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Phytotoxicity of Indole-3-acetic Acid Produced by the Fungus, *Pythium aphanidermatum*

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Pythium aphanidermatum **causes the serious disease of Pythium red blight on bentgrass. IAA, one of the metabolites that has been isolated from this fungus, showed the same symptom of Pythium red blight on bentgrass at a concentration of 1,000 mg/l. The IAA content in the foliage of bentgrass infected by this fungus was about 200 times that of an untreated control. These results suggest that IAA produced by this fungus was the causal substance of Pythium red blight on bentgrass.**

Key words: phytotoxicity; indole-3-acetic acid (IAA); *Pythium aphanidermatum*; bentgrass

Pythium red blight is one of serious diseases of bentgrass,[1] and is caused by infection with *Pythium aphanidermatum*.[2-5] *P. aphanidermatum* shows remarkable phytotoxicity in the *Pythium* species, but the metabolite of this fungus has not been clarified. In this paper, we describe the active principle causing Pythium red blight on bentgrass.

The fungus, *P. aphanidermatum*, was stationarily cultured in a potato extract medium containing sucrose (30 g/l) at 24°C for 21 days. The culture broth (10 l) was filtered, and the filtrate was adjusted to pH 2.0 with 2 N HCl, before being extracted twice with EtOAc. The combined solvents were concentrated *in vacuo*, and the resulting residue was fractionated by column chromatography on silica gel (benzene-acetone). The active fraction eluted with 15% acetone was further purified by preparative TLC ($CHCl_3$-acetone-AcOH, 90:10:0.3, v/v/v) to afford 15 mg of IAA (R_f: 0.22) as colorless needles, together with *p*-hydroxybenzaldehyde (13 mg, R_f: 0.48) as colorless needles and linoleic acid (17 mg, R_f: 0.71) as a colorless oil. The physicochemical properties of IAA are as follows: mp 161–162°C; UV λ_{max} (EtOH) nm (ε): 229 (10,400), 273 (9,900), 278 (10,000), 289 (8,700); IR ν_{max} (KBr) cm^{-1}: 3392, 3094, 2924, 1702, 1460, 1408, 1224, 1102, 932, 741; NMR δ_H (acetone-d_6, 270.05 MHz): 3.75 (2H, d, J=0.6 Hz), 7.02 (1H, ddd, J=1.2, 7.3, 7.7 Hz), 7.10 (1H, ddd, J=1.3, 7.3, 8.1 Hz), 7.29 (1H, d, J=2.3 Hz), 7.39 (1H, ddd, J=0.8, 1.2, 8.1 Hz), 7.60 (1H, br.d, J=7.7 Hz), 10.11 (1H, br.s); NMR δ_C (acetone-d_6, 67.80 MHz): 31.4 (t), 109.1 (s), 112.1 (d), 119.6 (2C, d), 122.2 (d), 124.5 (d), 128.5 (s), 137.5 (s), 173.3 (s); EIMS m/z: 175 (M^+). These data are identical with those of authentic IAA. The data for *p*-hydroxybenzaldehyde are as follows: mp 116–119°C; UV λ_{max} (EtOH) nm (ε): 220 (14,800), 280 (18,700), 330 (6,600); IR ν_{max} (KBr) cm^{-1}: 3176, 1669, 1603, 1520, 1456, 1292, 1220, 1164, 835; NMR δ_H (*d*-methanol, 270.05 MHz): 6.95 (2H, d, J=8.6 Hz), 7.81 (2H, d, J=8.6 Hz), 9.80 (1H, s); NMR δ_C (*d*-methanol, 67.80 MHz): 116.9 (2C, d), 130.3 (s), 133.4 (2C, d), 165.2 (s), 192.8 (d). These data are also identical with those of authentic *p*-hydroxybenzaldehyde. The data for linoleic acid are as follows: IR ν_{max} (KBr) cm^{-1}: 3016, 2920, 2854, 1711, 1464, 1443, 1412, 1296, 1230; NMR δ_H ($CDCl_3$, 270.05 MHz): 0.88 (3H, m), 1.26–1.31 (16H, br.s), 1.62 (2H, m), 2.05 (2H, m), 2.35 (2H, t, J=7.5 Hz), 2.77 (2H, m), 5.36 (4H, m); NMR δ_C ($CDCl_3$, 67.80 MHz): 14.1 (q), 22.7 (t), 24.7 (t), 29.1–29.7 (8C, t), 31.9 (t), 34.0 (t), 127.9 (d), 128.1 (d), 130.0 (2C, d), 179.8 (s). These data are also identical with those of authentic linoleic acid.

Inhibitory activities of IAA, *p*-hydroxybenzaldehyde and linoleic acid toward bentgrass were examined. Bentgrass (*Agrostis palustris* Huds.) was germinated and planted in commercial soil for grass (topdressing, 800 g) in 12-cm-diameter pots, and grown under artificial light (NEC FL40SEX-N-HG fluorecent lamp, 8,000 lx) at 24°C for seven days. The chemicals to be tested were each formulated as an aqueous solution containing 0.1% Tween-80 as a

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Abbreviation: IAA, indole-3-acetic acid

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Table 1. Inhibitory Evaluation of IAA, *p*-Hydroxybenzaldehyde and Linoleic Acid toward Bentgrass

Sample	Concentration (mg/l)	Inhibitory activity*
IAA	100	1
	300	2
	1000	4
p-Hydroxybenzaldehyde	100	0
	300	0
	1000	1
Linoleic acid	100	0
	300	1
	1000	1
Control		0

* Zero denotes no withered area; 4 denotes that an area of more than 75% was withered; an intermediate number denotes as intermediate degree of withered area.

wetting agent and 2% ethanol to aid their solubility. Each aqueous solution was sprayed on all the foliage of bentgrass with an atomizer at the rate of 10 ml per pot. Triplicate experiments were conducted. After the treatment, the bentgrass in each pot was placed inside a moist chamber, and was grown under artificial light (8,000 lx) at 30°C. Eight days after the treatment, the inhibitory activity toward the bentgrass treated with the chemicals was compared with the untreated control, the activity being evaluated as the proportion of withered area[6] (see Table 1 for details).

Three metabolites produced by this fungus were each applied at concentrations of 100, 300 and 1000 mg/l, respectively (Table 1). IAA showed weak inhibitory activity toward bentgrass at a concentration of 100 mg/l, while it completely withered bentgrass at a concentration of 1000 mg/l. The symptom induced by IAA isolated from this fungus was identical to that of Pythium red blight on bentgrass. On the other hand, *p*-hydroxybenzaldehyde and linoleic acid did not have any marked inhibitory activity toward bentgrass at the same concentration. These results suggested that IAA isolated from this fungus was the active principle causing Pythium red blight on bentgrass.

A quantitative analysis of the IAA[7,8] content of the foliage infected by this fungus was next examined. Bentgrass was germinated and planted as already mentioned. Seven days after seeding, the fungal broth (5 ml/pot), which had been cultured while shaking for 4 days at 24°C in a potato extract medium, was inoculated the foliage of the bentgrass. After this treatment, the bentgrass in each pot was placed inside the moist chamber and grown under artificial light (8,000 lx) at 30°C. The foliage of the bentgrass was harvested on the 4th and 8th days after the treatment. The foliage from each (0.3–0.8 g fresh weight/pot) was homogenized in 15 ml of 80% acetone-water with Polytron equipment (Kinematica Co.) at the medium-speed setting for 60 s, and the resulting homogenate was filtered through four layers of cheesecloth. The residue was suspended in an equal volume of an 80% acetone solution, and the suspension was filtered. The combined filtrates were concentrated *in vacuo* to give an aqueous solution. The aqueous fraction was adjusted to pH 3.5 with 0.1 N tartaric acid, and then washed with the same volume of petroleum ether, after which it was partitioned twice with the same volume of diethyl ether. The diethyl ether fraction was partitioned twice with the same volume of a 0.1 M potassium phosphate buffer solution (pH 8.0). The aqueous fraction, adjusted to pH 3.5 with 0.1 N HCl, was partitioned twice with the same volume of diethyl ether. The organic fraction was concentrated *in vacuo*, and the resulting residue was dissolved in acetonitrile. IAA in each sample was quantified chromatographically by HPLC (Shimadzu Co. Ltd., model LC-6A) with a 250 × 4.6 mm TSKgel ODS-80Ts column (Tosoh Co. Ltd.). Each sample was eluted with a 20% acetonitrile solution (pH 3.5, 20 mM sodium acetate buffer) at a flow rate of 0.8 ml/min. The eluate was monitored with a spectrofluorometer (Shimadzu Co. Ltd., model RF-550). The excitation wavelength was 280 nm, and the emission wavelength was 355 nm, triplicate experiments being conducted. Similarly to the quantitative analysis of IAA, the morbidity of the bentgrass was evaluated as the proportion of its withered area.[6]

The morbidity of bentgrass was about 25% on the 4th day after the treatment, and bentgrass had completely withered on the 8th day after the treatment. Penetration by the hyphae of the fungus into the foliage was observed under a microscope (Fig. 1). The IAA content of the untreated foliage of bentgrass on the 8th day was 50 ng/g fresh weight. On the other hand, the IAA contents of the foliage on the 4th and 8th days after the treatment were 1140 and 9760 ng/g fresh weight, respectively. In proportion to the morbidity of the bentgrass, an increase in

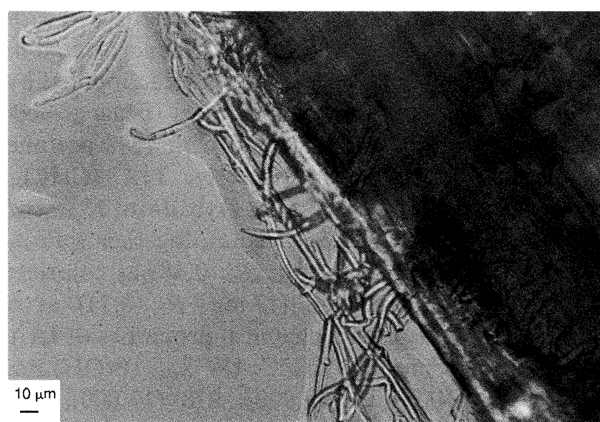

Fig. 1. Foliage of Bentgrass Infected by *Pythium aphanidermatum* on the 8th Day after Treatment.

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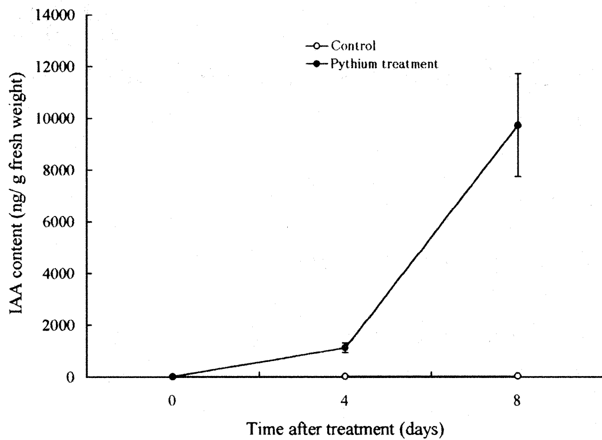

Fig. 2. Time-course Plot of the IAA Content in the Foliage of Bentgrass after the Treatment with *Pythium aphanidermatum*. Each bar shows the mean ± S.D. (n = 3).

the IAA content was observed in the foliage (Fig. 2), the IAA content on the 8th day after the treatment being about 200 times that of the untreated control. The relationship between the morbidity and IAA content of bentgrass suggested that IAA produced by this fungus was the causal substance of Pythium red blight on bentgrass.

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References

1) Sasano, I., Introduction of grass disease developed on golf courses. *Kongetsu no Nougyo* (in Japanese), **7**, 28–36 (1991).
2) Fitzpatrick, H. M., Generic concepts in the *Pythiaceae* and *Blastocladiaceae*. *Mycologia*, **15**, 166–173 (1923).
3) Drechsler, C., The cottony leak of cucumbers caused by *Pythium aphanidermatum*. *J. Agr. Res.*, **30**, 1035–1042 (1925).
4) Harter, L. L. and Whitney, W. A., A transit disease of snap beans caused by *Pythium aphanidermatum*. *J. Agr. Res.*, **34**, 443–447 (1927).
5) Takahashi, M., Tanaka Y., Ichitani, T., and Alicbusan, R. V., Ecologic and taxonomic studies on *Pythium* as pathogenic soil fungi. *Ann. Phytopath. Soc. Japan*, **38**, 306–312 (1972).
6) Tojo, M., Fujita, Y., Awad, H. M., and Ichitani, T., Preparation of *Pythium* inocula using bentgrass seeds for glasshouse studies. *Proc. Kansai Pl. Prot.*, **35**, 1–5 (1993).
7) Crozier, A., Loferski, K., Zaerr, J. B., and Morris, R. O., Analysis of picogram quantities of indole-3-acetic acid by high performance liquid chromatography-fluorescence procedures. *Planta*, **150**, 366–370 (1980).
8) Akiyama, M., Sakurai, N., and Kuraishi, S., A simplified method for the quantitative determination of indoleacetic acid by high performance liquid chromatography with a fluorometric detector. *Plant Cell Physiol.*, **24**, 1431–1439 (1983).

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