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Two compounds from allelopathic rice accession and their inhibitory activity on weeds and fungal pathogens

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Abstract

A flavone (5,7,4′-trihydroxy-3′,5′-dimethoxyflavone), a cyclohexenone (3-isopropyl-5-acetoxycyclohexene-2-one-1) and a liquid mixture of low polarity, containing long-chain and cyclic hydrocarbons, were isolated from leaves of allelopathic rice accession PI 312777 using column chromatography. Their structures and constituents were identified by means of HR-MS, NMR and GC/MS analyses, respectively. Bioassays showed that both the flavone and cyclohexenone significantly inhibited the growth of weeds *Echinochloa crus-galli, Cyperus difformis* and *Cyperus iris,* and the spore germination of fungal pathogens *Pyricularia oryzae* and *Rhizoctonia solani* at all tested concentrations. Moreover, the combination of the inactive mixture of low polarity and the active flavone or cyclohexenone significantly enhanced the inhibitory activities on weed growth. In addition, the two compounds and the mixture of low polarity from the leaves of PI312777 did not inhibit the rice growth at the same concentrations. It was also established that both compounds could be released into the soil, and was especially induced by *E. crus-galli*. The results suggest that 5,7,4′-trihydroxy-3′,5′-dimethoxyflavone and 3-isopropyl- 5-acetoxycyclohexene-2-one-1 may act as allelochemicals participating in the defense of rice against weeds and pathogens.

Keywords: *Oryza sativa*; Graminae; Rice; Allelopathy; Flavone and cyclohexenone; Bioactive compounds; Weed and fungal pathogens

1. Introduction

Rice (*Oryza sativa* L.) is one of the principal food crops in the world. Its production is characterized by heavy use of herbicides and fungicides which may cause environmental problems in the paddy ecosystem (Chung et al., 2001a; Kim and Shin, 2000). Accordingly, rice allelopathy can potentially be used to improve weed and pathogen management in rice production (Kim and Shin, 2000).

Extensive studies of allelochemicals in rice plants have led to identification of a range of phenolic compounds, including *p*-hydroxybenzoic, vanillic, *p*-coumaric and ferulic acids (Chung et al., 2001a; Kim and Kim, 2002; Mattice et al., 1998; Rimando et al., 2001). However, these phenolic acids are unlikely to explain the allelopathy of rice since their soil concentrations never reach phytotoxic levels (Olofsdotter et al., 2002b). More recently, an increasing number of studies have shown that some flavones, diterpenes and other types of compounds are potent allelochemicals in rice (Kato-Noguchi et al., 2002; Kato-Noguchi and Ino, 2003; Kong et al., 2002a; Lee et al., 1999). It is possible that there are still other allelochemicals in rice plants, especially in a few allelopathic rice accessions of numerous rice germplasm collections (Chung et al., 2001b; Dilday et al., 1994; Kong et al., 2002a; Olofsdotter et al., 1995). The objectives of this study were thus to isolate and identify other types of allelochemicals present an allelopathic rice accession (PI312777), and to evaluate their inhibitory activities on weed and fungal pathogen in rice production. Furthermore, the amounts of these allelochemicals released into the soil were examined.

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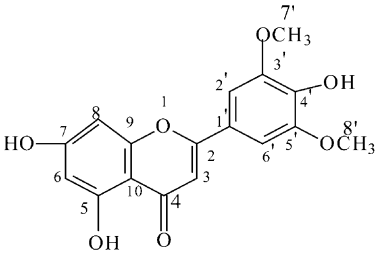
2. Results and discussion

Three fractions were obtained from leaf extracts of PI312777 by silica gel column chromatography. The first fraction **F1** was a liquid mixture of low polarity, whose chemical constituents were analyzed by GC–MS. The major components of the mixture were long-chain and cyclic hydrocarbons (Table 1), whereas two crystalline compounds were obtained from the second and third fractions, respectively. Their molecular formulae were determined by high resolution mass spectra, and their structures, a flavone **1** (5,7,4′-trihydroxy-3′,5′-dimethoxyflavone) and a cyclohexenone **2** (3-isopropyl-5-acetoxycyclohexene-2-one-1), were subsequently identified by analysis of their NMR spectra. Flavone **1** was originally isolated from rice husk and later found in rice leaves and straw as an antifungal agent (Kato et al., 1977; Liu et al., 1995), while cyclohexenone **2**, to the best of our knowledge, has never been reported in rice plants.



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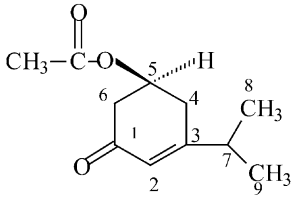

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Inhibitory effects of **1**, **2** and **F1** on growth of selected weeds and spore germination of pathogens were determined at different concentrations. Thus, since *Echinochloa crus-galli, Cyperus difformis* and *Cyperus iris* are major weeds associated with rice in South China, and *Pyricularia oryzae* and *Rhizoctonia solani* are common fungal pathogens causing rice disease, these were selected to evaluate the potential inhibitory effects of **1**, **2** and **F1** isolated from PI312777 leaves.

Both **1** and **2** significantly inhibited the growth of *E. crus-galli, C. difformis* and *C. iris* and spore germination of *P. oryzae* and *R. solani*. However, the mixture **F1** of containing low polarity constituents did not show any inhibitory effects, even at high concentrations (Table 2 and Fig. 1). In addition, neither **1**, **2** nor **F1** inhibited growth of rice itself, so none were autotoxic. The IC_{50} values of **1** on the growth of *E. crus-galli, C. iris* and *C. difformis* were ca 200, 150 and 100 $\mu g.g^{-1}$, and on spore germination of *P. oryzae* and *R. solani* were ca 50 and 70 $\mu g.g^{-1}$, respectively. The IC_{50} values (in $\mu g.g^{-1}$) of **2** on test weeds and pathogens were ca 150 (*E. crus-galli*

Table 1
Chemical constituents of the mixture **F1** with low polarity isolated from the leaves of PI312777

Retention time (min)	Chemical constituents	Relative amount (%)
1.89	2-methylhexane	8.77
5.40	4,6-dimethylundecane	2.48
6.28	2,2,6-trimethyldecane	2.35
7.26	2-hexyl-1-decanol	2.11
7.58	1-butyl-2-propylcyclopentane	8.45
8.19	2,6-dimethyl-decahydronaphthalene	22.45
8.40	*trans*, *trans*-1,10-dimethylspiro[4,5]decane	8.03
8.48	2,3-dimethyl-decahydronaphthalene	3.37
8.63	*trans*, *cis*-1,8-dimethylspiro[4,5]decane	8.19
8.78	1,2-dimethyldecahydronaphthalene	11.49
9.10	*cis*, *cis*-1,1-dimethylspiro[4,5]decane	11.60
10.18	1,4,6-trimethyl-1,2,3,4-tetrahydronapthalene	1.77
14.35	hexadecanoic acid, methyl ester	1.85
–	Other unknown components[a]	7.09

[a] A total of 11 components whose each relative amounts was less than 1%.

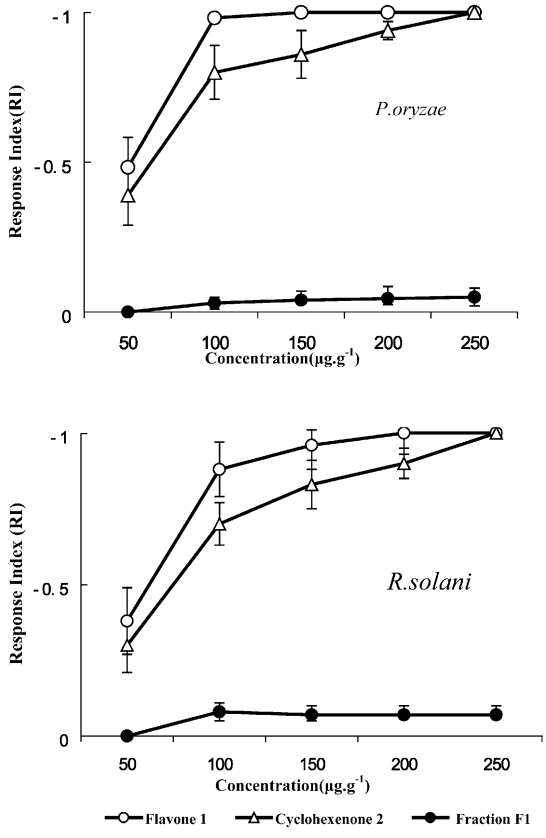

Fig. 1. Inhibitory activities of **1**, **2** and **F1** against spore germination of *P. oryzae* and *R. solani* at different concentrations.

Table 2
Inhibitory activities of **1** and **2** on the growth of associated weeds and rice

Compounds	Concentration ($\mu g.g^{-1}$)	Shoot dry weight per pot (RI±SE)			
		E. crus-galli	*C. iris*	*C. differmis*	PI312777
Flavone **1**	50	−0.18±0.03a	−0.23±0.04a	−0.26±0.05a	0.00a
	100	−0.38±0.04b	−0.41±0.06b	−0.47±0.04b	0.00a
	150	−0.46±0.07c	−0.49±0.05b	−0.68±0.07c	0.01±0.00a
	200	−0.49±0.05c	−0.67±0.09bc	−0.84±0.09d	0.00a
	250	−0.61±0.06d	−0.73±0.07c	−0.89±0.09d	−0.01±0.0a
Cyclohexenone **2**	50	−0.10±0.02a	−0.25±0.03a	−0.18±0.02a	0.02±0.00a
	100	−0.26±0.03b	−0.33±0.03b	−0.29±0.03a	0.00a
	150	−0.48±0.05c	−0.49±0.04b	−0.55±0.06b	0.01±0.00a
	200	−0.52±0.04cd	−0.64±0.05bc	−0.71±0.09c	0.00a
	250	−0.59±0.07d	−0.76±0.09c	−0.77±0.07cd	−0.01±0.00a

The mixture of low polarity was almost inactive against the growth of weeds and rice itself, even at high concentrations (RI values ranged from +0.01 to −0.01, not shown on the Table 2). Data in a column followed by the same letter are not significantly different at the 0.05 levels by Duncan's multiple-range test.

and *C. iris*), 130 (*C. differmis*), 75 (*P. oryzae*) and 95 (*R. solani*), respectively. At all concentrations tested, the inhibitory activity of **1** on both weeds and pathogens was slightly higher than that of **2**. Both **1** and **2** did not completely inhibit the growth of three weeds at 250 $\mu g.g^{-1}$, while complete inhibition of spore germination of two pathogens was observed at the same concentration. Interestingly, addition of the inactive low polarity mixture **F1** to flavone **1** or cyclohexenone **2** significantly enhanced their inhibitory effects on weed growth, but not on the spore germinations of *P. oryzae* and *R. solani* (Fig. 2). Perhaps the low polarity components acted as a sort of surfactant increasing **1** or **2** inhibitory activities on weed growth.

Rice allelopathy can be achieved by allelochemicals produced and released into the environment. The presence of both flavone **1** and cyclohexenone **2** with inhibitory effects on weeds and pathogens in rice leaves does not, however, establish that they are released into the environment and thus have allelopathic effects under natural conditions. Therefore, the amounts of **1** and **2** in soil containing PI312777 seedlings were examined by HPLC at various intervals. It was found that **1** and **2** occurred in the soil at day 15 after seedling emergence, and reached a total of 31.9 $\mu g.g^{-1}$ at day 30, with **2** in higher amounts than **1** (Table 3). Interestingly, the amounts of **1** and **2** in the soil increased significantly and reach a total of 43.7 $\mu g.g^{-1}$ at day 30, when PI312777 was sown in associated with *E. crus-galli* (Table 3). The amounts of **1** and **2** released into the soil were induced by this association. In agreement that rice allelopathic effect could be enhanced under higher density of *E. crus-galli* (Kim and Shin, 2000). It must be pointed out that **1** and **2** were not present in *E. crus-galli,* and thus were actually released from rice into the soil. The results thus prove that both **1** and **2** in PI312777 seedlings are released into the soil in substantial amounts, especially in the presence of *E. crus-galli,* although the mechanism of release is unknown. Allelochemicals in live rice seedlings are, however, known to be released from their root tissues (Olofsdotter et al., 2002a,b). Further research needs to be carried out and established how **1** and **2** are released into the soil by rice root exudation, leaves leaching or seed husk germination. Interestingly, the concentrations of **1** and **2** detected in the soil were below inhibition thresholds, even with *E. crus-galli* association. It can thus provisionally be explained that rice allelopathy depend on the combined effects of several allelochemicals, i.e. the concentration of a single allelochemical in the soil is generally below its inhibition threshold, and hence, allelochemical mixtures can accumulate to sufficiently high concentration to be bioactive on weeds (Chung et al., 2001a). Besides **1** and **2**, there may be still other allelochemicals in PI312777 seedlings awaiting detection and identification.

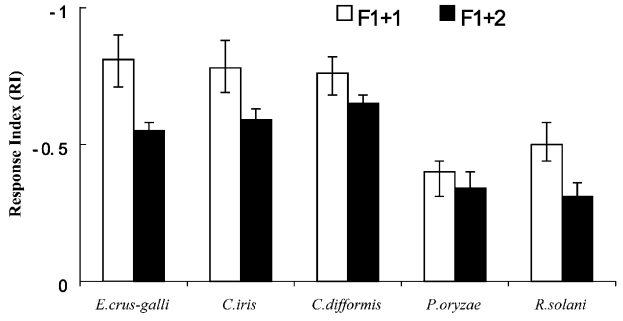

Fig. 2. Inhibitory activities of mixtures of **F1** with **1** or **2** on the growth of weeds and spores germination of pathogens. The weight ratio of both mixture **F1**+**1** and **F1**+**2** was 1:1, a total of concentrations of test material were 100 $\mu g.g^{-1}$.

Table 3
The amounts of **1** and **2** released into the soil at different intervals

Interval (days)	Compound (μg.g^{-1})			
	Flavone **1**		Cyclohexenone **2**	
5	0.0	(0.0)	0.0	(0.0)
10	0.0	(Trace)	0.0	(0.0)
15	2.6±0.9	(4.4±1.1)	Trace	(0.9±0.1)
20	9.1±1.9	(11.9±2.3)	5.4±1.1	(8.3±1.0)
25	12.8±1.5	(17.3±3.1)	8.9±1.9	(12.3±2.0)
30	20.1±3.2	(25.3±3.3)	11.8±1.7	(18.4±3.1)
35	19.2±2.9	(26.5±3.7)	12.2±2.3	(17.6±3.6)

Means±SE from three independent experiments with 5 rice seedlings in 100 g soils for each determination are shown. Data in parentheses are the amounts of **1** and **2** released into the soil growing PI 312777 seedlings associated with *E. crus-galli*.

The flavone **1** and cyclohexenone **2** had significantly inhibitory activities on both weeds and fungal pathogens and were be released into the soil in substantial amounts, suggesting that they may act as allelochemicals participating in the defense of rice against weeds and pathogens. So far, there has been limited success in finding allelochemicals that can really explain the overall allelopathic mechanism in rice (Olofsdotter et al., 2002a). Therefore, further clarification of the chemical basis and release mechanism on allelopathy in rice is warranted.

3. Experimental

The NMR spectra were measured in deuterated dimethyl sulfoxide or chloroform with a Brücher 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 rotations were measured with a Perkin-Elmer Model-241 MC polarimeter.

3.1. Rice plants, weeds and pathogens

PI312777, a well known allelopathic rice accession significantly suppressing the growth of major weeds in rice field (Dilday et al., 1994), was used. Seeds of PI312777 were sown on a plot (30 m^2) at South China Agricultural University (Guangzhou, China), where the organic matter contents and the N, P and K of the soils were as follows: organic matter, 17.7±0.5 g kg^{-1}; total N, 0.87±0.07 g kg^{-1}; available N, 80.1±0.5 mg kg^{-1}; total P, 0.28±0.03 g kg^{-1}; available P, 80.1±0.9 g kg^{-1}; total K, 102.9±0.8 g kg^{-1}; available K 102.5±0.05 mg kg^{-1}. The rice plants have previously been shown as demonstrating allelopathic potentials during their early growth stages (Ebana et al., 2001). Therefore, the aerial portion of PI312777 plants (5 kg) was randomly collected from the experimental plot at its 6th leaf stage. Experiments were carried out from April to May in 2003, no herbicides and fungicides were applied in the experimental plot during the experimental periods.

Ripe weed seeds were collected from rice fields in autumn in 2002. Seeds were dried in sunlight (48 h) and stored in a sealed glass jar. Fungal pathogens were obtained from Department of Plant Protection, South China Agricultural University.

3.2. Isolation and identification of compounds

Freeze-dried foliar parts of rice plants were ground, and powders (50 g) were extracted with MeOH–H_2O (7:3, 500 ml) over 24 h. The filtrate was concentrated in vacuo and the concentrated extract was partitioned three times with EtOAc (3×50 ml). The EtOAc phase was subsequently reduced in volume to ca 5 ml under a stream of nitrogen gas and then subjected to silica gel CC with *n*-hexane–EtOAc (9:1 and then 4:6; v/v) mixture, affording three fractions. The first fraction, **F1**, was a liquid mixture of low polarity (539 mg). The second was purified by CC with the same eluent and giving a white crystal (155 mg) on standing. The third was a crude solid that was re-crystallized with *n*-hexane/acetone (4:6; v/v) mixture and gave yellow crystals (209 mg).

The constituents of the fraction **F1** were analyzed by GC/MS on a Hewlett-Packard 5972 GC/MSD (Kong et al., 2002b). Liquid mixture (5 μl) was injected onto an HP-5 bonded stage fused-silica capillary column (30 m×2.5 mm) using the split mode. The initial oven temperature (70 °C) was maintained for 2 min following injection. The temperature was then increased to 150 °C at a rate of 20 °C/min and then to 180 °C at a rate of 10 °C/min. After having maintained the column at 180 °C for 5 min, the oven temperature was raised to 250 °C at a rate of 15 °C/min. Mass spectra were repetitively scanned from 35 to 450 amu every 2 sec. Ionization was set in the electron impact mode (EI) at 70 eV. Constituents were identified by peak matching against standards in the NIST 95 Computer Library or by spectral similarity to an authentic reference compound (Aldrich Chemical Co.). The relative amounts of constituents were calculated by integrating all peaks with areas greater than 1%.

The molecular formulae of both white and yellow crystals were determined by high-resolution mass spectral analysis and then their structures were further identified from analysis of NMR spectra.

Flavone **1**: yellow crystal, mp 279–280 °C, molecular formula: $C_{17}H_{14}O_7$ (found 330.0724, calcd 330.0756); ^{1}H NMR (d_6-DMSO): 12.95 (1H, *s*, exchangeable, OH on C-5), 10.80 (1H, *s*, exchangeable, OH on C-7), 9.31

(1H, *s*, exchangeable, OH on C-4′), 7.41 (2H, *s*, H-2′ and H-6′), 7.06 (1H, *s*, H-3), 6.66 (1H, *d*, $J = 1.6$ Hz, H-8), 6.31 (1H, *d*, $J = 1.7$ Hz, H-6), 3.98 (6H, *s*, 2×OCH_3); ^{13}C NMR (d_6-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-3), 98.7 (C-6), 94.2 (C-8), 56.4 (2C, C-7′ and C-8′).

Cyclohexenone **2**: white crystal, mp 135–137 °C, molecular formula: $C_{11}H_{16}O_3$ (found 196.1148, calcd 196.1114); $[\alpha]_D^{20}$ −55.6° ($CHCl_3$; c 0.009); ^1H NMR ($CDCl_3$): 5.69 (1H, *brs*, H-2), 4.33 (1H, *m*, H-5), 2.46 (1H, *ddd*, H-6a), 1.98 (1H, *ddd*, H-4a), 1.79 (1H, *ddd*, H-6b), 1.78 (3H, *s*, H-11), 1.60 (1H, *brs*, H-7), 1.55 (1H, *ddd*, H-4b), 1.46 (3H, *brs*, H-8), 1.27 (3H, *brs*, H-9); ^{13}C NMR ($CDCl_3$): 182.6 (C-1), 172.1 (C-10), 113.1 (C-2), 86.9 (C-3), 67.0 (C-5), 47.5 (C-6), 45.8 (C-4), 36.1 (C-7), 30.8 (C-11), 27.1 (C-9), 26.7 (C-8).

3.3. Bioassays

Seeds of *E. crus-galli, C. difformis* and *C. iris* were individually sown on 5 cm×5 cm pots (at least 50 seeds per pot), with each containing 150 g soil collected from a rice field (Foy, 1999; Weidenhamer et al., 1989). After emergence, seedlings were thinned to 10 plants per pot. **1**, **2** and **F1** (50, 100, 150, 200 and 250 $\mu g \cdot g^{-1}$) were each added to treated pots, respectively. Control pots received water only. All pots were placed in a controlled environmental chamber (3 m^3) with a 12 h day length and approximately 350 $\mu mol.m^{-2}.s^{-1}$ light intensities at pot level, 25–28 °C daytime temp and 70% relative humidity. Pots were watered and randomized once a week, and seedlings were harvested after four weeks. Shoots were clipped at the point of first root, dried for at least 48 h at 80 °C, and shoot dry weights were determined. Autotoxic effects of **1**, **2** and **F1** on the growth of PI312777 were also determined under the same conditions described above.

The inhibitory effects on *P. oryzae* and *R. solani* were evaluated by using the spore germination assay (Akatsuka et al., 1985; Suzuki et al., 1996). Pathogens were raised on an agar medium at 25 °C in the dark for a week, with spores collected by agitating with distilled water using 3-day-old cultures under fluorescent light after removal of the spores from the culture plate. The spore suspension was filtered through cheesecloth and diluted with fresh medium to 10–20 spores in a microscopic field. The filtered spore suspension (200 µl) was added to different concentrations of test material on a slide glass and incubated at 25 °C in a moist chamber for 12 h, and then the percentages of germinating and non-germinating spores were determined using a microscope.

The impacted effects of bioassays were evaluated using the following Response Index (RI) (Williamson and Richardson, 1988).

$$\text{If } T > C, \quad RI = 1 - C/T.$$

$$\text{If } 2T < C, \quad RI = T/C - 1.$$

where T (Treatment) is the shoot dry weight of weeds or percentage of spores germination of pathogens under **1**, **2** or **F1** and C (Control) is those under water only. RI values ranged from +1 to −1, positive values indicating stimulation, and negative values indicating inhibition. The absolute value of RI varied directly with the strength of the effect. All bioassays were performed five times under identical conditions. Data are presented as means (RI) ±SE. ANOVA with Duncan's multiple range-test was performed.

3.4. Quantification analysis of compounds in soil

A total of 42 pots (5 cm×5 cm), each containing 100 g soil collected from a rice field, were divided into two groups. In 21 pots were each sown with five pre-germinated PI312777 seeds. The others were each sown with five pre-germinated PI312777 and at least ten pre-germinated *E. crus-galli* seeds (after emergence, *E. crus-galli* were thinned to 5 plants). All pots were placed in a greenhouse where night and daytime temperatures ranged roughly from 20–30 °C. Pots were watered once a day. Three pots were randomly taken out from each group at various intervals and their soils individually extracted with MeOH (100 ml, agitated for 48 h at 25 °C, then centrifuged at 1200*g* for 30 min) after air-drying and carefully removal the root tissues. The extraction was performed beginning on the fifth day after emergence, and continued once every 5 days for a total of 35 days. All extracts were concentrated with a stream of nitrogen gas and the residues were respectively dissolved in MeOH–H_2O (1:1 v/v, 2 ml) and loaded onto a reversed phase C_{18} Sep-Pak cartridges (Waters, Co.). The cartridge was eluted with MeOH-H_2O (1:1 v/v, 5×3 ml) and then with MeOH (3×3 ml), with the MeOH fraction concentrated with a nitrogen gas stream to gain ca 100 µl concentrate for quantification analysis.

Quantification analysis of the flavone **1** and cyclohexenone **2** in soils was carried out with an HPLC Hitachii L7100 equipped with a C_{18} reversed column (Hypersil 125 mm×4.0 mm, 5 µm). HPLC determination conditions: mobile phase was MeOH–H_2O (3:1 v/v), eluted at a flow rate of 2.0 ml min^{-1}, with detection at 245 nm. The injection volume of the samples was 10 µl, with all samples filtered through a 0.25 µm nylon syringe pre-filter before analysis. The average recoveries of known amounts of **1** and **2** added to the soil were 87.9% and 88.3%, respectively. The amounts of **1** and **2** in the soils were respectively quantified by interpolating the peak height on the chromatograms of HPLC to a standard curve constructed by the peak height of pure **1** and

2 isolated from PI312777 plant described above in Section 3.2.

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