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Distribution and Function of Allantoin (5-Ureidohydantoin) in Rice Grains

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ABSTRACT: Despite increasing knowledge of allantoin as a phytochemical involved in rice, relatively little is known about its distribution and function in rice grains. In this study, allantoin was quantified in 15 Chinese rice grains, and its contents varied with grain fraction, cultivar, and genotype. Bran always had the highest allantoin level, followed by brown rice and milled rice. Hull contained the lowest allantoin content. Allantoin in japonica bran ranged from 70 to 171 μg/g but rarely exceeded 100 μg/g in indica bran. There was a positive relationship between allantoin level in grains and seedling survival in seedbeds under low temperature or water deficit. Exogenous allantoin stimulated plant growth, increased soluble sugar and free proline contents, and decreased malondialdehyde content in rice seedlings. However, allantoin did not show any antioxidant activity through free radical-scavenging capacity, reducing power, linoleic acid peroxidation inhibition, and chelating activity. The results suggest that allantoin in rice grains may play some roles in providing plant stress protection but not serving as a beneficial health antioxidant.

KEYWORDS: *rice grain, allantoin, function, seedling survival, plant stress protection, antioxidant activity*

INTRODUCTION

Rice (*Oryza sativa* L.) is a staple of the Chinese diet, and its significant importance has led to large areas of rice cultivation throughout China. This cereal is a source of energy, due to its high starch content, also providing nutrient composition including proteins, lipids, vitamins, and minerals.[1,2] In addition, rice grains contain an enormous variety of potentially valuable low molecular mass compounds, such as phenolic acids, flavonoids, steryl ferulate esters, and alkaloids.[3−6] These phytochemical constituents in rice grains contribute to antioxidant capacity, pharmacology, and other health-related benefits[4,6,7] and/or serve as an agent participating in the defense of rice against other organisms during seed germination and seedling growth.[8,9] Besides the compounds mentioned above, there may still be novel phytochemicals and their functions in rice grains to be discovered.

Allantoin (5-ureidohydantoin), a heterocyclic nitrogen compound derived from a purine, is well documented in many plant species, particularly in legumes.[10] An increasing number of studies have clearly shown that a wide variety of rice cultivars can produce considerable amounts of allantoin in their tissue sources and grains.[11−13] However, its distribution and, in particular, its potential functions and implications in rice grains remain obscure. A few studies have indicated that allantoin may act as an agent participating in chemical interactions between plants and other species including insects,[14] phytonematodes,[15] plants,[16] and microorganisms.[17] Applying allantoin on seedbeds and nurseries may improve the emergence and growth of rice seedlings in local practices in China.[18,19] However, the potential mechanisms are largely unknown.

Allantoin plays an essential role in the assimilation, metabolism, transport, and storage of nitrogen in plants.[10] In particular, the oxidation of ureides including allantoin and allantoate is involved in recycling of nitrogen from stressed, senescent tissues when ureides are accumulated in legumes under water deficit.[20] Allantoin has been well studied as a marker of oxidative stress in humans.[21,22] A series of studies from Russian scientists have shown that allantoin is an in vivo antioxidant as a vitamin.[23−25] Accordingly, allantoin as a phytochemical occurring in rice grains would be likely to play some roles in providing plant growth and stress resistance ability during seed germination and seedling stage, serving as a beneficial health antioxidant in humans, or both. Therefore, further studies are justified. Here we selected 15 Chinese rice cultivars of various genotypes to examine the distribution of allantoin in grains and to analyze the relationship between allantoin level in grains and seedling survival in paddy fields under low temperature or water deficit in early spring. Furthermore, allantoin's growth-stimulating effect on rice seedling and its antioxidant activity were evaluated in an attempt to further our understanding of the ecological functions and health-related implications of allantoin in rice grains.

MATERIALS AND METHODS

Rice Cultivars and Chemicals. Fifteen Chinese rice cultivars were used in this study (Table 1). These cultivars were selected on the basis of their various genotypes and commercial importance in the local rice industry. Among these cultivars, eight indica cultivars were planted in Guangzhou (southern China) and seven japonica cultivars were planted in Shenyang (northeastern China), which are their favorable growth regions in China.

Authentic allantoin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and vitamin C (ascorbic acid) were obtained from Sigma-Aldrich Co. Organic solvents and other chemicals were purchased from

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Table 1. Content and Distribution of Allantoin in Rice Grains of Various Cultivars and Genotypes[a]

cultivar	growth region, city	genotype	content (μg/g)			
			hull	bran	brown rice	milled rice
Huagan-1	Guangzhou	indica-inbred	2.68 ± 0.27bA	34.20 ± 4.11aC	14.15 ± 1.39cdeB	4.38 ± 0.24bcA
Huajingxian	Guangzhou	indica-inbred	0.92 ± 0.21aA	39.84 ± 4.21abC	15.49 ± 1.94cdeB	10.02 ± 1.26fhB
Huayou-354	Guangzhou	indica-hybrid	4.02 ± 0.62cA	95.60 ± 12.18defC	26.37 ± 3.01fB	4.89 ± 0.51cdA
Peizaruanxiang	Guangzhou	indica-hybrid	0.10 ± 0.01aA	50.6 ± 4.72abcD	7.45 ± 0.38abA	1.67 ± 0.12abA
Simiaoxuan	Guangzhou	indica-inbred	14.12 ± 3.61eA	108.02 ± 11.29efgB	15.92 ± 1.81cdeA	7.78 ± 0.96defA
Youyou-128	Guangzhou	indica-hybrid	0.10 ± 0.02aA	61.17 ± 3.79abcC	18.22 ± 1.92cdeB	5.63 ± 0.77cdeA
Yuexiangzhan	Guangzhou	indica-inbred	13.14 ± 1.52eA	70.49 ± 8.93bcB	16.56 ± 1.86cdeA	10.31 ± 1.24fhA
Zaoliangyou	Guangzhou	indica-hybrid	4.00 ± 0.27cA	46.34 ± 3.19abcB	6.49 ± 0.72aA	0.10 ± 0.01aA
Liaojing-9	Shenyang	japonica-hybrid	2.78 ± 0.23bA	78.28 ± 13.27cdeC	16.80 ± 1.78cdB	3.72 ± 0.46bcA
Liaojing-371	Shenyang	japonica-hybrid	7.93 ± 1.21dA	148.94 ± 12.14hiB	12.19 ± 0.16abcA	3.98 ± 0.40bcA
Liaoxing-1	Shenyang	japonica-inbred	1.27 ± 0.32aA	122.27 ± 26.53fghB	14.36 ± 0.64cdA	4.25 ± 0.29bcA
Liaoxing-8	Shenyang	japonica-inbred	1.12 ± 0.19aA	128.88 ± 22.40ghB	15.93 ± 1.27cdeA	4.79 ± 0.19cdA
Liaoxing-10	Shenyang	japonica-inbred	1.04 ± 0.28aA	171.26 ± 26.11iB	17.59 ± 0.49cdeA	8.00 ± 0.26efA
Qiuguang	Shenyang	japonica-inbred	1.12 ± 0.23aA	69.83 ± 11.18bcdC	19.59 ± 0.49cdeB	12.24 ± 1.29hB
Yanfeng-47	Shenyang	japonica-inbred	0.80 ± 0.12aA	135.77 ± 19.33bhiB	12.75 ± 0.27bcdA	4.39 ± 0.16bcA

[a]Data in a row followed by the same upper case letter between grain fractions within each cultivar or data in a column followed by the same lower case letter among cultivars are not significantly different at $P < 0.05$ according to analysis of variance (ANOVA) followed by Tukey's honestly significantly different tests.

commercial sources in Chinese markets and were of the highest purity available.

Experimental Sites. Two experimental sties were used for rice cultivation and field trials in this study. One is the Experimental Station of South China Agricultural University (Guangzhou, Guangdong province, southern China, N 23° 10′, E 113° 22′), and the other is Shenyang Experimental Station of Chinese Academy of Sciences (Shenyang, Liaoning Province, northeastern China, N 41° 31′, E 123° 24′). Guangzhou is in a subtropical climate zone, with a mean annual temperature of 21.8 °C and an annual rainfall of 1682 mm, whereas Shenyang is in a continental monsoon climate zone, with a mean annual temperature of 7.5 °C and an annual precipitation of 700 mm.

Sample Preparation for Rice Grains. During the 2008 growing season, fresh rice grains (4 kg bulk samples of each cultivar) were harvested from two experimental stations described above, respectively. Each of 15 sun-dried grains was each husked by hand to yield hull and brown rice. The brown rice was polished by a Cyclone Sample Mill (China Agricultural Machine Co., Beijing, China) to yield bran and milled rice. Hull, bran, brown rice, and milled rice were carefully isolated from the whole grain for each cultivar, and then they were ground and passed through a 1 mm sieve screen prior to solvent extraction and analysis by HPLC for the quantification of allantoin described below.

Brown rice and milled rice of a japonica cultivar (Liaoxing-8) and an indica cultivar (Youyou-128) described above were each placed in a series of glass bottles and stored in the dark at a temperature of 25 °C in a thermostatically controlled incubator. The bottles were randomly taken out from the incubator after various storage time intervals (3, 6, 9, or 12 months). Stored brown rice and milled rice were immediately ground and then screened to yield samples for quantitative analysis of allantoin described below.

Quantitative Analysis of Allantoin. Dry powder (5 g) of each for samples of hull, bran, and brown and milled rice described above was homogenized by a Waring Homogenate Machine (Waring Co., Hartford, CT, USA). Homogenates were extracted with 150 mL of 30% aqueous methanol (agitated for 24 h at a temperature of 25 °C, then centrifuged at 2400*g* for 30 min), respectively. The filtrates were each concentrated under vacuum at 40 °C to give an aqueous residue. The residues were dissolved in 50% aqueous methanol and loaded onto reversed phase C_{18} Sep-Pak cartridges (Waters Co.) equilibrated with water, which were eluted with 50% aqueous methanol and then methanol. The methanol fraction was concentrated with nitrogen gas to obtain 100 μL concentrates for quantitative analysis.

The quantification of allantoin in various samples was carried out with an HPLC HP-1100 (Agilent Co.) equipped with a C_{18} reverse phase column (Hypersil 100 mm × 4.0 mm, 5 μm) with a diode array detector. HPLC determination conditions were as follows: mobile phase was a mixture of H_2O/MeCN/HOAC (188:11:1, v/v/v), eluted at a flow rate of 0.85 mL/min at a temperature of 40 °C and detected at 216 nm. The injection volume of samples was 10 μL. Allantoin peaks were identified by their retention time (ca. 3.16 min.) and coelution with the authentic standard and quantified by comparison of the peak areas of samples with those of authentic standard. Working standard solutions ranging from 1 to 200 μg/g were prepared by dilution to establish a calibration curve. The quantification was achieved by regression analysis of the peak areas against standard concentrations.

Field Trials. Two rice fields were selected from the experimental stations in Guangzhou and Shenyang described above, respectively. The fields were each divided into numerous plots (2 × 3 m) that were in a completely randomized design with three replicates for each rice cultivar tested. Each plot was separated by trenches with at least 20 cm discard strips on each side. All plots received fertilizer treatment (N, P_2O_5, and K_2O at rates of 7.5, 9.0, and 8.5 g/m^2, respectively) 7 days before sowing. Seed viability of all cultivars was examined using germination percentage (>98%) according to the rules of the local rural administration, and each was sown into the plots at a density of 3.0×10^5 seeds/ha during the 2009 growing season.

One set of seedling survival under low temperature was investigated in the plots in Guangzhou. Eight indica cultivars were respectively sown on April 1, 2009. This sowing date was 2 weeks earlier than the recommended date (April 14) for direct seeding in local practices. A temperature of 18.6 °C on the sowing date was lower than a mean temperature of 22.2 °C in April (mean rainfall of 110 mm in April 2009). A second set was treated with water deficit in Shenyang. Seven japonica cultivars were respectively sown on the recommended date (May 15, 2009), but soil water content in the plots was maintained at 70% of soil water-holding capacity throughout the whole experiment. There was a mean temperature of 19.5 °C with a mean precipitation of 41 mm in May 2009. The percentages of seedling survival for each of rice cultivars were recorded 30 days after sowing in each plot in Guangzhou and Shenyang, respectively.

Greenhouse Experiments. An indica allantoin-poor rice grain, Huagan-1, and two japonica rice grains, Liaoxing-1 and Qiuguang, were used for evaluating the effect of exogenous allantoin on seedling growth. Presterilized rice seeds were soaked at a temperature of 25 °C in allantoin solution at a concentration of 1 mmol/L for 24 h. This

concentration was a typical dose used for seed-soaking practices in China.[18,19] Ten presoaked seeds with allantoin solution were sown into a plastic pot (8.5 cm × 10.5 cm) with 200 g of soils collected from the two experimental stations described above, respectively. An indica cultivar, Huagan-1, was sown in soil from Guangzhou, whereas two japonica cultivars, Liaoxing-1 and Qiuguang, were sown in soil from Shenyang. The control pots were sown with 10 seeds presoaked with water only.

All pots were placed in a greenhouse maintained at 20−30 °C night and daytime temperatures and 65−90% relative humidity. A randomized, complete-block design was used with six testing blocks and six replicates. Pots were randomized and irrigated with tap water once a day. After emergence, the seedlings were thinned to eight plants per pot. All pots were regularly hand-weeded during the experiments. The seedlings were harvested at the three-leaf stage. Biomass was recorded as dry weights dried for at least 48 h at a temperature of 80 °C. Soluble sugar content was measured according to the procedure of Mohsenzadeh et al.[26] Free proline content was determined as described by Yang et al.[27] Malondialdehyde (MDA) content was evaluated according to the Heath and Packer method.[28] The absorbance of MDA was measured at 532, 600, and 450 nm. The MDA content was calculated according to the formula: MDA (μM) = $6.45(A_{532} - A_{600}) - 0.56A_{450}$.

Assay for Antioxidant Activity. The in vitro antioxidant activity of allantoin was evaluated in a concentration-dependent manner through DPPH free radical-scavenging capacity, reducing power, linoleic acid peroxidation inhibition, and chelating activity. Allantoin was used at doses of 10, 30, 50, 100, and 150 mg/L methanol in the assay. Meanwhile, an authentic antioxidant vitamin C with the same doses as allantoin served as the control to confirm the usefulness of the assay. All measurements were done in triplicate. Effects were evaluated by comparison with the treated and control groups.

DPPH radical-scavenging activity was evaluated according to the procedure of Chung and Shin[6] with some modification. Briefly, 100 μL of allantoin or vitamin C at different concentrations was added to the freshly prepared 0.12 mM (47.3 mg/L) DPPH solution (1.9 mL), respectively. The blank containing methanol only served as the control. The mixture was kept at ambient temperature for 30 min prior to measurement of the absorbance at 517 nm.

The reducing power was determined as described by Aguilar-Garcia et al.[5] Samples (2.5 mL, 1.0 mg/mL) in phosphate buffer (2.5 mL, 0.2 M, pH 6.6) were added to potassium ferricyanide (2.5 mL, 1.0%), and the mixture was incubated at 50 °C for 20 min. Trichloroacetic acid (2.5 mL, 10%) was added, and the mixture (5.0 mL) was mixed with distilled water (5.0 mL) and ferric chloride (1.0 mL, 0.1%), and then the absorbance was recorded at 700 nm.

Inhibition on linoleic acid peroxidation was evaluated using the method developed by Dinis et al.[29] The reaction mixture containing each sample (0.5 mL, 2.0 mg/mL), a solution of linoleic acid emulsion (2.5 mL, 0.02 M), and 0.2 mL of phosphate buffer (pH 7.0, 0.2 M) was incubated at 37 °C for 72 h in the dark. The degree of oxidation was measured by sequentially adding ethanol (4.7 mL, 75%), ammonium thiocyanate (0.1 mL, 30%), sample solution (0.1 mL), and ferrous chloride (20 mM in 3.5% HCl) solution (0.1 mL). The absorbance at 500 nm was recorded as an index of the peroxide value.

The chelating of the sample on ferrous ions was estimated according to the method of Dinis et al.[29] Samples were added to a solution of 2.0 mM ferrous chloride (0.1 mL) and 3.7 mL of methanol. The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL), and the mixture was shaken vigorously and left standing at room temperature for 10 min. The absorbance of the resulting solution was measured at 562 nm.

Data Analysis. The data were presented as the mean ± standard error (SE) from independent experiments for each determination. The data were analyzed using Student's t test or one-way ANOVA, followed by Tukey's honestly significantly different tests. ANOVA and multiple comparisons were carried out with SPSS10.0 program. A simple Pearson's correlation was used to test whether the allantoin content of rice grain across samples correlated with the percent survival rate of rice seedlings in paddy fields. All graphs were performed by SigmaPlot 10 (Systat Software, Inc., USA).

RESULTS AND DISCUSSION

Allantoin was found in grains of all rice cultivars tested (Table 1). A few cultivars produced considerable amounts of allantoin in their grains. However, allantoin content and distribution in rice grains varied greatly with cultivar and genotype. There were higher allantoin contents in japonica rice than in indica rice (Table 1; Figure 1). Regardless of cultivar or genotype,

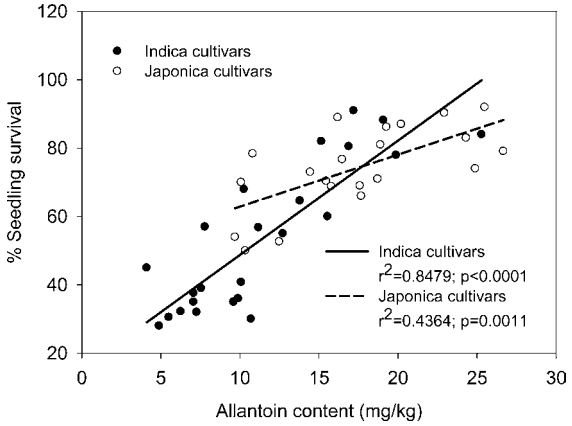

Figure 1. Relationship between seedling survival in paddy fields and allantoin content in grains of various rice cultivars and genotypes in early spring. Percent seedling survival of indica cultivars was investigated under low temperature in Guangzhou (southern China), whereas percent seedling survival of japonica cultivars was investigated under water deficit in Shenyang (northeastern China).

bran always had the highest allantoin content, followed by brown rice and milled rice. Hull contained the lowest allantoin content. In particular, most japonica bran contained allantoin at a high content of >100 μg/g, and the highest content of allantoin (171.26 μg/g) occurred in japonica Liaoxing-10, whereas indica bran rarely exceed 100 μg/g (Table 1). The results agree with an increasing number of studies that antioxidant and other biofunctional phytochemical constituents in rice grains usually occur in bran or brown rice rather than in milled rice.[3,5,7]

Rice growth requires a warm climate and adequate water. However, low temperature or drought occurs frequently during sowing and seedling stage in early spring. Even in some areas of China, a normal seedbed and nursery cannot be achieved due to low temperature or drought. In local practice, allantoin or allantoin-based regulators has been employed for seed-soaking before sowing, which may improve greatly emergence and growth of rice in seedbeds and nurseries under low temperature and drought in early spring.[18,19] However, the reasons for this practice are still being elucidated. To address this, seedling survival of indica cultivars under low temperature in Guangzhou and japonica cultivars under water deficit in Shenyang was investigated in paddy fields in early spring. Subsequently, higher seedling survival rates were observed in japonica rice cultivars and several allantoin-rich indica rice cultivars. There was a positive relationship between allantoin levels in rice grains and seedling survival in paddy fields, with a particularly high correlation (r^2 = 0.85, p < 0.0001) in indica rice cultivars (Figure 1). This result indicated a relationship between allantoin level in grains and rice cultivars resistant to

low temperature or drought during seed germination and early growth stages.

Further seed-soaking experiments in a greenhouse showed that application of allantoin exerted a positive effect on rice seedlings, but the effect was cultivar-dependent (Figure 2).

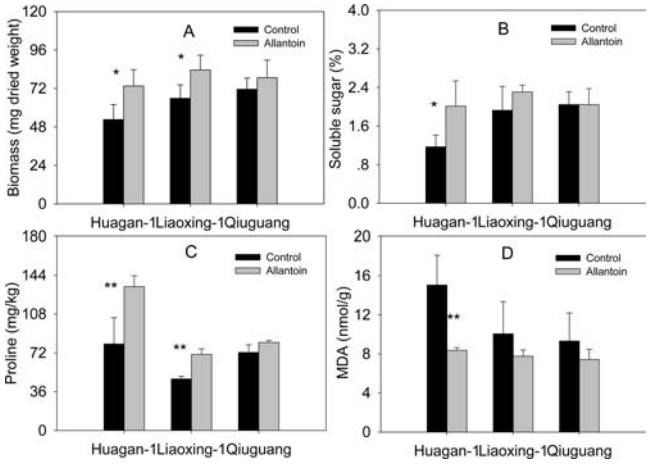

Figure 2. Effect of allantoin application on the growth and physiological parameters of rice seedlings. Rice seeds were soaked by allantoin at a concentration of 1 mmol/L before sowing. Seed-soaking with water only served as the control. * indicates significant difference at $P < 0.05$, and ** indicates significant difference at $P < 0.001$ by Student's t test.

Exogenous allantoin increased plant biomass (Figure 2A), soluble sugar content (Figure 2B), and free proline content (Figure 2C) and decreased MDA content (Figure 2D) in rice seedlings, especially for allantoin-poor indica Huagan-1, at significant rates (Figure 2). Soluble sugar, free proline, and MDA contents can be used as potential indicators of plant resistance to growth stress. These physiological parameters are considered to be sensitive to environmental change, particularly temperature and drought stress.[26,27] It appeared from the results that exogenous allantoin could provide allantoin-poor rice cultivars resistant to low temperature or water deficit. Actually, growth-stimulating allantoin causes changes in the chemical and biological properties of cultivated soils. When released from rice seeds or added to soil, in particular, allantoin stimulates shifts in microbial community composition and increases microbial diversity in the surrounding soil.[12,17] Through improvement of the soil microbial community structure, exogenous or endogenous allantoin may be at least, in part, responsible for rice seedling survival under environmental stress.

The presence of several phytochemical constituents in grains plays an important role in antioxidant activity.[3−6,30] A few studies have indicated the antioxidant capacity of allantoin, which would provide beneficial health effect in humans.[23−25] Allantoin is a unique phytochemical in rice grains; it is warranted to evaluate its antioxidant activity. The antioxidant activity of allantoin was determined in a concentration-dependent manner through its free radical-scavenging capacity, reducing power, linoleic acid peroxidation inhibition, and chelating activity. Subsequently, allantoin had no inhibitory effect on 2,2-diphenyl-1-picrylhydrazyl free radical and linoleic acid peroxidation even at a high concentration of 150 mg/L (Figure 3A,C). Similar results were observed in reducing power and chelating activity of allantoin (Figure 3B,D). Although the

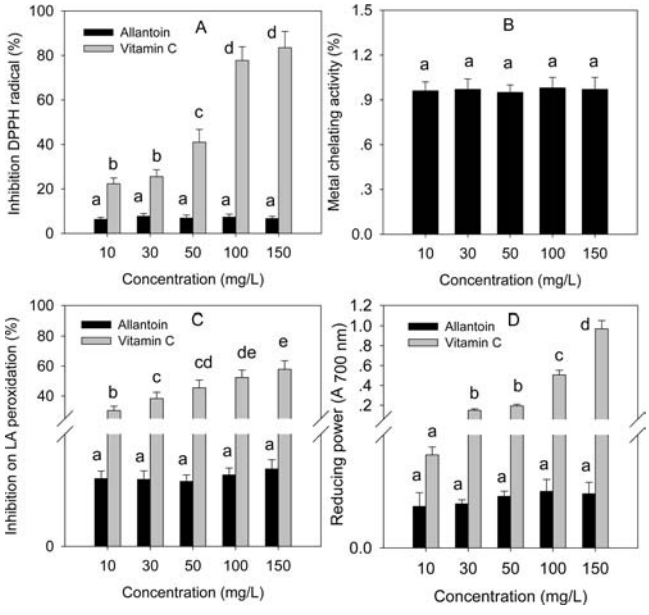

Figure 3. Antioxidant activities of allantoin in a concentration-dependent manner through free radical-scavenging activity (DPPH), reducing power, inhibition of linoleic acid peroxidation, and metal chelating activity (Fe^{2+}). Vitamin C at the same concentrations served as the control. Columns with the same letter are not significantly different at $P < 0.05$, one-way ANOVA, followed by the Tukey's honestly significant difference tests.

antioxidant effect of allantoin can be attributed to its ability to take a direct part in quenching of free radicals, or to reduce the lipid peroxidation rate,[23,24] the data generated in this study showed that allantoin did not show any antioxidant activity. Furthermore, storage led to a rapid decrease in allantoin content, and sufficient quantities of allantoin in both brown rice and milled rice disappeared after 3 months (Figure 4), whereas a normal antioxidant constituent in rice grains would be maintained even after a long storage time.[31,32] Accordingly, allantoin, given its short life in storage, is not appropriate for an antioxidant constituent in rice grains.

The phytochemical constituents in rice grains and their ecological functions and health-related implications have been extensively investigated.[7,9,32] A few phytochemicals in rice grains may offer both ecological functions and health-related benefits. Tricin (5,7,4′-trihydroxy-3′,5′-dimethoxyflavone), a naturally occurring flavone in rice grains, not only acts as an allelochemical inhibiting paddy weeds and soil pathogenic fungi[8,9] but also serves as an antioxidant and putative cancer chemopreventive agent.[7,33] Allantoin is an oxidation product and marker of oxidative stress in humans,[21,22] but its role in plant stress protection is more subtle. A recent study found that allantoin as an oxidation product of ureide oxidation is involved in recycling of nitrogen from stressed tissues under water deficit.[20] This study showed that allantoin in grains could provide rice resistant to low temperature or drought but did not serve as an antioxidant providing beneficial health effects in humans. However, potential mechanisms remain obscure. Further clarification of ecological functions and mechanisms of allantoin in rice grains as a phytochemical constituent of plant stress protection may offer many potential applications and implications in rice production.

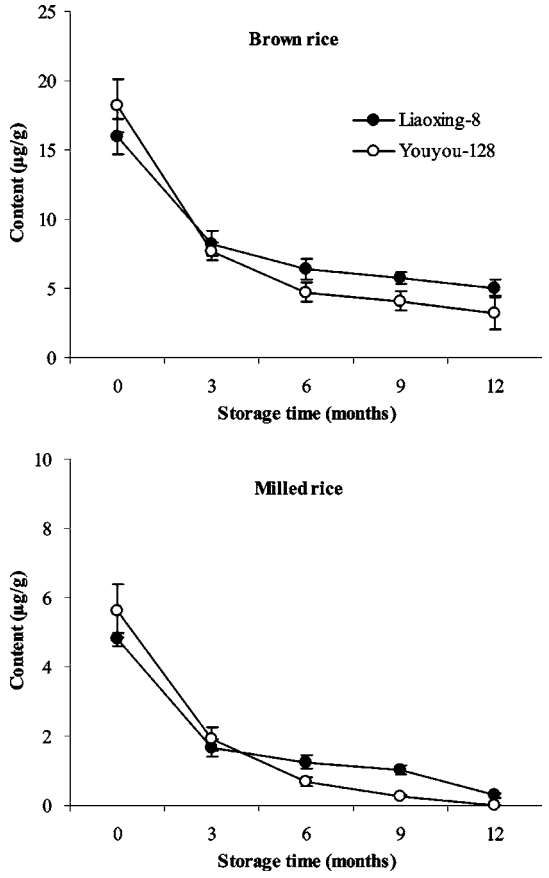

Figure 4. Dynamics of allaltoin contents in brown rice and milled rice with storage time.

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Notes

The authors declare no competing financial interest.

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