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ARTICLE

Plant-Growth Regulator, Imidazole-4-Carboxamide, Produced by the Fairy Ring Forming Fungus *Lepista sordida*

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Rings or arcs of fungus-regulated plant growth occur often on the floor of woodlands, in agricultural areas, and in grasslands worldwide. These rings are commonly called "fairy rings". A plant-growth regulating compound was isolated from a fairy ring forming fungus, *Lepista sordida*, and its chemical structure was identified as imidazole-4-carboxamide (ICA) by spectroscopic analyses including single-crystal X-ray diffraction techniques. ICA inhibited the growth of turfgrass and rice seedling. On the other hand, in a greenhouse experiment, this compound increased rice grain yield by 26% compared with control.

KEYWORDS: Plant growth regulator; fairy ring; fungus; *Lepista sordida*; turfgrass; rice

INTRODUCTION

The rings, ribbons, or arcs of stimulated plant growth or of the fruiting bodies of the larger fungi which often occur on the floors of woodlands or in agricultural or amenity grassland in most parts of the world are commonly called "fairy rings" (**Figure 1**) (*1*−*6*). The term "fairy rings" has its origin in the myths and superstitions associated with their occurrence in the Middle Ages. The rings are produced by the interaction between grasses and fungi (*4*). The mycelium of the fungus in the soil of a fairy ring grows in a centrifugal fashion as a fungal isolate would do in pure culture on a plate of agar media, because of the apical growth habit of the fungi (*6*). Actively growing hyphae are found at the periphery of the ring, and fruiting bodies, if present, are often found closer to the center of the ring (*1*−*6*). Fairy ring fungi are classified into three types according to their effect on grassland: type I, those in which the grass is ultimately killed or badly damaged; type II, those in which the grass is only stimulated; type III, those which apparently do not influence the growth of the turfgrass (*4*).

The reason for the plant-growth stimulation by the fungi has been explained as follows: through the saprophytic action of the fungus mycelium, the protein portion of nonliving organic matter in the soil is decomposed to ammonia. The ammonia combines with other compounds or is used as a substrate by successive bacteria to generate nitrites and nitrates. The resulting accumulation of nitrogen in the soil in a form readily available to higher plants causes the typical growth pattern of conspicuous bands of taller, darker green plants (*1*−*6*). However, we investigated the possibility of a specific plant growth-regulating substance(s) being produced by one fairy ring forming fungi, *Lepista sordida*, and found a "fairy" (plant-stimulating compound), 2-azahypoxanthine (AHX), as reported previously (*7*). *L. sordida* is widespread in northern temperate zones throughout the world (*8*). The growth-promoting activity of AHX was observed toward all of the plants tested regardless of their families and suggested the possibility of its practical use in agriculture. For example, when biomass crops, rice or potato, were cultivated with 5 or 50 μM

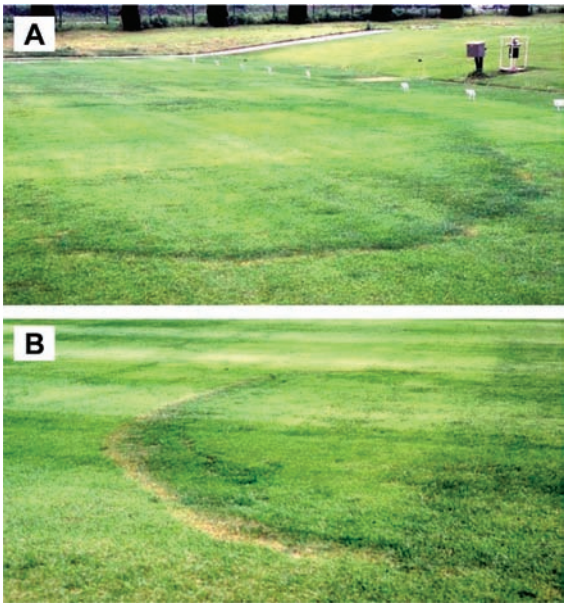

Figure 1. Fairy rings caused by *Lepista sordida*. Fairy rings occurred on the Turfgrass Study Field, Chiba Prefectural Agriculture and Forestry Research Center, Japan.

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AHX, the grain yield per plant increased by 25.5% (rice), 19.3% (potato, total yield), or 40.6% (potato, esculent size).

During further searches for other plant-growth regulator(s) from *L. sordida*, we succeeded in the isolation and structural identification of another active compound. We report the study here.

MATERIALS AND METHODS

Chemicals. All solvents used throughout the experiments were obtained from Kanto Chemical Co. (Tokyo, Japan). Ethyl imidazole-4-carboxylate (EIC) was purchased from Combi-Blocks (San Diego, CA). Imidazole, 5-aminoimidazole-4-carboxamide (AIC), and *N*-acetylimidazole were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Fungus and Plant Materials. *L. sordida* (Schuml: Fr.) Sing. was identified and kindly provided by Fumio Kobayashi of Nasu Biofarm, Ltd. (Nasu, Japan). The fungus strain was stored at 4 °C on PYG agar (0.3% polypepton, 0.3% yeast extract, 1% glucose, and 1.8% agar). Bentgrass (*Agrostis palustris* Huds.), a rice cultivar (*Oryza sativa* L. *cv*. Nipponbare), lettuce (*Lactuca sativa* L. cv. Great Lakes 366), and tea cells (*Camellia sinensis* L. cv. Yabukita) were used in this study.

General Apparatus. A JASCO grating infrared spectrophotometer was used to record the IR spectra (Tokyo, Japan). UV spectra were recorded on a UV−visible spectrophotometer (Ultrospec 2100 pro; Amersham Pharmacia Biotech, Piscataway, NJ). ^{1}H NMR spectra were recorded on a JEOL λ-500 spectrometer at 500 MHz, whereas ^{13}C NMR spectra were recorded on the same instrument at 125 MHz (JEOL, Tokyo, Japan). The HRESIMS spectra were measured on a JMS-T100LC mass spectrometer (JEOL). HPLC separations were performed with a JASCO Gulliver system using reverse-phase HPLC columns (Develosil C30-UG-15/30 and C30-UG-5, Nomura Chemical, Japan). A silica gel plate (F_{254}; Merck Co., Darmstadt, Germany) and ODS gel (Wakogel 50C-18; Wako Pure Chemical Industries, Ltd., Osaka, Japan) were used for analytical TLC and for flash column chromatography, respectively.

Purification of the Active Compound from the Fungus. Five pieces (5 × 5 × 3 mm) cut from 20-day-old mycelium cultures of *L. sordida* on PYG agar were inoculated into a 500 mL Erlenmeyer flask containing 300 mL of PYG liquid medium and incubated at 25 °C and 120 rpm for 4 weeks in the dark. The liquid-cultured fungus was filtered, and the filtrate (12 L, 122.7 g, dry weight) was partitioned between EtOAc and H_2O. The aqueous layer was dried under reduced pressure and extracted with EtOH. The EtOH-soluble components (2.1 g) were fractionated by ODS flash column chromatography (Wakogel 50C-18; Ø 30 × 520 mm; MeOH/H_2O 1:9, 1:1, MeOH, 1.0 L each) to obtain three fractions, and fraction 1 (1.5 g) was further separated by HPLC with a Develosil C30-UG-15/30 column (Ø 50 × 500 mm, 15−30 μm; Nomura Chemical, Japan; flow rate = 25 mL min^{-1}; injection volume = 35 mL; H_2O; UV detection at 230 nm; retention time = 85.2 min) being guided by the result of the bioassay to give nine fractions. Fractions 1−6 (27 mg) were further separated by HPLC with a Develosil C30-UG-5 column (Ø 20 × 250 mm, 5 μm; flow rate = 5 mL min^{-1}; injection volume = 0.5 mL H_2O; UV detection at 230 nm; retention time = 28.4 min) and then recrystallized to afford imidazole-4-carboxamide (ICA; 2.2 mg; 0.0017% from the filtrate). The structure of ICA was confirmed by single-crystal X-ray analysis (**Figure 2A**; Figure S1 and Tables S1−S71 in the Supporting Information). ICA for bioassays was synthesized according to the method reported previously (*9*).

Imidazole-4-carboxamide (ICA): IR, ν_{max} (KBr) cm^{-1} 3106, 2858, 2037, 1618; UV, λ_{max} nm 234, 204 (H_2O); ^{1}H NMR (CD_3OD) 7.72 (s, 1H), 7.68 (s, 1H); ^{13}C NMR (CD_3OD) 122.7, 135.5, 137.5, 167.2; ESIMS, *m*/*z* 134 [M + Na]$^+$; HRESIMS, *m*/*z* 134.0321 [M + Na]$^+$ (calcd for $C_4H_5N_3NaO$, 134.0330).

Crystal Structure Analysis of ICA. A plate-shaped colorless single crystal with dimensions of 0.19 × 0.12 × 0.04 mm was selected and mounted on a glass capillary. The diffraction data were collected using a Rigaku AFC-8 diffractometer equipped with a Saturn70 CCD detector with Mo Kα radiation by an oscillation method at 90 K. Bragg spots were integrated, scaled, and averaged to $\sin\nu/\lambda = 0.77$ Å^{-1} by the program CrystalClear (*10*) to give 1313 unique reflections with R_{int} of 0.054. The initial structure of ICA was solved by a direct method using the program SIR2004 (*11*) and refined by a full matrix least-squares method using the program SHELXL97 (*12*).

Crystal data: $C_4H_5N_3O\cdot H_2O$, FW = 129.13, triclinic, $P\bar{1}$, a = 6.539(2), b = 7.013(3) c = 7.293(3) Å, α = 74.810(16), β = 75.146(16), γ = 63.994(13)°, V = 286.17(19) Å^3; D_X = 1.499 Mg m^{-3}; Z = 2; μ(Mo Kα) = 0.122 mm^{-1}; $R(F)$ = 0.0496, wR(F^2) = 0.1072, and S = 1.045 for 1013 reflections with $I > 2\sigma(I)$.

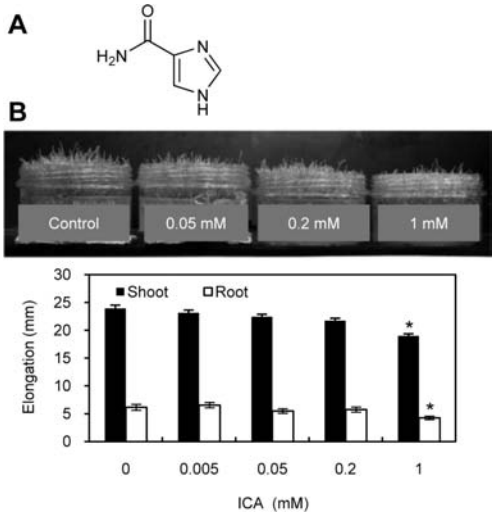

Figure 2. Structure of ICA and growth inhibitory activity of ICA toward bentgrass for 1 month. Results are the mean ± standard error (n = 50). The asterisk indicates significant difference from control (*, $P < 0.01$).

Bioassay. For fractionation, sterilized bentgrass seeds were sown on 100 mL of 0.8% agar (v/w) containing distilled water in a deep Petri dish and incubated in a growth chamber under an 18 h photoperiod at 25 °C for 5 days. After the incubation, 20 seedlings were transferred on the Advantec no. 2 filter paper soaked in 1 mL of sample solution (in distilled water) in a Petri dish (Ø 55 mm) and incubated in a growth chamber under an 18 h photoperiod at 25 °C for 5 days. The effect of each fraction on shoot and root elongations (n = 20) was measured using a ruler. For ICA evaluation toward bentgrass, ICA was dissolved at various concentrations in 100 mL of 0.8% agar (v/w) containing nutrient solution A (1 mM NH_4NO_3, 0.6 mM Na_2HPO_4, 0.3 mM K_2SO_4, 0.4 mM $MgCl_2$, 0.2 mM $CaCl_2$, 45 μM iron ethylenediaminetetraacetic acid (Fe-EDTA), 50 μM H_3BO_3, 9 μM $MnSO_4$, 0.3 μM $CuSO_4$, 0.7 μM $ZnSO_4$, and 0.1 μM Na_2MoO_4), and the solution was autoclaved. Sterilized bentgrass seeds (~600 mg) were sown on the ICA solution in a deep Petri dish and incubated in a growth chamber under an 18 h photoperiod at 25 °C for 30 days. The effect of ICA on shoot elongation (n = 50) was measured using a ruler. For ICA evaluation toward rice, sterilized seeds were germinated in the dark at 28 °C for 3 days and then transferred to a plastic dish (110 × 110 × 20 mm) containing half-strength nutrient solution A with various concentrations of ICA. The 15 seedlings were grown under an 18 h photoperiod at 28 °C for 20 days, and the nutrient solution was replaced daily. The effect of ICA on shoot and root elongations (n = 15) was measured using a ruler.

Greenhouse Pot Experiment. Pot experiments for rice, using soil culture, were conducted from June 10 to September 29 at the Faculty of Agriculture, Shizuoka University, in 2009. A 30-day-old seedling was transplanted into an individual Wagner pot (1/5000 a) containing fertilizers (N, 1440 mg; P_2O_5, 12 mg; K_2O, 760 mg; CaO, 806 mg) on June 10. Rice plants were watered with tap water (control) or 300 mL of 2 μM ICA daily. The panicle number and length were measured at harvest time, and the yields were evaluated after air-drying at 30 °C for 15 days.

Statistical Analysis. Data thus collected were analyzed statistically using Student's t test to determine significant difference in the data among the groups. P values of < 0.05 and < 0.01 were considered to be significant.

RESULTS AND DISCUSSION

Purification of Plant Growth-Regulating Compound. Isolation of the active compound from *L. sordida* was guided by its growth-regulating activity on bentgrass. The liquid-cultured fungus was filtered, and the filtrate was partitioned between EtOAc and H_2O. The aqueous layer was dried under reduced pressure and extracted with EtOH. The EtOAc-soluble part promoted the growth of bentgrass, and the EtOH-soluble part and the residue after the

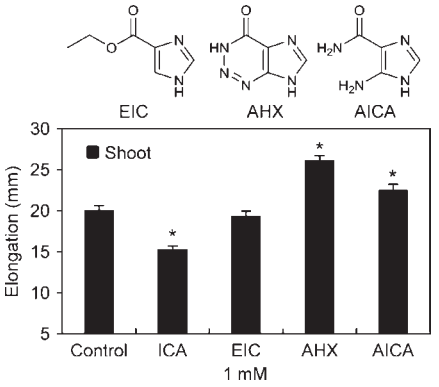

Figure 3. Effect of ICA and its analogues on bentgrass growth for 1 month. Results are the mean ± standard error ($n = 40$). AHX, 2-azahypoxanthine; EIC, ethyl imidazole-4-carboxylate; AICA, 5-aminoimidazole-4-carboxamide. The asterisk indicates significant difference from control (∗, $P < 0.01$).

extraction with EtOH showed no effect on the growth (Figure S2A). A growth-promoting compound, AHX, has been obtained from the EtOAc-soluble part as reported previously (*7*). To search for other plant-growth regulating compound(s), the EtOH-soluble part and the residue were fractionated by ODS flash column chromatography, giving three and five fractions, respectively. Because fraction 1 of the EtOH-soluble part inhibited the growth of bentgrass (Figure S2B), the fraction was further separated by successive chromatography. As a result, ICA was isolated as the inhibitory principle (**Figure 2A**; Figure S1). Although ICA has been synthesized (*9*) and has been reported as an inhibitor of guanine deaminase (*13*), this is the first isolation of ICA from a natural source.

Plant Growth-Regulating Activity. ICA exhibited growth-inhibitory activity against shoots and roots of bentgrass seedlings at 1 mM (**Figure 2B**). To elucidate the structure−activity relationship, ICA, AHX, ethyl imidazole-4-carboxylate (EIC), which is the precursor of ICA in chemical synthesis, and 5-aminoimidazole-4-carboxamide (AICA), which is the precursor of AHX in chemical synthesis, were tested toward bentgrass (**Figure 3**). Only ICA inhibited the growth of bentgrass, and AHX and AICA exhibited growth-promoting activity. In the previous study, the examination concerning the structure−activity relationship among AHX and its analogues (xanthine and hypoxanthine) revealed that only AHX had a promoting activity similar to this study (*7*).

The plant-growth regulating of ICA was also investigated using rice (*O. sativa* L. cv. Nipponbare), which belongs to the same family as the turfgrass. The rice seedlings were incubated with various concentrations of ICA. The compound suppressed shoot and root elongations significantly (**Figure 4**; Figure S3). In addition, we elucidated the structure−activity relationship among various ICA analogues toward rice (**Figure 5**). The results were similar to those toward bentgrass; only AHX showed root-promoting effect, and only ICA exhibited growth-inhibitory effect on shoot and root elongations. The growth-inhibitory activity of ICA was also observed toward lettuce, which belongs to a family different from that of the turfgrass or rice (**Figure 6**).

In the previous study, when rice was cultivated with 5 μM AHX, the grain yield per plant increased by 25% (*7*). We thought that ICA also might affect rice yield, because both of the compounds have structural similarity to each other and will relate to each other in their biosynthesis and/or their metabolism in rice. Therefore, we examined the effect of ICA on rice grain yield in a greenhouse experiment (**Table 1**). The soil cultivation of rice with 2 μM ICA for about 3 months resulted in grain yield's increment from 36.9 g (control) to 46.5 g (rate of increase, 26%), and the seed weight per 100 seeds did not change as AHX did (*7*).

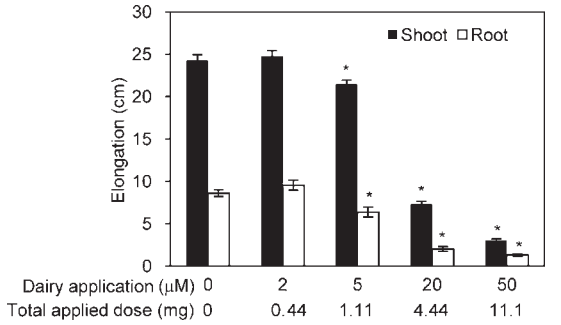

Figure 4. Effect of ICA on the growth of rice. Twenty-day-old seedlings were treated with or without ICA. Results are the mean ± standard error ($n = 15$). The asterisk indicates significant difference from control (∗, $P < 0.01$).

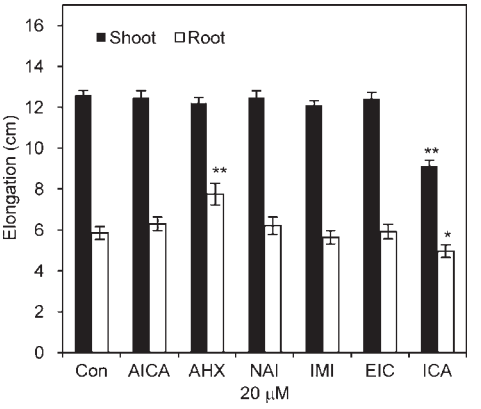

Figure 5. Effect of ICA and its analogues on rice growth. Sterilized rice seeds were sown on 0.85% w/v agar in 50 mL of nutrient solution A containing various samples at a concentration of 20 μM in a deep Petri dish and incubated in a growth chamber under an 18 h photoperiod at 28 °C for 12 days. Results are the mean ± standard error ($n = 20$). NAI, *N*-acetylimidazole; IMI, imidazole. The asterisks indicate significant difference from control (∗, $P < 0.05$; ∗∗, $P < 0.01$).

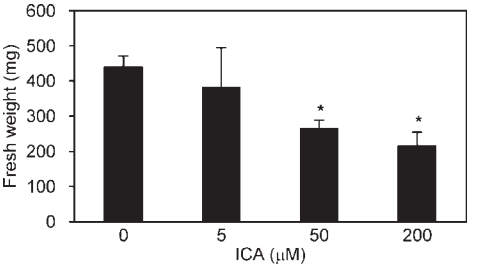

Figure 6. Effect of ICA on fresh weight of lettuce. Sterilized lettuce seeds were sown on 100 mL of 0.85% agar (v/w) containing nutrient solution A with various concentrations of ICA in a deep Petri dish and incubated in a growth chamber under an 18 h photoperiod at 25 °C for 40 days (mean ± standard error, $n = 6$). The asterisk indicates significant difference from control (Student's *t* test; ∗, $P < 0.01$).

This result indicated that the weight per seed in the ICA-treated group was the same as the control, but the number of the seeds in the treated group increased.

It has been reported that various imidazole derivatives, in which AHX and ICA were not included, showed growth-promoting activity toward some plants and that the derivatives acted as the substitute for cytokinin (*14*). Therefore, we examined the relationship between cytokinin and ICA using tea cells. Tea cells were incubated in the presence of a cytokinin, 6-benzylaminopurine (BAP), an auxin, naphthaleneacetic acid (NAA), and/or

Table 1. Effect of ICA on Greenhouse-Grown Rice (*Oryza sativa* L. cv. Nipponbare) in Soil Culture[a]

soil culture trait	control	2 μM ICA (rate of increase, %)
grain yield		
grain yield (g plant^{-1})	36.9 ± 6.43	46.5 ± 6.27* (26%)
wt (g 100 seed^{-1})	2.19 ± 0.04	2.21 ± 0.05
plant size		
panicle length (cm)	20.7 ± 0.56	22.1 ± 3.06
stem length (cm)	84.6 ± 6.52	87.2 ± 5.05
no. of panicles plant^{-1}	27.3 ± 3.78	30.5 ± 2.35
wt of shoot (g plant^{-1})	133.6 ± 15.5	152 ± 7.32

[a] Results are the mean ± standard deviation (n = 6). The asterisk indicates significant difference from control (*, $P < 0.05$).

ICA. Both hormones were necessary for normal cell proliferation of the cells. In the presence of both hormones, ICA inhibited cell proliferation at 200 μM (Figure S4A). When ICA was used instead of BAP or NAA, ICA did not act as a substitute for cytokinin (Figure S4B,C). ICA in the absence of both of the hormones also showed no influence for cell proliferation (Figure S4D). In the previous paper, a similar experiment done using AHX and AHX promoted cell proliferation dose-dependently in the absence of both of the hormones (*7*). These results suggest that the effect of ICA differs from those of the imidazole analogues reported previously and AHX.

The mycelium of the fairy ring forming fungus *Marasmius oreades* interferes with plant−water relationships and produces hydrogen cyanide, polyacetylene, and sesquiterpene, which are capable of damaging grass roots (*15*, *16*). The fungus used in this study, *L. sordida*, also causes a withered zone of dead grass occasionally (**Figure 1B**). The relationship between the inhibitory activity of ICA against turfgrass and the dead grass zone is unclear at the present time.

ICA and AHX have been chemically synthesized from 5-aminoimidazole-4-carboxamide (AICA) (*17*−*19*). AICA-riboside and its monophosphate exist in various organism species (*20*). A previous paper has described that AICA-riboside promoted infection of *Rhizobium* spp. purine auxotrophs and enhanced the development of bean root nodules elicited by the auxotrophs (*21*), and the conversion of AICA-riboside into its monophospate and then inosine monophospate in *Rhizobium etli* has been reported (*20*). In addition, the crystal structure and function of an enzyme that catalyzes formylation of AICA-nucleotide, 5-formaminoimizadole-4-carboxamide ribonucleotide synthetase, from the bacteium *Methanocaldococcus jannaschii* have been published (*22*). Although we have not detected AICA, AICA-riboside and its monophosphate in *L. sordida* yet, it might be possible that the biosynthetic pathway from AICA or its nucleoside to ICA and/or AHX exists in the fungus.

Many challenges, such as the role of ICA in the fairy rings and the fungus, the biosynthetic pathway of ICA and AHX in the fungus, and the metabolism of the compounds in the fungus and plants, remain unsolved. However, the results mentioned above allowed us to conclude that this compound has the possibility for practical use in agriculture.

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Supporting Information Available: X-ray analysis and effect on rice and tea cell. This material is available free of charge via the Internet at http://pubs.acs.org.

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