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Association of Induced Disease Resistance of Rhododendron Seedlings with Inoculation of *Streptomyces* sp. R-5 and Treatment with Actinomycin D and Amphotericin B to the Tissue-culture Medium

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Seedlings of rhododendron were treated by adding *Streptomyces* sp. strain R-5, actinomycin D and/or amphotericin B to the tissue-culture medium. HPLC analysis showed that all of the treated seedlings contained these antibiotics at concentrations higher than the suppressive levels to mycelial growth of *Pestalotiopsis sydowiana*, a major pathogen of rhododendron. Occurrence of disease caused by this fungus in the seedlings was suppressed by treatment of the medium surface with strain R-5, but not by treatment with these antibiotics, suggesting that growth of strain R-5, an antibiotic producer, could be essential for induction of disease resistance in tissue-cultured seedlings of rhododendron.

We previously reported that the endophytic *Streptomyces* sp. strain R-5 isolated from field-grown rhododendron had intense antimicrobial activities against filamentous fungi, bacteria and yeast[1]. When the surface of tissue-culture medium in which seedlings of rhododendron were growing was treated with a suspension of this strain, the seedlings were protected from Pestalotia disease[2]. Furthermore, our preliminary experiment revealed production of actinomycins and polyene antifungal antibiotics by strain R-5 in liquid medium. From these results, it was assumed that production of antibiotics by strain R-5 might be an important factor of disease resistance in rhododendron seedlings. In this study, we investigated this possibility.

Materials and Methods

Cultivation of Strain R-5 and *Pestalotiopsis*

The inoculum of *Streptomyces* sp. R-5 was prepared as follows. Strain R-5 was grown on the IMA-2 slant medium consisting of glucose, 0.5 g; soluble starch, 0.5 g; beef extract, 0.1 g; yeast extract, 0.1 g; NZ-case, 0.2 g; NaCl, 0.2 g; $CaCO_3$, 0.1 g; agar powder, 0.15 g; distilled water, 10 ml at 30°C for several days. Ten ml of 20%-glycerol solution consisting of glycerol, 20 g; sterilized distilled water, 90 ml; DMSO, 10 ml was poured into this slant and spores of strain R-5 were suspended. Subsequently 500 μl of the spore suspension was added into 50 ml of IMA-2 liquid medium and grown on a rotary shaker (200 rpm) at 30°C for 12 hours.

Pestalotiopsis sydowiana (Bresadola) Sutton (MAFF No. 305755, provided by the National Institute of

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Agrobiological Resources), a causal agent of Pestalotia disease of rhododendron, was cultured on potato dextrose agar (PDA: Difco Laboratories, Detroit, MI) medium at 25°C for 10 days. Mycelial disks of 4 mm diameter were punched out from the colony margin with a cork-borer and used as inocula.

Extraction and HPLC Analysis of Antibiotic(s) from Rhododendron Seedlings and Medium Pretreated with Strain R-5

A one ml of mycelial suspension of strain R-5 was poured on the surface of tissue-culture medium in which rhododendron seedlings were growing. Seedlings mock-treated with IMA-2 liquid medium served as controls. Treated seedlings were incubated in a growth chamber at 25°C and 14 hours photoperiod (4,000 lux) for 10 days. Seedlings were harvested from four flasks and rinsed in water, followed by air-drying and weighing. They were homogenized in liquid nitrogen with mortar and pestle. The homogenate was mixed with 200 ml of 100% methanol and stirred. To remove plant debris, it was filtered through a cotton ball packed funnel. The plant debris was washed with methanol repeatedly until its green color faded. These washes were added to the above filtrate and served as a crude extract. Using a rotary evaporator the extract was concentrated to half volume at 35°C to obtain the aqueous phase. In order to eliminate chlorophyll from the aqueous phase, it was diluted with methanol and partitioned four times against hexane. The final methanol fraction was dried in a rotary evaporator. The dried material was dissolved in a small amount of ethyl acetate. The ethyl acetate soluble fraction was mixed well with an equal volume of deionized water. The ethyl acetate phase was dried over Na_2SO_4 and then evaporated. The dried material attached to the flask wall was washed with an appropriate volume of acetone and then evaporated again. The final product was weighed and dissolved in methanol to the concentration of 1 mg/ml. The solution was filtered with a PVDF-filter [Cosmonice filter W, pore-size 0.45 μm, Nakarai tesque] before applied to HPLC.

The tissue-culture agar medium was harvested from four flasks and was stirred in 400 ml of methanol in a beaker. This mixture was centrifuged at 8,000 rpm for 3 minutes and supernatant was filtered with a filter paper (No.1, 90 mm diameter, Advantec, Japan). The filtrate was evaporated at 35°C and the residue was lyophilized. The lyophilized material was dissolved in 100 ml of deionized water and partitioned against ethyl acetate. The ethyl acetate soluble fraction was concentrated to dryness with an evaporator. The dried material was treated as described above until applied to HPLC.

Condition of HPLC analysis was as follows: an isocratic HPLC using a column (4.6×250 mm, Cosmosil, acetonitrile - phosphate buffer=60 : 40; injection volume 25 μl, flow rate, 1.0 ml/minute).

The concentration of the antibiotics was calculated based on the standard curve of commercial available actinomycin D and amphotericin B. The concentration of actinomycin D in test specimens was expressed as μg per ml of water in seedlings that was estimated from the difference of their wet and dry weights.

Extraction of Antibiotics from Seedlings Treated with Commercial Antibiotics

The antibiotic solution was prepared as follows: 2 mg of actinomycin D [Wako Pure Chemical Industries, Ltd., Japan] or amphotericin B [Wako Pure Chemical Industries, Ltd., Japan] was dissolved in 100 μl of dimethylsulfoxid (DMSO) and then diluted with 900 μl of distilled water. Subsequently 1 ml of each solution was applied singly or in combination (mixed 500 μl each) to the surface of tissue-culture medium in flasks in which the seedlings were growing. The treated seedlings were incubated in a growth chamber conditioned as above for 10 days. Harvested seedlings were rinsed in water and air-dried, followed by weighing. They were homogenized in liquid nitrogen with mortar and pestle. Preparation of methanol soluble fraction and elimination of chlorophyll from the fraction were conducted as above. The final methanol phase was concentrated to half its volume using an evaporator and vacuum-filtrated to remove insoluble materials. The filtrate was mixed with a small amount of acetonitrile and then dried using an evaporator. The dried material was dissolved in 2 ml of 50% methanol and centrifuged at 15,000 rpm for 5 minutes to eliminate insoluble residues again. The supernatant was filtrated with a PVDF-filter before applied to HPLC analysis.

The condition of HPLC analysis was as follows: 0~5 minutes, isocratic (acetonitrile - phosphate buffer=40 : 60); 5~10 minutes, gradient (40 : 60~60 : 40); 10~30 minutes, isocratic (60 : 40).

The concentration of the antibiotics in seedlings was evaluated using standard curves and water content of seedlings estimated as above.

Bioassay of Disease Resistance

Pestalotiopsis sydowiana (Bresadola) Sutton, a causal pathogen of rhododendron Pestalotia disease (provided by the National Institute of Agrobiological Resources, stock No. 305755), was cultured on potato dextrose agar (PDA)

at 25°C for 5 days. Mycelial disks of 4 mm diameter were punched out from colony margin.

The surface of medium with growing seedlings were treated with i) actinomycin D and/or amphotericin B as above, ii) 1 ml of 1% DMSO (a solvent of the antibiotics) as controls, and iii) suspension of strain R-5 as above. The seedlings were incubated for 10 days. The upper 4th leaves of all of these seedlings were inoculated with a mycelial disk of *P. sydowiana* and incubated in the same growth chamber. Seven and 14 days after inoculation, symptoms on the seedlings were observed with naked eye and categorized as follows: i) no change on inoculated 4th leaves, ii) brownish symptom on inoculated leaves, iii) brownish symptom on 3rd~5th leaves, iv) browning of entire seedling. The incidence for each category was expressed as a percentage of the number of seedlings in the category per total number of observed seedlings.

Table 1. UV-absorbing peaks of actinomycin D and the corresponding compound(s) in growth medium and rhododendron seedlings, analyzed by HPLC.

Sample	Retention time (minutes)	λ max (nm)
Actinomycin D	12.3	242, 427(sh), 444
Extraction of growth medium pretreated with strain R-5	12.9	241, 428(sh), 443
Extraction of rhododendron seedlings pretreated with strain R-5	12.8	241, 427(sh), 443

sh: shoulder

Table 2. UV-absorbing peaks of actinomycin D, amphotericin B and the corresponding compound(s) in rhododendron seedlings, analyzed by HPLC.

Sample	Retention time (minutes)	λ max (nm)
Amphotericin B	6.2	283, 348, 365, 385, 408
Actinomycin D	22.5	242, 428(sh), 443
Extract of rhododendron seedlings pretreated with amphotericin B	22.5	241, 428(sh), 444
Extract of rhododendron seedlings pretreated with actinomycin D	6.2	283, 347, 365, 384, 408
Extract of rhododendron seedlings pretreated with both actinomycin D and amphotericin B	6.1	270, 348, 365, 384, 408
	22.5	242, 427(sh), 443

sh: shoulder

Table 3. Incidence of Pestalotia-disease caused by inoculation of *P. sydowiana* in rhododendron seedlings pretreated with strain R-5 and antibiotics.

Treatment	Symptom	Incidence (%) after inoculation	
		7 days	14 days
Control (DMSO)	No change on inoculated 4th leaves	12	8
	Brownish symptom on inoculated leaves	32	8
	Brownish symptom on 3rd – 5th leaves	56	20
	Browning of entire seedling	0	64
Strain R-5	No change on inoculated 4th leaves	6	8
	Brownish symptom on inoculated leaves	94	92
	Brownish symptom on 3rd – 5th leaves	0	0
	Browning of entire seedling	0	0
Actinomycin D	No change on inoculated 4th leaves	17	3
	Brownish symptom on inoculated leaves	47	7
	Brownish symptom on 3rd – 5th leaves	37	33
	Browning of entire seedling	0	57
Amphotericin B	No change on inoculated 4th leaves	24	9
	Brownish symptom on inoculated leaves	39	9
	Brownish symptom on 3rd – 5th leaves	36	30
	Browning of entire seedling	0	52
Actinomycin D	No change on inoculated 4th leaves	13	0
+	Brownish symptom on inoculated leaves	20	7
Amphotericin B	Brownish symptom on 3rd – 5th leaves	67	13
	Browning of entire seedling	0	80

Results and Discussion

Antibiotic(s) Detected in Rhododendron Seedlings and Medium Pretreated with Strain R-5

In HPLC analysis, a peak of which retention time (about 12.8 minutes) and UV spectrum were consistent with those of the standard actinomycin D was detected in the preparations of both seedlings and medium pretreated with strain R-5 (Table 1). The concentrations of actinomycins were calculated as *ca.* 24 and *ca.* 7 μg/ml in seedlings and medium, respectively. However, a similar peak was never detected in untreated controls. These data indicate that actinomycins produced by strain R-5 in the medium could move to the seedlings and/or that strain R-5 grown in the seedlings could produce this antibiotic within tissues. Any polyene antifungal antibiotics were not detected in the seedlings.

Antibiotics Detected in Seedlings Treated with Commercial Antibiotics

The peaks which were consistent with those of standard amphotericin B and/or actinomycin D were detected at the

expected retention time, *ca.* 6.2 minutes and *ca.* 22.5 minutes, respectively, in the extract of seedlings treated with these commercial antibiotics (Table 2). In a single treatment with either antibiotics, the concentrations of amphotericin B and actinomycin D were *ca.* 67 and *ca.* 86 μg/ml in the seedlings, respectively. On the other hand, in a combination treatment with the mixture of these antibiotics the concentrations were *ca.* 51 and *ca.* 34 μg/ml, respectively. These data indicate that both antibiotics are absorbed by the seedlings from the medium.

Bioassay of Disease Resistance

As shown in Table 3, when seedlings were treated with strain R-5, only inoculated leaves turned brownish in more than 90% of seedlings by 7 and 14 days after inoculation. Stems and leaves lower and upper than the inoculated leaves kept green within these days. However, more than 50% of seedlings treated with commercial actinomycin D and/or amphotericin B were killed by 14 days after inoculation.

An additional experiment showed that mycelial growth of *P. sydowiana* was suppressed significantly by 0.1 μg/ml of amphotericin B or 10 μg/ml of actinomycin D on PDA. As described above, seedlings contained 7~86 μg/ml of actinomycins and 51~67 μg/ml of amphotericin B, when the medium with growing seedlings were treated with suspension of strain R-5 or test antibiotics, although any polyene antifungal antibiotics were not detected by unknown reasons. Thus, both antibiotics accumulated in the seedlings were more than sufficient in quantity to suppress the mycelial growth of *P. sydowiana*.

Many reports demonstrated that the antibiotics, such as 2,4-diacetylphloroglucinol, or other secondary metabolites produced by biocontrol agents play an important role in suppression of plant diseases[3~6]. However, our present data indicate that these antibiotics when added to the medium cannot suppress development of the pathogen in the seedlings, and that growth of strain R-5 in/on the seedlings is essential in disease resistance.

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