Release and Activity of Allelochemicals from Allelopathic Rice Seedlings

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3-Isopropyl-5-acetoxycyclohexene-2-one-1 (1), momilactone B (2), and 5,7,4′-trihydroxy-3′,5′-dimethoxyflavone (3) were isolated and identified from an allelopathic rice accession PI312777. These three compounds at low concentrations could inhibit the growth of weeds Echinocloa crusgalli and Cyperus difformis associated with rice, especially mixtures of the compounds had stronger inhibitory activity than did individual compounds. Studies with hydroponic culture, continuous root exudates trapping system (CRETS), and direct resin adsorption methods showed that a total of 7.6 n moles 1, 2, and 3 were exuded from living roots of each seedling into the environment at 10 days after seedlings were transplanted. Furthermore, 1, 2, and 3 were found in the soil growing PI312777 seedlings at day 15 after seedlings emergence and reached a total of 39.5 μg/g soil at day 30. The results indicated that PI 312777 seedlings could release sufficient quantities of 1, 2, and 3 into the environment to act as allelochemicals inhibiting the growth of associated weeds. Investigations on the distribution of 1, 2, and 3 in PI 312777 plant, and its root exudates showed that the levels of 1, 2, and 3 were significantly higher in the shoots and root exudates than in the roots, and only trace 1 was observed in the roots. The results suggest that the roots of rice seedlings are not major site of synthesis or accumulation 1, 2, and 3, but a pathway for their release into the environment. The levels of 1, 2, and 3 in the root exudates were over 2-folds higher under direct resin adsorption than under hydroponic culture and CRET, and hence, it is the preferred method to collect and identify active allelochemicals in rice exudates in future studies on rice allelopathy.

KEYWORDS: Oryza sativa L.; allelochemical; release; root exudates; weed-suppressive activity

INTRODUCTION

Weeds pose an important biological constraint to rice productivity, and rice production is characterized by heavy use of herbicides, which may cause environmental and health problems. Fortunately, rice allelopathy, which results from the allelochemicals synthesized and released from rice itself, may be an alternative to the chemical control of weeds in paddy ecosystem (1). Therefore, searching for allelochemicals from rice has been extensively studied (2–6). A range of phenolic acids, such as p-hydroxybenzoic, vanillic, and p-coumaric and ferulic acids, were identified as potent allelochemicals from rice tissues and the root exudates (2, 4, 6, 7). More recently, an increasing number of studies have shown that a few flavones, diterpenoids, and other types of compounds are also the potent allelochemicals from rice (3, 5, 8).

Rice allelopathy occurs if the compounds are not only biosynthesized in seedlings but are also released by the living plants into their surroundings at ecologically relevant concentrations with inhibitory activity on associated weeds. Allelochemicals in living rice plants can usually be released from their root tissues (9, 10), but only a few studies have identified the allelochemicals excreted by living rice root systems. Several classes of secondary metabolites including carbohydrates, amino acids (11), phenolic acids (7, 12), cytokinins (13, 14), alkyl resorcinols (15), momilactone B (3, 16), and flavones (5) have been determined from rice root exudates, but it is not clear whether these compounds possess allelopathic function and which rice tissues synthesize and release them. Recent studies have shown that phenolic acids are unlikely to account for the allelopathy in rice because their concentrations in paddy never reach phytotoxic levels (12), and living rice seedlings may release allelopathic momilactone B or several flavones into the environment through their root tissues (3, 5, 16).

In our laboratory, many kinds of compounds were isolated and identified from an allelopathic rice accession PI 312777, which significantly suppressed the growth of various weeds in
rice fields (17). Among them, a cyclohexeneone, a diterpenoid, and a flavone could significantly inhibit the growth of weeds commonly found in paddies. However, the presence of these compounds in a rice plant does not necessarily mean that they can be released into the environment to demonstrate their allelopathic effects at ecologically relevant concentrations under natural conditions. This study is aimed to approach whether compounds in a rice plant does not necessarily mean that they and a flavone could significantly inhibit the growth of weeds.

**MATERIALS AND METHODS**

**Instruments.** The NMR spectra were measured in deuterated dimethyl sulfoxide or chloroform with a Bruker AC-P300Q spectrometer (400 MHz for $^1$H, 100 MHz for $^{13}$C). All chemical shifts are reported as δ values relative to TMS. High-resolution mass experiments were carried with a JEOL JMS-01SG-2 mass spectrometer (EI, 70 eV). Optical rotation was measured in chloroform with a Perkin-Elmer Model-241 MC polarimeter.

**Test of Allelopathic Rice Seedlings.** The rice variety PI 31777 was selected as a model allelopathic rice accession (17), and its seeds were the gift from Dr. John D. Mattice (USDA, ARS, USA). The seeds were sown on a rice field plot (30 m$^2$) at South China Agricultural University (Guangzhou, China), with 17.7 ± 0.5 g/kg soil organic matter, 80.1 ± 0.5 mg/kg soil available N, 80.1 ± 0.5 kg/g soil available P, and 102.5 ± 0.05 mg/kg available K. The experiments were carried out from April to May in 2003, and no herbicides were applied during experimental periods.

**Isolation and Identification of Allelochemicals.** The above-ground part of PI31777 plants was randomly collected from the experimental plot at the 6th leaf stage, and foliage was freezing-dried and ground. The powder (50 g) was extracted with 70% methanol over 24 h. The filtrate was concentrated in vacuo, and the concentrated extract was partitioned three times with EtOAc. The EtOAc phase was subsequently evaporated under nitrogen gas, and then subjected to silica gel CC with n-hexane/EtOAc (9:1) and then 4:6, v/v mixture, affording seven fractions.

3-Isopropyl-5-acetoxycyclohexene-2-one-1 (1). Removal of solvent from fraction 2 afforded (247 mg) of white crystals, mp 135–137 °C. Its molecular formula was determined to be C$_{15}$H$_{22}$O$_2$ by high-resolution mass spectrum, and accurate mass was calculated based on C = 12.000000, H = 1.007825, and O = 15.994915 (found, 196.1108; calcld, 196.1099). [α]$_{20}^D$ = 55.6 (CHCl$_3$, c 0.009). UV max (EtOH) nm: 346, 269. IR νmax (KBr) cm$^{-1}$: 3400 (OH), 1630 (C=O), 1610 (C=C), 1569 (C=C), 1430 (C=C). $^1$H NMR (d$_2$-DMSO): 12.95 (1H, s, exchangeable, OH on C-5), 10.80 (1H, d, J = 1.6 Hz, H-8), 6.31 (1H, d, J = 1.7 Hz, H-6), 3.96 (6H, s, 2 × OCH$_3$), $^{13}$C NMR (d$_2$-DMSO): 181.7 (C-4), 163.9 (C-2), 163.6 (C-7), 161.4 (C-5), 161.0 (C-9), 157.3 (2C, C-3' and C-5'), 148.1 (C-4'), 120.4 (C-1'), 104.6 (2C, C-2', and C-6'), 103.7 (C-10), 103.3 (C-9), 98.7 (C-6), 94.2 (C-8), 56.4 (2C, C-7', and C-8').

**Collection of Root Exudates.** The collection of the root exudates from living PI 31777 seedlings was carried out with hydroponic culture, continuous root exudates trapping system (CRETS) and direct resin adsorption, respectively. Collection was conducted three times under identical manipulations.

**Hydroponic Culture (12, 16).** Seedlings with uniform root length and shoot height were selected from the experimental plot at the 3rd leaf stage. Five seedlings were wrapped with foam, inserted into holes in a Styrofoam float, and transplanted into Hoagland’s solution in a plastic pot (5 × 5 cm). The pot was wrapped with aluminum foil to restrict light and inhibit algae growth and placed in a sterile environment growth chamber (1 m$^3$). Seedlings were grown for 12 h photoperiod. The hydroponic solution in the pot was kept at the same level by adding distilled water at 24 h intervals, and only the seedling roots were immersed in the solution during the incubation. The hydroponic solution in the pot was filtered after 10 days, and the filtrate was loaded onto a column (2 × 20 cm) of XAD-8 resin (Aldrich Co.) and eluted with methanol. The root exudates were obtained after methanol was removed in a vacuum at 50 °C. CRETS. After water-falling, five seedlings at the 3rd leaf stage were transplanted into the pot of the continuous root exudates trapping system (CRETS) designed by Tang and Young (18). The column was attached (2 × 20 cm) and packed with XAD-8 resin, the reservoir was connected, the Hoagland’s solution was circulated, and chemical trapping was begun. All the resin pots and reservoirs were covered with aluminum foil to restrict light and deters algae growth. After 10 days, the column was detached, washed with distilled water, and then eluted with methanol. Finally, methanol was removed and the root exudates were obtained.

**Direct Resin Adsorption.** Five seedlings at the 3rd leaf stage were transplanted into a previously autoclaved pot (9 × 7 cm) containing XAD-8 resin and 500 mL 1 mM Mes-Tris buffer (pH 5.5) + 0.5 mM CaSO$_4$, and the pot was placed in a sterile environment growth chamber (1 m$^3$). The seedlings were grown at 25 ± 1 °C with a 12 h photoperiod, and the pot was watered once a day to maintain the volume of the solution. After 10 days, the seedlings were carefully separated from the pot, and then the resin and solution were subjected to a column (5–10 cm). The column was first removed, washed with distilled water, and then eluted with methanol. Removed methanol, and gave the root exudates.

The root exudates collected with hydroponic culture, CRETS, and direct resin adsorption were subjected to quantification analysis of allelochemicals as described the following section.

**Quantification Analysis of Allelochemicals.** Five rice seedlings with uniform root length and shoot height at the 3rd leaf stage were transplanted into the pot of the direct resin adsorption described above. After 10 days, 416 ± 31 mg (fr wt) of shoots and 327 ± 19 mg (fr wt) of roots were harvested, and 149 ± 15 mg of root exudates were collected. The fresh shoots and roots were each homogenized with 300 mL of methanol, and the homogenate was filtered. The filtrate was concentrated in a vacuum at 40 °C to give an aqueous residue.

The shoots and roots residues and root exudates were dissolved in 50% aqueous methanol (v/v, 2 mL) and loaded onto reversed phase C$_{18}$ Sep-Pak cartridges (Waters, Co.). The cartridge was eluted with 50% aqueous methanol (5–3 mL) and then methanol (3–3 mL), and the methanol fraction was concentrated with nitrogen gas to obtain the concentrate (100 µL) for quantification analysis.

Quantification analysis of allelochemicals in the shoots, roots, and root exudates of rice seedlings was carried out with an HPLC Hitachi L 7100 equipped with a C$_{18}$ reversed column (Hypersil 125 × 4.0-
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RESULTS AND DISCUSSION

On the basis of the data of HR-MS and various spectra, three compounds isolated from PI312777 seedlings were identified as 3-isopropyl-5-acetoxyhexene-2-one-1 (1), momilactone B (2), and 5,7,4′-trihydroxy-3,5′-dimethoxyflavone (3), respectively (Figure 1). Compounds 2 and 3 were originally isolated from rice husks and later found in rice leaf and straw as a phytoalexin to participate in defense of rice against pathogens (3, 8, 20, 21), while 1, to the best of our knowledge, has never been reported in rice plants. Compounds 1, 2, and 3 could significantly inhibit the growth of weeds E. crusgalli and C. difformis associated with rice (Figure 2). Their inhibition threshold, lowest concentration required to initiate inhibition (22), are 11.3 and 6.8 μg/g soil (1 on E. crusgalli and C. difformis), 8.4 and 5.4 μg/g soil (2 on E. crusgalli and C. difformis), 3.6 and 2.7 μg/g soil (3 on E. crusgalli and C. difformis), respectively. Interestingly, the mixtures with 1, 2, and 3 had much stronger inhibitory activity than when applied alone, especially a total of 3.0 μg/g soil mixture with 1, 2, and 3 initiating inhibition on the growth of E. crusgalli and C. difformis (Figure 3). The results agree with some previous studies that the concentration of a single allelochemical is generally below its inhibition threshold, and the mixture of allelochemicals may synergistically inhibit weed growth (2, 23, 24).

It is possible that compounds 1, 2, and 3 in PI312777 seedlings may demonstrate allelopathic effect if they are released at the concentrations over inhibition threshold into the environment through living root tissues. Determination of 1, 2, and 3 in root exudates showed that they occurred under all collections, and there was more of 3 than of 1 or 2 (Figure 4), indicating that these compounds could be excreted into the environment by the living root tissues of PI312777 seedlings in quantitative amounts. It is noteworthy that the levels of 1, 2, and 3 in the root exudates were almost 2-fold higher under direct root adhesion than under hydroponic culture and CRETS conditions (Figure 4), which suggests that direct root adhesion is much better than hydroponic culture and CRETS for collecting rice root exudates.

Investigations on the distribution of 1, 2, and 3 in rice seedlings and their root exudates collected by direct root adhesion showed that they had higher levels in shoots and root exudates but decreased greatly in roots where the levels of 2 and 3 decreased more than over 70%, and only trace 1 was determined. In addition, 1, 2, and 3 always had higher levels in both shoots and root exudates than in the root (Figure 5), showing that 1, 2, and 3 were primarily synthesized in the shoots of rice seedlings and released into the environment through their root tissues. Generally, allelochemicals were distributed differentially in rice seedlings with root tissues normally containing higher levels of phenolic acids than shoot tissues (2, 4, 6, 7). However, the levels of 1, 2, and 3 in rice shoots and root exudates were significantly greater than those in the root tissues; in particular, only trace 1 was determined in rice roots (Figure 5). Recent studies showed that rice seedlings could release 2 through their roots into the environment, and the level of 2 accumulated in the culture was much higher than those in their shoots and roots during days 12–15 (16). The root extracts of PI312777 seedlings was less allelopathic than the leaf extracts (25), and it was postulated that the allelochemicals in rice seedlings were released immediately after translocation from the leaves to the roots, or that they were not released from roots but leached directly from leaves (25). Our results show that 1, 2, and 3 had much lower levels in rice roots than in their shoots, and root exudates agree with less allelopathic effects of the root extracts of PI312777 seedlings, which indicated that the roots of rice seedlings were not major site of synthesis or accumulation 1, 2, and 3, but a pathway for their release into the environment. However, it is still unclear how 1, 2, and 3 were released after translocation from rice shoots to root exudates.
When 1.8 nmol of 2 are released from a rice seedling to the culture solution, it may act as an allelochemical that inhibits the growth of neighboring plants (16). In this study, 1.1 nmol of 1, 2.9 nmol of 2, and 3.6 nmol of 3, a total of 7.6 nmol, were exuded from per rice seedlings (Figure 5). Furthermore, the amounts of 1, 2, and 3 in the soil growing PI312777 seedlings were examined by HPLC at various intervals. It was found that 1, 2, and 3 occurred in the soil at day 15 after seedlings emergence, and reached 7.8 µg/g soil of 1, 11.9 µg/g soil of 2, and 19.8 µg/g soil of 3, a total of 39.5 µg/g soil at day 30 (Figure 6). The amounts of 1, 2, and 3 released into the soil were over inhibition threshold enough to initiate inhibition on associated weeds at day 30 after rice emergence. All results showed that 1, 2, and 3 actually were released into the environment in sufficient quantities with inhibitory activities, and could act as the allelochemicals to inhibit the growth of weeds associated with rice, which agrees with an increasing number of recent studies that other type of compounds in rice seedlings are allelochemicals (3, 5, 8, 16), and common phenolic acids were unlikely primary allelochemicals in rice (12).

Actually, rice can release many kinds of secondary metabolites through its root tissues. These metabolites in rice exudates possess multiple functions on the chemical interactions among organisms in the environment (11), and allelopathy is one of the multiple functions among metabolites (10). Unfortunately, active allelochemicals in rice root exudates were often performed under arbitrary conditions, rather than collecting from the intact and
living rice seedlings (18). Our research showed that the levels of 1, 2, and 3 in rice root exudates were correlated with collection conditions, suggesting that it is a priority to determine how to trap and identify the active allelochemicals in rice exudates in the future research on rice allelopathy. So far, there has been limited success in finding allelochemicals that can really explain the allelopathic action in the field (9). Further clarification of the chemical basis and release mechanism on rice allelopathy is warranted.

ABBREVIATION USED

CRETS, continuous root exudates trapping system.

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