

Uptake and Exudation of Phenolic Compounds by Wheat and Antimicrobial Components of the Root Exudate

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In the course of our study, it was found that phenyl propenoic acid derivatives were readily taken up by wheat. Leaf leachate components were chosen for the feeding experiments and *p*-coumaric acid, ferulic acid and caffeic acid were found to be quickly taken up into the plants via the roots. The analytical study revealed that the exudate contained potent antimicrobial compounds together with amino acids and sugars.

Besides the primary metabolites, 4-hydroxystyrene, 3-methoxy-4-hydroxystyrene and 3-methoxy-4-acetoxystyrene were identified as exudate components from wheat roots in sterile hydroponic culture. This indicates that these antimicrobial components may play a significant role in the defense system as allelochemicals for the rhizosphere.

Introduction

Plant-microbe interactions start with distant chemical signaling in the rhizosphere. Substances interchanged at this stage modulate the early response and define conditions for the initiation of symbiosis. Plants release a variety of organic compounds into the environment by leaf leachates, root exudates, and through decomposition of litter. Growth of microorganisms in the rhizosphere can be profoundly controlled by these compounds. A certain class of these compounds, predominantly phenolic acids, has been shown to be allelopathic to seed germination, seedling growth, and soil bacteria. However, little is known about other biologically active compounds involved in the plant-microbe interaction in the rhizosphere (Bouillant *et al.*, 1994). On the other hand, microbial metabolites may affect plant growth.

In this study we were especially interested in the compounds predominantly taken up by plants and

in those secreted from the roots. We chose the wheat plant as a model. A preliminary study indicated that wheat roots took up simple phenolics selectively, so we focused on phenyl propenoic acid analogues, i.e., *p*-coumaric acid, ferulic acid and caffeic acid which are specifically taken up into the plant (Fig. 1).

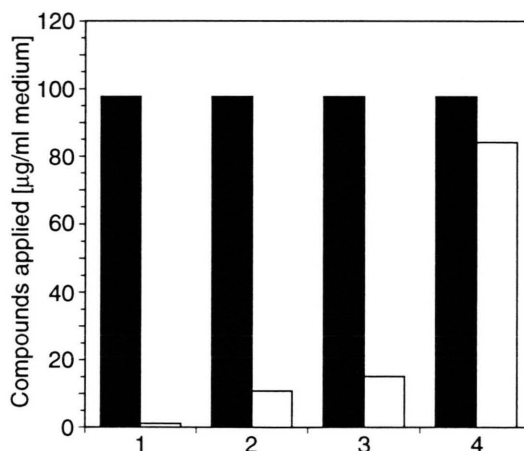


Fig. 1. Uptake of phenolic compounds by wheat roots. The amount of phenolic acids in the culture medium was monitored by HPLC. 1: *p*-coumaric acid, 2: ferulic acid, 3: caffeic acid, 4: resorcinol. Black column (■) shows the original content of the chemicals, white column (□): the content of the remaining chemicals in the culture medium.

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We found that the exudates resulting from the feeding experiments showed a pronounced antimicrobial activity and contained amino acids and sugars. Chromatographic separation of the ethyl acetate phase of the exudates enabled us to isolate 3 antimicrobial compounds.

In the present paper, the identification of the antimicrobial constituents is reported together with the possibility that plants possess a decarboxylase which converts some phenyl propenoic acids into the corresponding styrenes.

Results

Identification of exudate components

Wheat plants were aseptically grown on Gelrite medium at 20 °C for 8 days and the roots were then soaked in 500 µg/ml of phenolic acid solutions. After 18 h-incubation at 20 °C, the plants were removed from the test solution, which was then evaluated by chromatographic detection (Fig. 2) and antimicrobial assays. Thin-layer and

high performance chromatographies indicated that the resulting culture medium contained antimicrobial compounds (**1**, **2** and **3**) together with amino acids and sugars. The medium was partitioned with ethyl acetate (EtOAc). Antimicrobial activity was found in the EtOAc phase, and amino acids and sugars were identified in the aqueous phase. In the feeding experiment with *p*-coumaric acid, the acid was quickly taken up by the roots and most of the acid fed disappeared from the medium. The culture medium obtained after an 18 h-treatment gave a single component which was characterized as a UV-absorbing spot on silica gel TLC (**1**: R_f 0.46, benzene-acetone-methanol; 7:2:1,v/v). The ^1H NMR spectrum indicated the presence of a vinyl group (δ 5.02, 1H, dd, J =1.01, 10.8 Hz; δ 5.58, 1H, dd, J =1.01, 17.6 Hz; δ 6.63, 1H, dd, J =10.8, 17.6 Hz) and *p*-disubstituted benzene ring (δ 6.79, 2H, d, J =8.7 Hz; δ 7.27, 2H, d, J =8.7 Hz). The presence of a major fragment at m/z 120 in EI-MS enabled to identify this component to be 4-hydroxystyrene (Scheme).

In the feeding experiment ferulic acid was readily taken up by the roots and *ca.* 95% of the acid initially applied was removed from the culture medium within 14 h. Besides the acid, the medium gave two spots on the silica plate (**2**: R_f 0.53, **3**: R_f 0.63, benzene-acetone-MeOH; 7:2:1). In the ^1H NMR of **2** the presence of a vinyl group was confirmed (δ 5.03, 1H, d, J =10.7 Hz; δ 5.60, 1H, d, J =17.7 Hz; δ 6.64, 1H, d, J =10.7, 17.7 Hz) and the splitting pattern of the aromatic protons was identical to that of ferulic acid. These findings as well as the molecular ion peak (m/z 150) in EI-MS suggested that **2** was 3-methoxy-4-hydroxystyrene (Scheme). In the ^1H NMR the only difference of the splitting pattern of **3** from that of **2** was the presence of a 3H singlet at δ 2.31. This fact together with the presence of a molecular ion peak at m/z 192 and a prominent fragment at m/z 150 ($\text{M}^+ - \text{CH}_3\text{CO}$) indicated that **3** is 3-methoxy-4-acetoxystyrene (Scheme). These styrenes were subjected to antimicrobial assays and found to possess antimicrobial activities in a wide range spectrum.

However, there is no direct evidence that ferulic acid / *p*-coumaric acid are converted into the corresponding styrenes by the wheat plant.

So, we conducted a feeding experiment with ^{13}C -labeled *p*-coumaric acid under the same con-

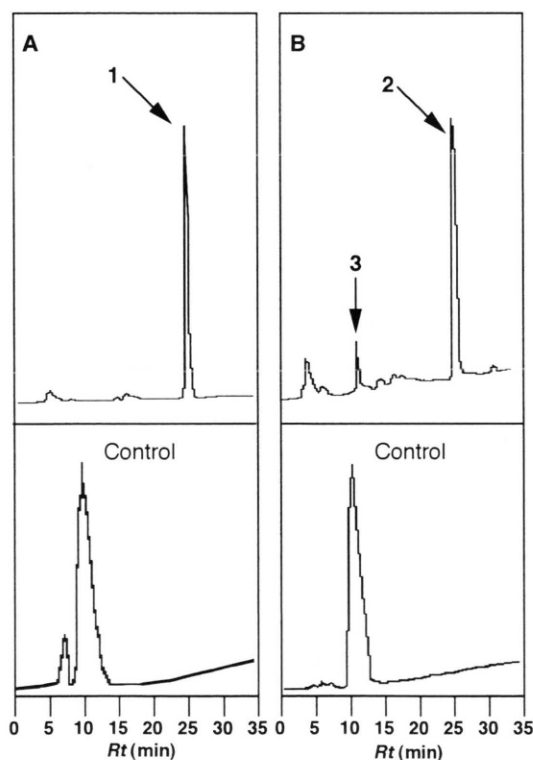
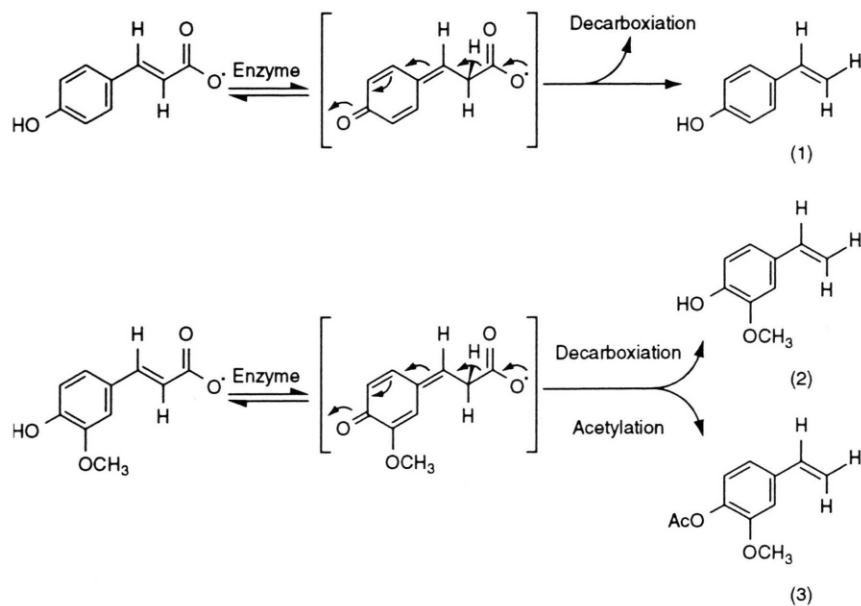


Fig. 2. HPLC profiles of wheat root exudates. A: treatment with *p*-coumaric acid (500 µg/ml). B: ferulic acid (500 µg/ml). The major peaks in controls are *p*-coumaric acid (A) and ferulic acid (B).



Scheme. A possible mechanism of enzymatic conversion of 4-hydroxyphenyl propenoic acids to 4-hydroxystyrenes by plants.

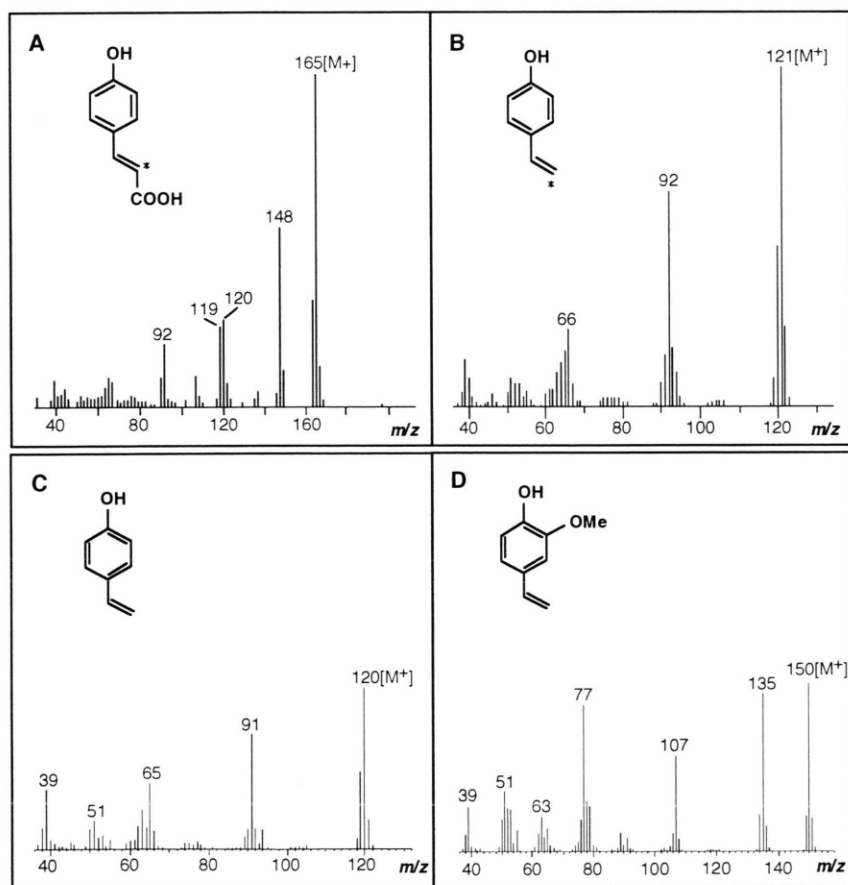


Fig. 3. MS spectra of ^{13}C labeled *p*-coumaric acid and decarboxylated products. A: $[2-^{13}\text{C}]$ -*p*-coumaric acid prepared by chemical synthesis. B: $[1-^{13}\text{C}]$ -4-hydroxystyrene-derived from $[2-^{13}\text{C}]$ -*p*-coumaric acid. C: 4-hydroxystyrene found in sterile wheat callus fed with *p*-coumaric acid. D: 3-methoxy-4-hydroxystyrene derived from ferulic acid. *, ^{13}C atom.

dition as in the wheat culture, and found ^{13}C -labeled 4-hydroxystyrene as a major antimicrobial component.

The HPLC chromatographic separation of the exudate from the *p*-coumaric acid-feeding experiment was carried out, and a peak (R_t 23.5) was collected and then subjected to GC-MS. The presence of a molecular ion peak at m/z 121, supported that ^{13}C -labeled 4-hydroxystyrene was formed in the callus system (Fig. 3).

This indicates that plants possess a specific enzyme which decarboxylates the $\text{C}_6\text{-C}_3$ unit consisting of benzene and propenoyl moieties into the corresponding styrenes.

Confirmation of the direct conversion of phenyl propenoic acids into styrenes

Wheat calli were aseptically grown on MS medium at 25 °C for 2-months. (Murashige and Skoog, 1962) and the clumps were then soaked in 500 $\mu\text{g/ml}$ of ^{13}C -labeled *p*-coumaric acid and ferulic acid solutions. After a 24 h-incubation at 25 °C, the clumps were removed from the test solution. The resulting solution was subjected to

HPLC (Fig. 4) and GC-MS analysis. The fragmentation patterns were identical to those of 4-hydroxystyrene and 3-methoxy-4-hydroxystyrene (Fig. 3).

These compounds markedly inhibited the growth of some unidentified bacteria isolated from the rhizosphere.

Biological activities of 1, 2, and 3

Compounds, **1**, **2**, and **3** were subjected to some biological tests such as antimicrobial tests with *Escherichia coli* and *Bacillus subtilis* and spore germination tests with *Aspergillus candidus* and *Cladosporium herbarum*, a phytotoxicity test with *Medicago sativa*. Three styrenes (**1**, **2**, and **3**) which had a variety of activities are shown in the Table.

Compounds, **1** and **2** exhibited the highest activity and both inhibited the spore germination of *Aspergillus candidus* and *Cladosporium herbarum* at a minimum inhibitory concentration (MIC) of 32 $\mu\text{g/ml}$.

However, these styrenes were not active in a seed germination inhibition test.

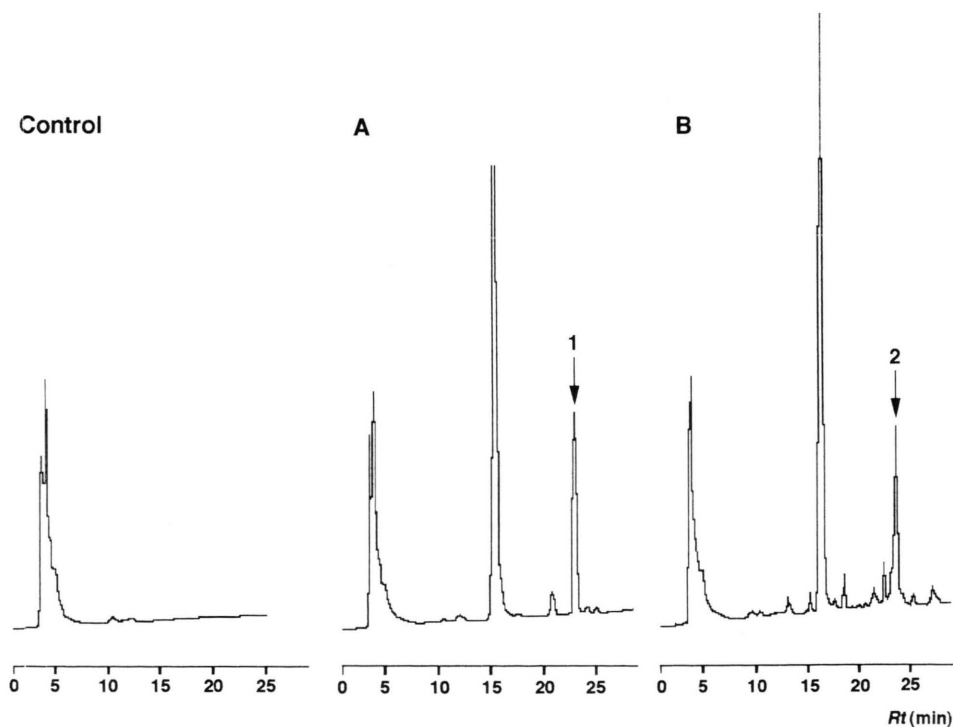


Fig. 4. HPLC profiles of exudate compounds of wheat callus treated with *p*-coumaric acid (A: 500 $\mu\text{g/ml}$) and ferulic acid (B: 500 $\mu\text{g/ml}$).

Table. Anti-microbial activity and phytotoxicity of decarboxylated compounds.

Test organism	MIC [$\mu\text{g/ml}$]			<i>p</i> -Coumaric	Ferulic acid
	1	2	3		
<i>Bacillus subtilis</i> (IFO 3108) ^a	500	500	65	1000	1000
<i>Escherichia coli</i> (IFO 3301, K-12) ^a	500	500	>1000	1000	>1000
<i>Aspergillus candidus</i> (IFO 4036) ^b	125	32	125	500	1000
<i>Cladosporium herbarum</i> (IFO 6374) ^b	32	65	125	250	500
<i>Medicago sativa</i>	>1000	>1000	>1000	>1000	>1000

^a Dilution method; ^b Spore germination test.

1: 4-hydroxystyrene; 2: 3-methoxy-4-acetoxystyrene; 3: 3-methoxy-4-hydroxystyrene.

Discussion

Root exudates are defined here as low molecular weight compounds which are released into the surrounding medium by living and intact roots (Rovira, 1969). Our interest in rhizosphere chemicals is confined to the biologically active secondary metabolites which may bear more relevance to allelopathy than do water-soluble exudates, such as common sugars and amino acids. The chemistry of the bioactive compounds in the rhizosphere is of fundamental importance to understand the interactions between the plant root system and other living organisms.

Our preliminary study showed that the mother compounds of **1**, **2**, and **3** were present in leaf leachates and such styrenes may possibly be converted from the corresponding precursors by microbial decarboxylases (Hashidoko *et al.*, 1993).

These compounds were found to show specific antimicrobial activity to some bacteria, suggesting that the growth of certain microbes can be affected under the environmental condition containing such compounds.

The decarboxylation of *p*-coumaric acid, caffeic acid and ferulic acid to the corresponding styrenes has been studied in fungal and bacterial metabolisms (Finkle *et al.*, 1962; Indahl *et al.*, 1968). So, the possibility was not excluded that the three exudate components may be produced by some bacteria attached on the wheat root surface. To verify whether plants contain a decarboxylase, feeding experiments with [2-¹³C] labeled *p*-coumaric acid were carried out under strict aseptic conditions with wheat and petunia calli. This finding strongly suggests that plants contain a decarboxylase which catalyzes *p*-hydroxycinnamic acid derivatives to yield the corresponding styrenes.

The root exudate obtained from phenyl propenoic acid treatment was found to contain an appreciable amount of amino acids and a large amount of sugars. The root exudates were found to contain 17 amino acids and 4 sugars (data will be shown in a following paper). This indicates that these compounds are released into the rhizosphere as response to leaf leachate components such as 4-hydroxycinnamic acid derivatives.

These findings may suggest a unique physiological role of phenolic compounds in the microbe-root interactions.

Materials and Methods

General experimental procedure

¹H NMR spectra were obtained with a Varian VXR-500 instrument. Mass spectra were recorded with a JEOL JMS-SX102A instrument. IR and UV spectra were measured with a Nicolet 710 FT-IR and a Shimadzu UV-3000 spectrophotometer, respectively.

Plant materials

Wheat (*Triticum aestivum* L. Norin no. 24) seeds were used in the uptake experiment. The seeds were peeled and sterilized by immersing them in 70% EtOH for 60 sec., followed by 5% H₂O₂ for 20 min.

The treated seeds were rinsed three times with sterilized water. Five surface-sterilized seeds were transferred onto a medium containing 0.1% (w/v) MgCl₂ and 0.2% (w/v) Gelrite in test tubes and incubated in the light (3000 Lux) at 20 °C for 8 days. The roots of 8-day-old plants were soaked into each test solution containing 500 $\mu\text{g/ml}$ of *p*-coumaric acid, ferulic acid, caffeic acid and ¹³C-

labeled *p*-coumaric acid, respectively at 20 °C for 18 h. The plants were removed from the test solutions, and an aliquot of the solutions was then subjected to an antimicrobial test and HPLC analysis (L-6200 intelligent pump; L-4200 UV-VIS detector (254 nm); AS-2000 autosampler; L-5020 column oven; D-2500 chromato-integrator; HITACHI, Tokyo) using an Inertsil ODS (4.6 x 250 mm, 5 µm; GL Sciences, Tokyo) and a flow rate of 0.8 ml/min. A linear gradient of 30% MeOH aq. in 1% AcOH to 90% MeOH aq. in 1% AcOH was employed. The HPLC was monitored at 254 nm. Three decarboxylated compounds were obtained as oils. 4-Hydroxystyrene, 3-methoxy-4-hydroxystyrene and 3-methoxy-3-acetoxystyrene were induced by phenyl propenoic acid treatments (Scheme).

Chemicals

p-Coumaric acid, ferulic acid, and caffeic acid were purchased from Nacalai Tesque Inc., Kyoto. [2-¹³C]-Malonic-acid was obtained from Sigma Chemical Company, St. Louis, USA.

Extraction and isolation of styrenes

The test solution (2 liters) was extracted twice with EtOAc. The organic phases were combined and concentrated *in vacuo* to afford a brownish oil (175 mg), which was chromatographed on a silica gel column (Wakogel C-100) eluted in stepwise mode with 65 ml each of 20, 40, 70, 100% EtOAc / *n*-hexane. The active fraction (40% EtOAc eluate) was further purified by a silica gel column to give styrenes, **1** (30 mg), **2** (5 mg), and **3** (2 mg).

Properties of **1**

EI-MS *m/z*: 120 [M+H]⁺, UV λ max (MeOH) nm (log ε): 225 (3.56), 286 sh (3.37), 299 sh (3.41), 313 (3.45); ¹H NMR δ (500 MHz, *d*₆-Acetone): 5.02 (1 H, dd, *J*=1.01, 10.8 Hz), 5.58 (1 H, dd, *J*=1.01, 17.6 Hz), 6.63 (1 H, dd, *J*=10.8, 17.6 Hz), 6.79 (2 H, d, *J*=8.7 Hz), 7.27 (2 H, d, *J*=8.7 Hz), 8.46 (1 H, s).

Properties of **2**

EI-MS *m/z*: 150 [M+H]⁺, UV λ max (MeOH) nm (log ε): 211 (4.25), 287 (4.01), 300 sh (3.56), 312 (3.95); ¹H NMR δ (500 MHz, CDCl₃): 3.86 (3 H), 5.03 (1 H, d, *J*=10.7 Hz), 5.60 (1 H, d, *J*=17.7

Hz), 6.64 (1 H, dd, *J*=10.7, 17.7 Hz), 6.77 (1 H, s), 6.89 (1 H, s), 7.09 (1 H, s).

Properties of **3**

EI-MS *m/z*: 192 [M+H]⁺, UV λ max (MeOH) nm (log ε): 207 (4.15), 250 (3.56), 287 (3.46), 315 sh (3.34); ¹H NMR δ (500 MHz, CDCl₃): 2.31 (3 H, s), 3.85 (3 H, s), 5.23 (1 H, d, *J*=10.7 Hz), 5.68 (1 H, d, *J*=17.4 Hz), 6.67 (1 H, dd, *J*=10.7, 17.4 Hz), 6.98 (3 H, m).

Culture conditions

Wheat (*Triticum aestivum* L. Norin no. 24) callus cultures were initiated from embryos excised from surface-sterilized seeds. For callus culture medium consisted of MS inorganic nutrients, 1 mg/l thiamine-HCl, 100 µg/l meso-inositol, 2% sucrose, 0.7% Bacto-Agar and 2,4-D 1.0 mg/l (Purnhauser, 1987). The cultures were maintained at 25 °C (16 h dark). After four weeks the callus clumps were cut into 0.5 cm pieces and transferred onto the same medium.

Application of phenyl propenoic acid to wheat calli

Phenyl propenoic acids (1.5 mg) were dissolved in 3 ml of distilled water and sterilized by autoclaving for 5 min. Ten-mm sections of the 2-month old callus were transferred into the test tubes. The tubes were incubated at 25 °C on a rotating cultivator (2 rpm). After 24 h-incubation, the sections were weighed and returned into the original tubes. After filtration the filtrate was subjected to HPLC (Fig. 4) and GC-MS analysis (MS-GC 200 gas chromatography, Hewlett Packard 5890, U.S.A.; MS-AM 20 quadrupole mass spectrometer, JEOL, Japan; MS-PC 386 D1, Data System; MA-PR 1124 Printer) using a 15 m x 0.25 mm I.D. capillary column coated with a 0.25 mm film of TC-FFAP (GL Science, Japan); Helium was used as carrier gas, and the flow rate was 30.5 ml/min; the temperature of the interface was 250 °C, that of ion source 170 °C and that of the injection port 250 °C; the temperature program began at 80 °C, ramped to 200 °C at a rate of 10 °C/min, and then held for 10 min. The molecular ions at *m/z* 120 (121 for ¹³C labeled sample) and *m/z* 150 were monitored for 4-hydroxystyrene and 3-methoxy-4-hydroxystyrene, respectively (Fig. 3).

Antimicrobial assay and seed germination inhibition test

Antimicrobial assays and seed germination inhibition tests were carried out as described (Kobayashi *et al.*, 1993, 1994).

Synthesis of ^{13}C -labeled *p*-coumaric acid

In a round-bottomed flask, fitted with a reflux condenser and a thermometer, were placed 109 mg (1.05 mmol) [^{13}C] malonic acid, 87 mg (0.71 mmol) of *p*-hydroxybenzaldehyde, and 1.5 ml of pyridine. The [^{13}C] malonic acid was warmed by an oil bath and dissolved by stirring. Piperidine (20 μl) was then added, and the mixed solution heated to 80 °C. An internal temperature of 80–85 °C was maintained for 1 h, and finally heated

under reflux (109–115 °C) for additional 3 h. After cooling the reaction mixture was poured into a beaker containing 5 ml of cold water, and then acidified by slow addition of 2 N hydrochloric acid. The resulting solution was partitioned with EtOAc, and then dried over anhydrous Na_2SO_4 . After a silica gel column the labeled *p*-coumaric acid was obtained in powder form.

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