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Identification of two phytotoxins, blumenol A and grasshopper ketone, in the allelopathic Japanese rice variety Awaakamai

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ABSTRACT

Aqueous methanol extracts of the traditional rice (*Oryza sativa*) variety Awaakamai, which is known to have the greatest allelopathic activity among Japanese traditional rice varieties, inhibited the growth of roots and shoots of cress (*Lepidium sativum*), lettuce (*Lactuca sativa*), timothy (*Phleum pratense*), *Digitaria sanguinalis*, *Lolium multiflorum* and *Echinochloa crus-galli*. Increasing the extract concentration increased the inhibition, suggesting that the extract of Awaakamai contains growth inhibitory substances. The extract of Awaakamai was purified and two main growth inhibitory substances were isolated and determined by spectral data as blumenol A and grasshopper ketone. Blumenol A and grasshopper ketone, respectively, inhibited the growth of cress shoots and roots at concentrations greater than 10 and 30 μmol/L. The concentrations required for 50% growth inhibition on cress roots and shoots were 84 and 27 μmol/L, respectively, for blumenol A, and 185 and 76 μmol/L, respectively, for grasshopper ketone. These results suggest that blumenol A and grasshopper ketone may contribute to the growth inhibitory effect of Awaakamai and may play an important role in the allelopathy of Awaakamai.

Introduction

Rice has been extensively studied with respect to its allelopathy as part of a strategy for sustainable weed management options, such as breeding allelopathic rice strains (Olofsdotter, 2001; Takeuchi et al., 2001; Olofsdotter et al., 2002; Rimando and Duke, 2003). The first observation of allelopathy in the living rice was made in field examinations in Arkansas, U.S.A., and in which about 190 of 5000 rice accessions inhibited the growth of *Heteranthera limosa* (Dilday et al., 1989). This finding led to a large field screening program. More than 16,000 rice accessions from 99 countries in the germplasm collection of USDA-ARS have been screened. Of these, 412 rice accessions inhibited the growth of *H. limosa* and 145 rice accessions inhibited the growth of *Ammannia coccinea* (Dilday et al., 1994; 1998). In Egypt, 1000 rice varieties were screened for suppressive ability against *Echinochloa crus-galli* and *Cyperus difformis* under field conditions, and inhibitory activity was found in more than 40 varieties (Hassan et al., 1998). Similar attempts have been made in other countries, and many rice varieties have been found to inhibit the growth of several plant species (Kim et al., 1999; Olofsdotter et al., 1999; Azmi et al., 2000; Kato-Noguchi and Ino, 2001). These findings suggest that the living rice plant may produce and release alleochemical(s) into the neighboring environment, thus encouraging the exploration of allelochemicals in rice.

Fujii et al. (2001) found that Awaakamai showed the greatest allelopathic activity among Japanese traditional rice varieties. Awaakamai recorded 96.2% growth inhibition of lettuce seedlings in the bioassay of plant-box-method and inhibited the growth of several weed species, such as *E. crus-galli*, *Monochoria vaginalis* and *Schoenoplectus juncoides*, under field conditions (Fujii et al., 2001; Araya et al., 2003) and laboratory conditions (Kanesawa et al., 2009). However, allelopathic substances in Awaakamai have not yet been determined. The objective of this study was to isolate growth inhibitors causing the allelopathic effect of Awaakamai.

Materials and methods

Plant materials

Japanese traditional rice variety Awaakamai (*Oryza sativa* L.) was grown on the field of University Farm, Kagawa University for 50 days. Leaves and stems of rice plants were then harvested and stored at −20 °C until extraction. Cress (*Lepidium sativum* L.), lettuce (*Lactuca sativa* L.), timothy (*Phleum pratense* L.), *Digitaria sanguinalis* L., *Lolium multiflorum* L. and *Echinochloa crus-galli* (L.) Beauv were used for bioassays as test plants to evaluate the allelopathic activity of Awaasamai.

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Extraction and bioassay

Leaves and stems of rice plants (100 g fresh weight) were extracted with 500 mL of 80% (v/v) aqueous methanol for two days. After filtration using filter paper (No. 2; Toyo, Tokyo, Japan), the residue was extracted again with 500 mL of methanol for two days and filtered, and the two filtrates were combined.

An aliquot of the extract (final assay concentration was 0.01, 0.03, 0.1 or 0.3 g fresh weight rice plant equivalent extract per mL) was evaporated to dryness, dissolved in a 0.2 mL of methanol and added to a sheet of filter paper (No. 2; Toyo Ltd.) in a 3-cm Petri dish. Methanol was evaporated in a draft chamber. Then, the filter paper in the Petri dishes was moistened with 0.8 mL of a 0.05% (v/v) aqueous solution of Tween 20. After germination in the darkness at 25 °C for 16–120 h, 10 seedlings of cress, lettuce, timothy, *D.*, *L. multiflorum* or *E. crus-galli* were sown on the Petri dishes. The length of their shoots and roots was measured after 48 h of incubation in the darkness at 25 °C.

For control treatments, methanol (0.2 mL) was added to a sheet of filter paper in the Petri dish and evaporated as described above. After germination, control seedlings were then placed into the filter paper moistened with the aqueous solution of Tween 20 without the methanol extract. The bioassay was repeated five times using a randomized design with 10 plants for each determination. Significant differences between treatment and control plants were examined by Weich's *t*-test for each test plant species.

Purification of active substances in aqueous methanol extract

Rice plants (1 kg fresh weight) were extracted as described above and the extract was concentrated at 40 °C in vacuo to produce an aqueous residue. The aqueous residue was adjusted to pH 7.0 with 1 mol/L phosphate buffer, partitioned five times against an equal volume of ethyl acetate. The ethyl acetate fraction was evaporated to dryness and chromatographed on a column of silica gel (100 g, silica gel 60, 70–230 mesh; Merck), eluted stepwise with *n*-hexane containing increasing amounts of ethyl acetate (10% per step, v/v; 100 mL per step) and methanol. The biological activity of the fractions was determined using a cress bioassay as described above, and activity was found in fractions obtained by elution with methanol. After evaporation, the residue was purified by a column of Diaion HP-20SS (100 g, Mitsubishi Chemical, Tokyo, Japan), and eluted with 60, 70, 80 and 90% (v/v) aqueous methanol (100 mL per step) and methanol (200 mL). The active fraction was eluted by 70% aqueous methanol and evaporated to dryness. The residue was dissolved 20% (v/v) aqueous methanol (2 mL) and loaded onto reverse-phase C_{18} Sep-Pak cartridges (Waters). The cartridge was eluted with 20, 40, 60, 80% (v/v) aqueous methanol and methanol (15 mL per step). Active fraction was eluted by 20% aqueous methanol and evaporated to dryness. The residue was finally purified by reverse-phase HPLC (20 mm i.d. × 25 cm, ODS_5 C_{18}-PAC; Nacalai, Kyoto, Japan) eluted at a flow rate of 7.5 mL/min with 25% aqueous methanol, detected at 240 nm. Inhibitory activity was found in a peak fraction eluted between 41–42 and 43–44 min, respectively, yielding two active components, inhibitor **2** (9.7 mg) and inhibitor **1** (13.2 mg) as colorless oil. The active substances were characterized by ^{1}H NMR spectra.

Results and discussion

Allelopathic potential of the rice

Aqueous methanol extract of the Japanese traditional rice variety Awaakamai inhibited root and shoot growth of all test plant species, and increasing the extract concentration increased the inhibition (Fig. 1). The extract obtained from 0.3 g fresh weight rice plants inhibited the root growth of cress, lettuce, timothy, *Digitaria sanguinalis*, *Lolium multiflorum* and *E. crus-galli* to 14, 12, 17, 31, 21 and 7.1% of control root growth, respectively, and inhibited the shoot growth of cress, lettuce, timothy, *D. sanguinalis*, *L. multiflorum* and *E. crus-galli* to 15, 6.5, 7.2, 31, 6.8, and 9.1% of control shoot growth, respectively. The extract of rice plants therefore had an inhibitory effect on a wide range of plant species, both dicotyledonous plants (cress and lettuce) and monocotyledonous plants (timothy, *D. sanguinalis*, *L. multiflorum* and *E. crus-galli*). These results suggest that the extract of Awaakamai may contain growth inhibitory substances.

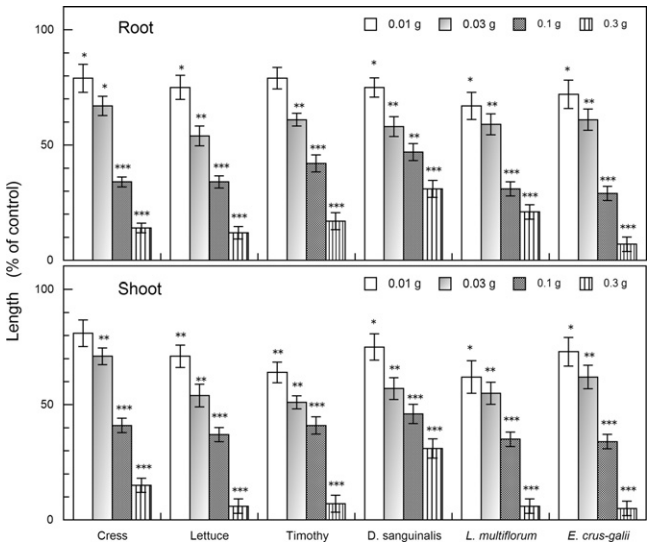

Fig. 1. Effects of aqueous methanol extract of rice plants on root and shoot growth of cress, lettuce, timothy, *D. sanguinalis*, *L. multiflorum* and *E. crus-galli*. Concentrations of tested samples corresponded to the extract obtained from 0.01, 0.03, 0.1 and 0.3 g fresh weight rice plants per mL. Means ± SE from 5 independent experiments with 10 plants for each determination are shown. Asterisk indicates significant difference between control and treatment: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Identification of growth inhibitory substances and biological activity

Two growth inhibitory substances, inhibitors **1** and **2**, isolated from the aqueous methanol extract of rice plants were characterized as follows. The ^{1}H NMR spectrum of inhibitor **1** (400 MHz, CD_3OD, TMS as internal standard) showed δ 5.85 (1H, s, H-8), 4.34 (1H, m, H-3), 2.29 (1H, ddd, J = 12.9, 4.0 and 2.1 Hz, H-4a), 2.18 (3H, s, CH_3-10), 1.99 (1H, ddd, J = 12.7, 4.2 and 2.1 Hz, H-2a), 1.43 (3H, s, CH_3-13), 1.38 (3H, s, CH_3-11), 1.38–1.47 (1H, m, H-4b), 1.32–1.37 (1H, m, H-2b), 1.16 (3H, s, CH_3-12). From the comparison of these data with those reported in the literature (Hwang et al., 2004), this inhibitor was identified as grasshopper ketone (MW 224; Fig. 2).

The ^{1}H NMR spectrum of inhibitor **2** showed δ 5.91 (1H, brs, H-4), 5.87 (1H, dd, J = 15.7 and 5.1 Hz, H-8), 5.79 (1H, d, J = 15.7 Hz,

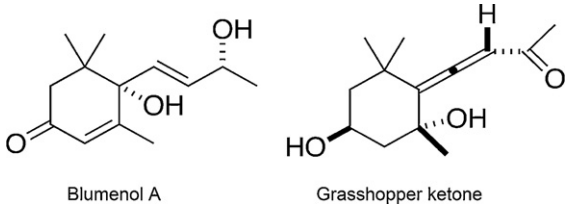

Fig. 2. Chemical structures of two phytotoxins in Awaakamai isolated by bioassay-guided fractionations.