliage of plants in which roots were treated with the bacterium. Although strain B2 was detected at 10^8 cfu g^{-1} soil in the rhizosphere, the bacteria were not detected from the endorhizosphere.

Levels of the enzymes POX, PAL, TAL, β-1,3-glucanase, β-1,4-glycosidase, *N*-acetylhexosaminidase, chitinase and LOX

The activities of POX, PAL, TAL, β-1,3-glucanase, β-1,4-glycosidase, *N*-acetylhexosaminidase and chitinase were not significantly higher in leaves inoculated with *S. marcescens* strain B2 than in control leaves, either before or after the challenge inoculation. In contrast, LOX activities increased rapidly from the first day of strain

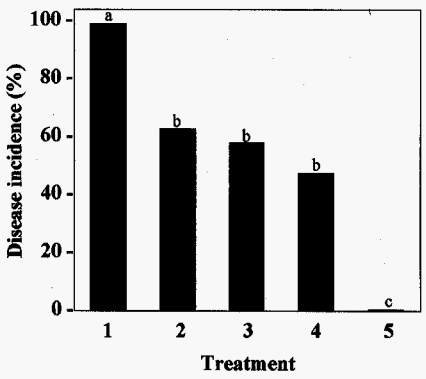

Fig. 2. Effect of *S. marcescens* strain B2 on rice blast caused by *P. oryzae*. The treatments were as described in Fig. 1. Disease incidence was calculated 1 week after inoculation. The letters indicate statistical significance as identified by Tukey's method ($p=0.05$).

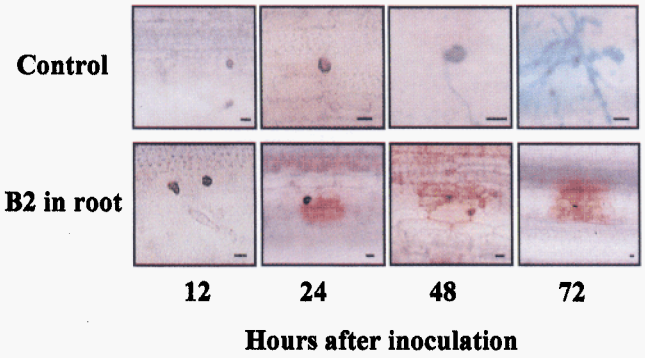

Fig. 3. Characteristic changes in epidermal cells of leaf tissues of rice plants at an early stage of infection by *P. oryzae*. Upper row: Control, lower row: B2 in root. Scale: 20 μm.

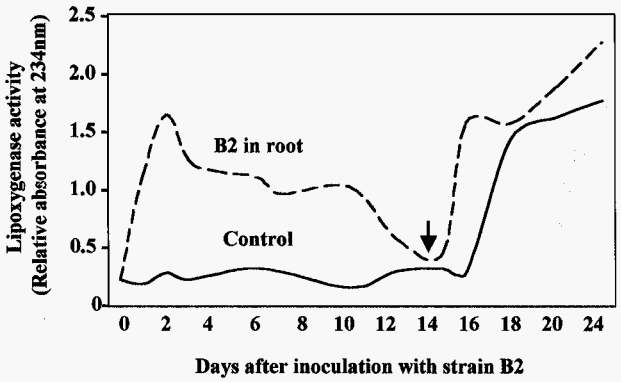

Fig. 4. Changes in lipoxygenase activity in leaves of rice seedlings after *S. marcescens* strain B2 was added into the rhizosphere soil (B2 in root) or treated with sterile water (Control). Seedlings were challenge-inoculated with *P. oryzae* 14 days after bacterial inoculation (arrow indicates site of inoculation with pathogen). The figure is based on the data from the average of three repeated experiments.

B2-inoculation, reached a maximum 2 or 3 days later, and then declined from 7 to 10 days after inoculation (Fig. 4). Fourteen days after *S. marcescens* strain B2 application, rice plants were inoculated with rice blast pathogen. In response to the inoculation, LOX activities increased rapidly beginning 12 hours after the challenge inoculation. LOX activity increased significantly earlier in leaves inoculated with *S. marcescens* strain B2 than in control leaves after the challenge inoculation (Fig. 4).

Detection of antifungal compounds in rice leaves

The fungal growth of *P. oryzae* was inhibited by extracts from leaves inoculated with *S. marcescens* strain B2 (Fig. 5). Light microscopy showed abnormal *P. oryzae* mycelia, including swelling and bursting, in the extracts from leaves inoculated with *S. marcescens* strain B2.

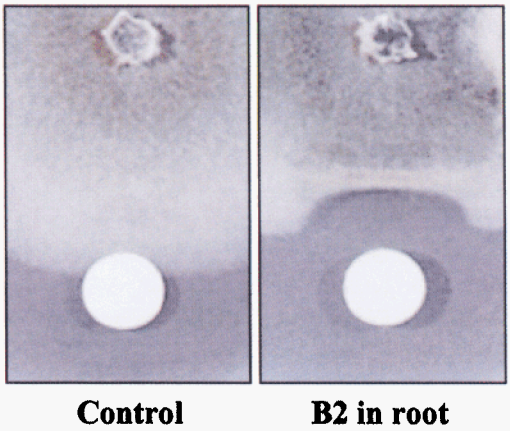

Fig. 5. Comparison of antifungal activity between extracts from leaves of control rice plants (Control), and of plants cultivated in soil with *S. marcescens* strain B2 (B2 in root) after 3 days. Inhibition zones appear in right panel (B2 in root).

DISCUSSION

In the present paper we reported that an antagonistic bacterium, *Serratia marcescens* strain B2 induced resistance in rice plants to rice blast pathogen, *Pyricularia oryzae*. *S. marcescens* strain B2 has been previously reported to have antifungal activity against various phytopathogenic fungi, including *P. oryzae*[1,9,28–30]. *S. marcescens* produces lytic enzymes such as chitinases, antibiotics such as prodigiosin, and siderophores[28–30]. We previously reported that *S. marcescens* strain B2 indirectly controlled cyclamen Fusarium wilt by inducing systemic resistance in the plants, rather than directly affecting the pathogen[29]. In the present study, lesions on rice leaves markedly decreased in size in plants treated with strain B2 into the rhizosphere. However, *S. marcescens* strain B2 was not detected at the infection site on leaves from root-treated plants. We found that lesions on plants treated with strain B2 consistently were smaller and encircled by a brown material, which resembles a brown polyphenolic material around pathogen infection sites produced by resistant plant cultivar[10]. These brown materials were observed 24 hr after infection for strain B2 treatment. Although the brown materials were often observed in control plants, the accumulation was observed in the tissues 48 hr after the challenge inoculation. We are currently histochemically analyzing the infection site on leaves inoculated with *S. marcescens* strain B2.

Early induction and accumulation of resistance-associated proteins, such as POX, PAL, TAL, LOX, β-1, 4-glycosidase, and the pathogen-related proteins chitinase and β-1,3-glucanase have been associated with induced

resistance in infected plants[4,6,11,17,21,23,25,32,35]. However, Smith *et al.* reported that although nonpathogenic *Pseudomonas syringae* pv. *syringae* induces systemic resistance to *P. oryzae* in rice, the systemically acquired resistance was not associated with an increase in the activities of PAL, coniferyl alcohol dehydrogenase, POX, β-1,3-glucanase or chitinase[27]. In the present study, LOX activity increased rapidly in leaves of rice plants with the biocontrol bacterium, *S. marcescens* strain B2 added to the rhizosphere. We demonstrated a correlation between an increase in LOX activity and the appearance of resistant lesions on the rice leaves. The increase in LOX activity was indicative of the production of antifungal compounds such as unsaturated fatty acid hydroperoxides. However, we have not yet identified the antifungal compound responsible for the resistance observed in the present study. The increase in LOX activity suggests the formation of unsaturated fatty acids that have antimicrobial activity and induce the formation of rice blade phytoalexins[25]. Our data indicate that the change in LOX activity in rice leaves inoculated with *S. marcescens* strain B2 was closely related to rice blast resistance. We observed more rapid elevation of LOX activity in leaves inoculated with *S. marcescens* strain B2 than in control plants 2 weeks prior to the challenge inoculation, and concluded that the inoculation enhanced the response of susceptible cultivars in a manner similar to probenazole or benzothiadiazole[5,7,12].

Several biotic stimuli, such as PGPR, have been reported to trigger an induced systemic resistance (ISR) response against infection by phytopathogenic microbes[19]. In contrast to classic, pathogen-induced systemic acquired resistance (SAR), which is characterized by an early increase in endogenously synthesized salicylic acid (SA)[13,15], this rhizobacteria-mediated ISR response is independent of SA accumulation and pathogenesis-related gene activation. Press *et al.* reported that SA produced by *S. marcescens* 90-166 is not the primary determinant of ISR in cucumber and tobacco[20]. In contrast, *P. aeruginosa* 7NSK2 produces SA and activates the SAR pathway in bean[3]. Recently, rhizobacteria-mediated ISR has been reported to follow a novel signaling pathway in which components from the jasmonate and ethylene response successively trigger a defense reaction[18,19]. The biosynthesis of jasmonate is closely correlated with LOX function in plants[33] and may be related to the systemic resistance we observed in response to *S. marcescens* strain B2 in the present study. The research on plant systemic resistance has mainly been carried out in dicotyledon plants, such as cucumber, carnation and arabidopsis. We have shown here that monocotyledons such as rice also have mechanism for systemic resistance.

Chitinolytic enzymes released by *S. marcescens* strain B2[1,9,28-30] hydrolyze the cell wall of chitinous microbes to produce chitooligosaccharides, which induce defensive responses in various plants including rice[8,22,34]. Other products released from the fungal cell wall by lytic enzymes include lipopolysaccharides and siderophores, which may also elicit defense reactions. However, further studies are required to identify how *S. marcescens* strain B2 elicits the response we observed here.

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