JARQ 49 (1), 45 - 49 (2015) http://www.jircas.affrc.go.jp

Effect of 2-Azahypoxanthine (AHX) Produced by the Fairy-Ring-Forming Fungus on the Growth and the Grain Yield of Rice

Tatsuo ASAI[1#], Jae-Hoon CHOI[2,3#], Takashi IKKA[3], Keiji FUSHIMI[4], Nobuo ABE[3], Hidekazu TANAKA[3], Yasuhiro YAMAKAWA[3], Hajime KOBORI[4], Yoshikazu KIRIIWA[3], Reiko MOTOHASHI[3], Vipin Kumar DEO[2], Tomohiro ASAKAWA[5], Toshiyuki KAN[5], Akio MORITA[3] and Hirokazu KAWAGISHI[2,3,4*]

[1] Center for Education and Research in Field Sciences, Faculty of Agriculture, Shizuoka University (Shizuoka, Shizuoka 422-8529, Japan)
[2] Research Institute of Green Science and Technology, Shizuoka University (Shizuoka, Shizuoka 422-8529, Japan)
[3] Graduate School of Agriculture, Shizuoka University (Shizuoka, Shizuoka 422-8529, Japan)
[4] Graduate School of Science and Technology, Shizuoka University (Shizuoka, Shizuoka 422-8529, Japan)
[5] School of Pharmaceutical Sciences, University of Shizuoka (Shizuoka, Shizuoka 422-8526, Japan)

Abstract

To examine the effect of 2-azahypoxanthine (AHX) on rice plant (*Oryza sativa* L. cv. Nipponbare) growth, we carried out pot and field experiments. AHX was applied at 50 µM for two weeks at four growth stages (transplanting, tillering, panicle formation and ripening stages) in the pot experiment, and 1 mM AHX at three stages [at rising of seedling in nursery boxes (seedling treatment), transplanting and panicle formation stages] in the field experiment. Both pot and field experiments showed a tendency toward increased panicle number (PN), culm length (CL) and plant dry weights with AHX treatments. Brown rice yields were also improved by AHX treatments, especially when applying at stages of tillering and panicle formation and seedling and transplanting during pot and field experiments, respectively. In the latter, yield increased drastically up to 18.7, 15.8, 9.6 and 5.8% of control. However, panicle length and 1000-grain weight were not affected by AHX application. These results suggested that AHX increased the brown rice yield through its effects on PN and/or CL.

Discipline: Agricultural chemicals
Additional key words: fairy chemical, *Lepista sordida*

Introduction

Fairy rings are zones of stimulated turf-grass growth (Shantz & Piemeisel 1917), which appear as virtually continuous, circular bands of turf-grass that are dark green and faster-growing than adjacent plants of the same species. The rings are produced by interaction between grasses and fungi (Shantz & Piemeisel 1917) and fruiting bodies of fungi (mushrooms) usually appear on the rings after the simulation. The term “fairy rings” originates in the myths and superstitions associated with their occurrence in the Middle Ages, which was a phenomenon attributed to “fairies” before our study. In the previous paper, we investigated the possibility of a specific plant growth-regulating substance(s) being produced by one of the fairy-ring forming fungi, *Lepista sordida*, and succeeded in isolating and identifying a “fairy” (plant-stimulating compound), 2-azahypoxanthine (AHX), in the fungus (Fig. 1) (Choi et al. 2010). The growth-promoting activity of AHX was observed toward all plants tested, regardless of their families (turf-grass, rice, potato and lettuce). In addition, when biomass crops, rice or potato, were cultivated with 5 or 50 µM AHX, the yield per plant increased by 25.5% (rice), 19.3 %

[#]These authors contributed equally to this work.
*Corresponding author: e-mail achkawa@ipc.shizuoka.ac.jp
Received November 5 2013; accepted April 30 2014.

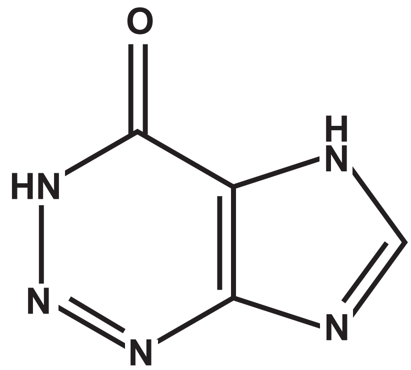

Fig. 1. Structure, chemical formula and molecular weight of AHX

(potato, total yield) or 40.6% (potato, esculent size) in greenhouse experiments (Choi et al. 2010). These groundbreaking results encouraged us to do further study.

In this report, we describe details of the above result concerning rice yield in the greenhouse experiment and further investigate the effects of AHX on the growth and grain yield of rice in a field experiment as the first step toward practically using the "fairy".

Materials and methods

1. Synthesis of AHX

AHX was synthesized according to the method reported previously with slight modification (Vaughan et al. 2002). Briefly, 5-aminoimidazole-4-carboxamide hydrochloric acid salt (62 g) was dissolved in water (750 ml) and conc. hydrochloric acid (40 ml), cooled to 0 °C and then slowly added to a well-stirred ice-cold solution of sodium nitrite (100 g) in water (350 ml). After the mixture had been stirred for 30 min, orange precipitates were collected by filtration. After the filtrate was washed with water, the dried precipitates were suspended in tetrahydrofuran (THF) (400 ml) and stirred for 1 h. The THF solution was removed by filtration. The orange filtrate was re-suspended in water (500 ml) and treated with 28% aqueous ammonia (300 ml). The resulting clear brown solution was stirred for a few minutes. The mixture was evaporated to dryness and the residue was dissolved in hot MeOH. After cooling to room temperature, the generated red precipitates were removed by filtration. Activated carbon powder was added to the yellow filtrate and allowed to stand for 3 h. The mixture was filtered and evaporated under reduced pressure, eliciting AHX as an off-white solid.

2. Pot experiment

After surface-sterilization, one thousand rice seeds (*Oryza sativa* L. cv. Nipponbare) were sown on May 10, 2009 and germinated and grown in a nursery box (30 × 60 cm) filled with artificially prepared fertilized soil (N 1500 mg, P_2O_5 1500 mg, K_2O 1500 mg per 2.5 kg soil per nursery box). On June 10, one seedling was transplanted into a 1/5000 Wagner pot filled with artificially fertilized soil (N 1440 mg, P_2O_5 1200 mg, K_2O 760 mg and CaO 806 mg per pot). At the panicle formation stage, the soil was dressed with 300 mg of nitrogen [as $(NH_4)_2SO_4$] per pot as the fertilizer. Surface irrigation was applied after transplanting to retain water 5 cm deep until harvest. The seedlings were cultivated in a greenhouse at the Faculty of Agriculture in Shizuoka University, Shizuoka, Japan. The fertilizers and fertilized soil used in this study were purchased commercially (JA Ooigawa, Japan).

The seedling were divided into five experimental groups (four kinds of treatments and control); four liters of 50 μM AHX was applied for two weeks at (1) transplanting (June 10-24), (2) tillering (June 30 to July 13), (3) panicle formation (July 29 to August 12) and (4) ripening (August 31 to September 13) stages, respectively. The control was applied tap water instead of AHX solution and each treatment was carried out with five replicates.

3. Field experiment

Surface-sterilized rice seeds (*O. sativa* L. cv. Nipponbare) were sown on April 29 in 2010, and germinated and grown in nursery boxes filled with artificially prepared fertilized bed soil, as in the pot experiment. Their plants were grown in the experimental paddy field at the Center for Education and Research in Field Science of Shizuoka University in Fujieda (34°9'N, 138°3'E), Shizuoka, Japan.

On June 4, the seedlings were transplanted into the paddy field, with a single plant per hill, spaced at 15 × 30 cm and 22 plants per row. The treatment block was ten rows (3.0 × 3.3 m = 9.9 m^2). According to a conventional cultivation method, a commercial chemical fertilizer (as described above) was applied at a rate of 46, 55 and 46 kg of N, P_2O_5 and K_2O per ha as a basal dressing fertilizer on June 1, and 28, 12 and 36 kg of N, P_2O_5 and K_2O per ha respectively as a topdressing fertilizer at the panicle formation stage on July 21. Surface irrigation was applied after applying the basal fertilizer, and then mid-season drainage was performed from July 7-26. After the drainage, full irrigation was reapplied until grain harvesting.

AHX was applied in three stages: when the seedlings rose in nursery boxes (seedling treatment), transplanting, and panicle formation stages. During the seedling treatment, one liters of 1 mM AHX solution per nursery box was applied daily for two weeks before the transplanting. During transplanting and panicle formation treatments, 50 liters of 1 mM AHX solution was applied per block (9.9 m^2) at transplanting (June 4) and during the panicle formation stage (July 21), respectively. The control treatment involved applying tap water instead of AHX solution. The AHX application rate, 1 mM, was decided on as follows. The water volume of surface irrigation in one block (9.9 m^2) was approximately 1000 liters, assuming irrigation around 10 cm deep, which meant the amount of AHX in 50 liters of 1 mM AHX corresponded to 1000 liters of 50 μM AHX as in the pot experiments. To mitigate the effect of the neighboring block, corrugated tinplates (30 cm high) were placed into the ground 5 cm deep along the borderlines between each block before transplanting and each experiment was carried out in triplicate.

4. Measurements of agronomic characters and yield

Rice was harvested on September 30 in 2009 in the pot experiment and September 29 in the field experiment. After the matured stage, the culm length (CL), panicle length (PL) and panicle number (PN) of each plant were measured. The rice was threshed by hand and dried in the greenhouse, whereupon the dry weight of each plant, 1000-grain weight and brown rice yield were measured.

5. Eating quality of brown rice

The seeds harvested in the field experiment were subjected to a rice-eating quality test and the values of the eating quality and the contents of free fatty acid, amylase, protein and water were measured by a rice-grain taste analyzer (RCTA11A, Satake, Tokyo, Japan).

6. Statistical analysis

Data were statistically analyzed using Tukey's test to determine significant differences in the data among the groups using Microsoft Excel 2003 (Microsoft). P values less than 0.05 were considered significant. The values are represented as mean ± S. D.

Results

Table 1 shows the result of the pot experiment, showing no significant differences in CL, PL and 1000-grain weight among all treatments. Conversely, the dry weight of plant significantly increased by AHX treatment at the ripening stage. Moreover, brown rice yields in all AHX treatment groups exceeded those in control. In particular, AHX application at tillering and panicle formation stages drastically increased the yield up to 18.7 and 15.8% of the control, respectively. During cultivation, no difference in leaf colors emerged among the treatments (data not shown), nor were necrosis and chlorosis in rice leaves observed in any of the treatments (data not shown).

The field experiment results are shown in Table 2. The PL and 1000-grain weight for all the groups remained the same. The culm tended to become longer in AHX treatments than control treatment, but the only significant difference compared to the control was observed in the panicle formation treatment. The PN of each plant in the seedling and the transplanting treatments were significantly higher than that of control. Plant dry weight was obviously heavier in all the AHX treatments than in control. Brown rice yield was also higher for all the AHX treatments, with AHX application during seedling and transplanting in particular greatly increased up to 9.6 and 5.8% of control, respectively.

The eating quality and contents of free fatty acid, amylase, protein and water of brown rice in the field experiment were shown in Table 3; proving that AHX treatments did not affect the values.

Table 1. Effect of applying AHX on the growth of rice cultured in soil-filled pots

Treatment	Panicle number per plant	Culm length (cm)	Panicle length (cm)	Dry weight of plant (g/plant)	Weight of 1000 grains (g)	Brown rice yield (g/plant)
Control	28.8 ± 1.3[a]	82.5 ± 4.5[a]	20.9 ± 0.4[a]	136.4 ± 4.4[a]	21.9 ± 0.5[a]	38.6 ± 3.4[a]
AHX Application						
Transplanting	31.0 ± 2.0[ab]	87.0 ± 4.3[a]	20.7 ± 0.2[a]	140.3 ± 3.8[ab]	21.6 ± 0.5[a]	40.5 ± 3.4[ab]
	(107.6)	(105.5)	(99.0)	(102.9)	(98.6)	(104.9)
Tillering	31.8 ± 1.5[b]	86.5 ± 2.9[a]	20.6 ± 1.1[a]	145.8 ± 12.0[ab]	22.1 ± 0.3[a]	45.8 ± 4.7[b]
	(110.4)	(104.8)	(98.6)	(106.9)	(100.9)	(118.6)
Panicle formation	28.8 ± 5.5[a]	84.3 ± 5.8[a]	20.6 ± 1.5[a]	141.8 ± 11.0[ab]	22.5 ± 0.5[a]	44.7 ± 3.6[b]
	(100.0)	(102.2)	(98.6)	(104.0)	(102.7)	(115.8)
Ripening	28.6 ± 3.4[a]	86.1 ± 4.0[a]	21.2 ± 0.8[a]	147.7 ± 6.0[b]	22.0 ± 0.6[a]	43.5 ± 6.5[ab]
	(99.3)	(104.4)	(101.4)	(108.3)	(100.5)	(112.7)

Values are means (n = 5)
Data followed by the same letter within columns do not differ significantly according to Turkey's test ($P < 0.05$)

Table 2. Effect of applying AHX on the growth of rice cultured in paddy fields

Treatment	Panicle number per plant	Culm length (cm)	Panicle length (cm)	Dry weight of plant (g/plant)	Weight of 1000 grains (g)	Brown rice yield (g/plant)
Control	11.4 ± 2.5[a]	65.6 ± 2.5[a]	21.0 ± 1.4[a]	50.9 ± 4.5[a]	21.6 ± 0.2[a]	18.2 ± 2.1[a]
AHX Application						
Seedling	13.1 ± 2.3[b]	66.1 ± 2.5[ab]	21.5 ± 1.5[a]	54.4 ± 5.6[b]	21.6 ± 0.4[a]	20.0 ± 2.0[b]
	(114.9)	(100.7)	(102.7)	(106.9)	(100.0)	(109.9)
Transplanting	13.1 ± 2.5[b]	66.9 ± 2.9[ab]	20.9 ± 1.5[a]	54.0 ± 3.7[b]	21.8 ± 0.5[a]	19.3 ± 1.0[b]
	(114.6)	(102.1)	(99.7)	(106.1)	(100.9)	(106.0)
Panicle formation	11.8 ± 2.4[ab]	67.3 ± 2.2[b]	21.0 ± 1.2[a]	54.0 ± 3.6[b]	21.9 ± 0.2[a]	19.3 ± 1.9[ab]
	(103.5)	(102.6)	(100.1)	(106.1)	(101.4)	(106.0)

Values are means (n = 3)
Data followed by the same letter within columns do not differ significantly according to Turkey's test ($P < 0.05$)

Table 3. Effect of applying AHX on the eating quality of brown rice

Treatment	Eating quality Value	Concentration (mg g-1)			Free fatty acid value (KOH mg kg^{-1})
		Amylose	Protein	Water	
Control	66.7 ± 1.5[a]	197.7 ± 1.2[a]	90.0 ± 0.0[a]	117.7 ± 1.5[a]	172.0 ± 14.7[a]
AHX Application					
Seedling	66.3 ± 1.5[a]	197.7 ± 1.5[a]	90.7 ± 0.6[a]	115.3 ± 0.6[a]	164.7 ± 18.8[a]
Transplanting	66.3 ± 0.6[a]	198.0 ± 1.0[a]	90.3 ± 1.2[a]	118.3 ± 1.2[a]	176.3 ± 15.9[a]
Panicle formation	66.3 ± 0.6[a]	197.7 ± 0.6[a]	91.3 ± 0.6[a]	116.7 ± 2.1[a]	162.7 ± 8.6[a]

Values are means (n = 3)
Data followed by the same letter within columns do not differ significantly according to Turkey's test ($P < 0.05$)

Discussion

In the previous paper, we disclosed the "fairy (plant growth principle, AHX)" from the fairy-forming fungus *Lepista sordida*. Furthermore, we also reported that the grain yield of rice increased following AHX-treatment in greenhouse experiments as supplemental data (Choi et al. 2010). In this study, we showed details of the greenhouse experiment and the effects of AHX on rice in a field experiment.

In both pot and field experiments, AHX treatments tended to raise the PN, CL and dry weight of plants compared to the control (Tables 1 and 2). Furthermore, AHX increased the brown rice yield; at field as well as pot levels. Conversely, AHX application did not affect PL, 1000-grain weight or the eating quality of rice (Table 3). These results indicated that AHX does not affect the size of rice grain and contents, and the increase in brown rice yield attributable to AHX treatment might be due to the increase in PN and/or CL per plant. AHX has a high content of nitrogen (51%) in the molecule (Fig. 1), while rice-grain production can be increased by nitrogen fertilizer (Mae 1997). However, adding $(NH_4)_2SO_4$ corresponding to the nitrogen content in the AHX molecule did not affect the rice-grain yield at all in the pot experiment (data not shown). Previously, the activity of AHX on rice was analyzed by oligo-DNA microarrays containing 44,000 genes and reverse transcriptase-polymerase chain reaction (RT-PCR) (Choi et al. 2010). The most presumable result was the induction of *glutathione S-transferase* (*GST*), *aquaporin*, *OsTIP2;1* and *Bowman-Birk-type proteinase inhibitor* (*BBI*) expressions in both 50 and 200 μM AHX-treated seedlings of rice. It has been reported that *GST* genes were transferred into rice and other plants to increase tolerance for various stresses such as low temperature or salt (Takesawa et al. 2002, Zhao & Zhang 2006). BBI has also been reported to increase plant tolerance to salt stress (Shan et al. 2008). In the previous study, AHX-treatment of rice enhanced its germination under low-temperature stress (15 °C) and shoot growth under salt stress (0.1 M NaCl) (Choi et al. 2010). In addition, tonoplast intrinsic proteins (TIPs) are the major components of vacuolar membranes and the most abundant aquaporins in plants (Loque et al. 2005). In addition to the water-channel function of the *Arabidopsis thaliana* aquaporins, AtTIP2;1 and AtTIP2;3 and the *Triticum aestivum* (L), TaTIP2 have been demonstrated to transport ammonia/ammonium ions (Jahn et al. 2004). AHX increased ^{15}N absorption from $^{15}NH_4NO_3$ into rice plants, especially into the root where *OsTIP2;1* was up-regulated by AHX, while when $NH_4{}^{15}NO_3$ was used, ^{15}N absorption into the plant did not increase at all (Choi et al. 2010). These results suggest that the effect of AHX on the rice grain was not due to the nitrogen in the AHX molecule that possibly might be used as a nitrogen fertilizer. In both pot and field experiments, the brown rice yields and plant dry weights increased by AHX treatment, but the rates of increase among them did not differ significantly, which suggested that AHX could increase the growth and yield of rice, irrespective of when applied. Furthermore, this also suggested that promoting AHX activity toward the growth and the yield of rice remained for a long period from seedling stage. Accordingly, the AHX function might be the turning point; acting as a switch that stimulates vegetable and reproductive growth in the early-growth stages, such as a plant hormone. However, many hormone-related genes were unaffected by AHX treatments when analyzed by microarray and RT-PCR (Choi et al. 2010). It thus suggested that AHX might have promoted growth through a pathway other than those known; involving plant hormones. However, its mechanism remains unknown.

When AHX was applied at the seedling stage, although only in the field experiment, CL was the lowest among AHX treatments, but the brown rice yield significantly exceeded that of control. The rice plant susceptibility to lodging rose with increasing plant height. In addition, the total AHX needed for the seedling treatment was much lower compared to other treatments. These results allow us to conclude that AHX treatment at the seedling stage is the best and most practical in agriculture among all treatments tried in this study. We examined only one rice cultivar, Nipponbare, to elucidate AHX activities on rice growth and yield to date. Between subspecies and cultivars of rice, differences in nutrient responses and stress tolerances may emerge. To bring AHX into practical use in agriculture, it is necessary to examine its effects on other cultivars, including subspecies indica cultivars.

Acknowledgment

This work was partially supported by a Grant-in-Aid for the Programme for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry and a Grant-in-Aid for Scientific Research on Innovative Areas “Chemical Biology of Natural Products” from The Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

Choi, J. H. et al. (2010) Disclosure of the “Fairy” of fairy-ring-formation fungus *Lepista sordida*. *ChemBioChem*., **11**, 1373-1377.

Jahn, T. P. et al. (2004) Aquaporin homologues in plants and mammals transport ammonia. *FEBS Lett*., **574**, 31-36.

Loque, D. et al. (2005) Tonoplast intrinsic proteins AtTIP2;1 and AtTIP2;3 facilitate NH_3 transport into the vacuole. *Plant Physiol*., **137**, 671-680.

Mae, T. (1997) Physiological nitrogen efficiency in rice: Nitrogen utilization, photosynthesis, and yield potential. *Plant Soil*, **198**, 201-210.

Shan, L. et al. (2008) A Bowman-Birk type protease inhibitor is involved in the tolerance to salt stress in wheat. *Plant Cell Environ*., **31**, 1128-1137.

Shantz, H. L. & Piemeisel, R. L. (1917) Fungus fairy rings in eastern Colorado and their effect on vegetation. *J. Agric. Res*., **11**, 191-245.

Takesawa, T. et al. (2002) Over-expression of ζ glutathione *S*-transferase in transgenic rice enhances germination and growth at low temperature. *Mol. Breed*., **9**, 93-101.

Vaughan, K. et al. (2002) A ^{15}N NMR investigation of a series of benzotriazinones and related antitumour heterocycles. *Magn. Reson. Chem*., **40**, 300-302.

Zhao, F. & Zhang. H. (2006) Salt and paraquat stress tolerance results from co-expression of the *Suaeda salsa* glutathione *S*-transferase and catalase in transgenic rice. *Plant Cell Tiss. Organ Cult*., **86**, 349-358.