Phytotoxic Substances in Bangladeshi Allelopathic Rice BR 17

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Abstract: Aqueous methanol extracts of Bangladeshi rice (Oryza sativa L.) cultivar BR 17 inhibited the growth of roots and coleoptile of Echinochloa crus-galli extract-concentration dependently. The extracts were then purified by several chromatographic runs, and a phytotoxic active substance was isolated and identified by spectral analysis as (–)-3-hydroxy-β-ionone. The concentration of (–)-3-hydroxy-β-ionone in BR 17 was higher than that of two other phytotoxic substances, 9-hydroxy-4-megastigmen-3-one and 3-oxo-α-ionol, found in BR 17. The novel isolated (–)-3-hydroxy-β-ionone inhibited the growth of E. crus-galli at concentrations higher than 10 μM. Those phytotoxic substances may contribute to the allelopathic effect of BR 17. Therefore, the rice cultivar BR 17 may be potentially useful for weed management as a weed suppressing agent when incorporated into the soil or included in a rice-based cropping system.

Key words: Allelopathy, Echinochloa crus-galli, Growth inhibitor, Oryza sativa, Weed management.

Evidence for allelopathy has accumulated in the literature over many years (Rice, 1984; Putnam and Tang, 1986; Inderjit, 1996; Bais et al., 2006; Belz, 2007). Given the agricultural importance of rice, rice allelopathy has been extensively studied to develop ecological weed control strategies by selecting allelopathic rice cultivars (Olofsdotter et al., 1995; Chung et al., 1997, 2000; Ahn and Chung, 2000). A large field experiment conducted at the University of Arkansas, USA screening 16,000 rice accessions from 99 countries for allelopathic potential, revealed 412 rice accessions inhibiting the growth of one or more weed species (Dilday et al., 1994, 1998). Similar screening programs have been carried out in other countries, and certain rice varieties were found to inhibit the growth of various other plant species (Hassan et al., 1998; Kim et al., 1999; Olofsdotter et al., 1999). These findings indicate that rice is allelopathic, and presumably produces and releases allelopathic substances into the neighboring environment.

The allelopathic activity of 102 Bangladesh rice (60 traditional and 42 high yielding cultivars) was determined against the seedling growth of cress, lettuce, Echinochloa crus-galli and E. colonum. High yielding rice cultivar, BR17 among them, marked the strongest inhibitory activity with an average of 59% growth inhibition (Kato-Noguchi et al., 2009). The methanol extracts of BR 17 also had the highest inhibitory activity against the growth of cress, timothy and crabgrass (Salam and Kato-Noguchi, 2010). Two phytotoxic substances, 3-oxo-α-ionol and 9-hydroxy-4-megastigmen-3-one, have already been isolated from the water extract of BR 17 and characterized (Salam and Kato-Noguchi, 2011). In this study, another potent phytotoxic substance was isolated from an aqueous methanol extract of BR 17 and characterized. The inhibitory activity of the phytotoxic substance was tested against E. crus-galli and the concentrations of the 3 phytotoxic substances in BR 17 were also determined.

Materials and Methods

1. Plant materials

Bangladeshi rice (Oryza sativa L. cv. BR17) was grown on the research farm of Kagawa University in Kagawa from May 15 to July 5, 2009 for 50 d. The shoots (leaves and stems) were then harvested and kept in a refrigerator at −20°C until extraction.

2. Extraction and bioassay

Rice plants (100 g dry weight of rice shoots) were cut into small pieces (about 1 cm) and extracted with 500 mL of 80% (v/v) aqueous methanol for 2 d. After filtration using filter paper (No. 2, Toyo, Tokyo, Japan), the residue was extracted again with 500 mL of methanol for 1 d and filtered. The 2 filtrates were combined.

An aliquot of the extract (final assay concentration was...
0.03, 0.1 or 0.3 g dry weight rice plant equivalent extract mL\(^{-1}\)) was evaporated to dryness, dissolved in 0.2 mL of methanol and added to a sheet of filter paper (No. 2; Toyo, Tokyo) in a 3-cm Petri dish. The methanol was evaporated in a fume hood and the filter paper in the Petri dish was moistened with 0.8 mL of a 0.05% (v/v) aqueous solution of Tween 20. Then, uniform 10 seedlings of *Echinocloa crus-galli* (L.) Beauv after germination in the dark at 25°C for 36 hr, were sown on the filter paper in the Petri dish. The length of their roots and coleoptiles was measured after 48 hr of incubation in the dark at 25°C. Controls were treated as described above with the exception that 0.2 mL methanol was used instead of the extract. The bioassay was repeated 5 times using a completely randomized design with 10 plants for each determination. Significant differences between treated and control plants were examined by Welch's *t*-test.

### 3. Purification of active substances

Rice plants (1 kg dry weight of rice shoots) 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 M phosphate buffer, partitioned 5 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). The biological activity of the fractions was determined using an *E. crus-galli* bioassay as described above, and activity was found in a fraction obtained by elution with 80% ethyl acetate in *n*-hexane. After evaporation, the residue was dissolved in 20% (v/v) aqueous methanol (2 mL) and loaded onto reverse-phase C18 cartridges (YMC Ltd., Kyoto, Japan). The cartridge was eluted with 20, 40, 60 and 80% (v/v) aqueous methanol and methanol (15 mL per step). The active fraction was eluted with 40% aqueous methanol and evaporated to dryness. The residue was finally purified by reverse-phase HPLC (10 mm i.d. × 50 cm, ODS AQ-325; YMC Ltd.) eluted at a flow rate of 2 mL min\(^{-1}\) with 45% aqueous methanol and detected at 220 nm. Inhibitory activity was found in a peak fractions eluted between 95 – 95 min, yielding an active substance as colorless residue. The substance was characterized by the analyses of \(^1\)H-NMR spectrum (400 MHz, CD\(_3\)OD) and the specific rotation.

### 4. Bioassay of isolated substance

The isolated substance was dissolved in a 0.2 mL of methanol and added to a sheet of filter paper (No. 2) in a 3-cm Petri dish. Ten *E. crus-galli* seedlings were arranged on the filter paper in each Petri dish and grown in the dark at 25°C for 48 hr. The inhibitory activity was determined after 48 hr as described above. The bioassay was repeated 5 times using a randomized design with 10 plants for each determination. Significant differences were examined by Welch’s *t*-test.

### 5. Quantification of phytotoxic substances

For quantification of (–)-3-hydroxy-\(\alpha\)-ionone, rice shoots (10 g dry weight) were extracted and partitioned against ethyl acetate and the ethyl acetate fraction was purified by a silica gel column and reverse-phase C18 cartridges as described above. The active fraction was chromatographed by HPLC (4.6 i.d. × 150 mm, ODS Hydrosphere C\(_{18}\); YMC; eluted at a flow rate of 0.8 mL min\(^{-1}\) with 25% aqueous methanol, detected at 220 nm). Retention time of (–)-3-hydroxy-\(\alpha\)-ionone was 27.4 min.

For quantification of (+)-3-oxo-\(\alpha\)-ionol and 9-hydroxy-4,7-megastigmadien-9-one, rice shoots (10 g dry weight) were extracted with 80% aqueous methanol and partitioned against ethyl acetate and the ethyl acetate fraction as described above. The ethyl acetate fraction was then purified by a silica gel column and reverse-phase C\(_{18}\) cartridges as described by Salam and Kato-Noguchi (2011). The active fraction was chromatographed by HPLC as described above. The retention time of (+)-3-oxo-\(\alpha\)-ionol and 9-hydroxy-4,7-megastigmadien-9-one was 14.7 and 16.3 min, respectively. These phytotoxic substances were quantified by measuring their peak areas on the chromatogram of HPLC. Experiments were repeated 5 times with 3 quantifications for each experiment.

### Results and Discussion

Aqueous methanol extracts of a rice cultivar BR 17 inhibited the root and coleoptile growth of *E. crus-galli* concentration dependently (Fig. 1). The extract obtained from 0.3 g dry weight of rice plants inhibited the root and coleoptile growth of *E. crus-galli* by 8.6, and 15.6% that of...
control, respectively. Therefore, the extract of BR 17 had an inhibitory effect on *E. crus-galli*. *E. crus-galli* was used as the test plant because it is one of the most significant biological constraints on rice production (Xuan et al., 2005).

A growth inhibitory substance was isolated from the extract of BR 17 by columns of silica gel and C$_{18}$ cartridges, and HPLC. The $^1$H NMR spectrum of the substance (400 MHz, CD$_3$Cl$_3$, TMS as internal standard) showed $\delta_H$ 1.09 (3H, s), 1.10 (3H, s), 1.47 (1H, d, $J$ = 12.2 Hz), 1.75 (3H, br s), 1.78 (1H, m), 2.07 (1H, dd, $J$ = 18.6, 10.3 Hz), 2.28 (3H, s), 2.42 (1H, dd, $J$ = 18.6, 5.9 Hz), 4.00 (1H, m), 6.09 (1H, d, $J$ = 16.1 Hz) and 7.19 (1H, d, $J$ = 16.1 Hz). The specific rotation of the substance was $\alpha_{25}^d$ = –71.4 (c = 0.02, CHCl$_3$). From comparison of these data with those reported in the literature (Güldner and Winterhalter, 1991; Dietz and Winterhalter, 1996; Mathieu et al., 2005), the substance was identified as (–)-3-hydroxy-$\beta$-ionone (MW 231; Fig. 2).

(–)-9-Hydroxy-$\beta$-ionone inhibited the growth of *E. crus-galli* roots and coleoptiles at concentrations higher than 10 $\mu$M (Fig. 3). The concentrations required for 50% inhibition of the growth of *E. crus-galli* in the assay (defined as $I_{50}$), as determined by a logistic regression analysis, were 36.7 and 146 $\mu$M, respectively. Two other phytotoxic substances, 3-oxo-$\alpha$-ionol and 9-hydroxy-4-megastigmen-3-one, were also isolated from BR 17 as described by Salam and Kato-Noguchi (2011). 3-oxo-$\alpha$-ionol inhibited the root and coleoptile growth of *E. crus-galli* at concentrations higher than 3 and 1 $\mu$M, respectively, and 9-hydroxy-4-megastigmen-3-one inhibited the root and coleoptile growth of *E. crus-galli* at concentrations higher than 0.3 and 30 $\mu$M, respectively. The $I_{50}$ values of 3-oxo-$\alpha$-ionol for the root and coleoptile growth of *E. crus-galli* were 16.3 and 134 $\mu$M, respectively, and those values of 9-hydroxy-4-megastigmen-3-one for the root and coleoptile growth were 30.2 and 180 $\mu$M, respectively. The concentration of (–)-9-hydroxy-$\beta$-ionone in BR 17 was higher than that of the other 2 phytotoxic substances (Table 1). The overall recovery of (–)-3-hydroxy-$\beta$-ionone, (+)-3-oxo-$\alpha$-ionol and 9-hydroxy-4,7-megastigmadien-9-one added to the extraction before filtration was 85 ± 13%, 79 ± 12% and 83 ± 11% (mean ± SE), respectively.

Phytotoxic substances in plants can be released into the soil, as exudates from living plant tissues and/or by decomposition of plant residues, and act as allelopathic substances which inhibit seed germination, seedling establishment and plant growth (Bais et al., 2006; Bonanomi et al., 2006; Belz, 2007). Decomposition of 1 kg BR 17 rice plants in 1 L soil water would produce (–)-3-hydroxy-$\beta$-ionone, 3-oxo-$\alpha$-ionol, 9-hydroxy-4-megastigmen-3-one and 3-oxo-$\alpha$-ionol at the concentrations of 92.3, 75.4 and 67.7 $\mu$M, respectively, based on Table 1. The threshold of (–)-3-hydroxy-$\beta$-ionone, 3-oxo-$\alpha$-ionol and 9-hydroxy-4-megastigmen-3-one for growth inhibition of *E. crus-galli* was 10, 3 and 0.3 $\mu$M, respectively (Fig. 3; Salam and Kato-Noguchi, 2011). Thus, the estimated concentrations of these phytotoxic substances in the soil water exceed the threshold of the growth inhibition. The present findings suggest that those phytotoxic substances may contribute to the allelopathic effect caused by BR 17.

![Chemical structure of (–)-3-hydroxy-$\beta$-ionone.](image)

**Fig. 2.** Chemical structure of (–)-3-hydroxy-$\beta$-ionone.

![Effects of (–)-3-hydroxy-$\beta$-ionone on root and coleoptile growth of *E. crus-galli*.](image)

**Fig. 3.** Effects of (–)-3-hydroxy-$\beta$-ionone on root and coleoptile growth of *E. crus-galli*. Means ± SE from 5 independent experiments with 10 seedlings for each determination are shown. Asterisks indicate significant difference between control and treatment: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

| Phytotoxic Substance      | Concentration ($\mu$mol kg$^{-1}$) |
|---------------------------|------------------------------------|
| (–)-3-Hydroxy-$\beta$-ionone | 92.3 ± 7.6                          |
| 3-Oxo-$\alpha$-ionol       | 75.4 ± 6.5                          |
| 9-Hydroxy-4-megastigmen-3-one | 67.7 ± 5.2                        |

Means ± SE from 5 independent experiments with 3 quantifications for each determination are shown.

Table 1. Concentrations of (–)-3-hydroxy-$\beta$-ionone, 3-oxo-$\alpha$-ionol and 9-hydroxy-4-megastigmen-3-one in rice cultivar BR 17.
extracts. When released into the soil by exudation and/or by decomposition of plant residues, those phytotoxic substances may act as allelopathic substances. Therefore, the rice cultivar BR 17 may be potentially useful for weed management as a weed suppressing agent when incorporated into the soil or included in a rice-based cropping system.

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