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Production of 2-(2-phenylethyl) chromones in cell suspension cultures of *Aquilaria sinensis*

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Abstract

2-(2-Phenylethyl) chromones are the major constituents responsible for the quality of agarwood, which is one of the most valuable non-timber products used as incenses, perfumes, traditional medicines and other products. In this study, cell suspension culture of *Aquilaria sinensis* (Lour) Gilg was used to monitor the eliciting effects of crude fungal extracts on cell growth and chromones production. Crude extracts of *Melanotus flavolivens* (B. etc) Sing. prepared with different solvents were used to elicit the production of 2-(2-phenylethyl) chromones in cell suspension cultures of *A. sinensis*. Four 2-(2-phenylethyl) chromones, 6,7-dimethoxy-2-(2-phenylethyl) chromone (1), 6,7-dimethoxy-2-[2-(4′-methoxyphenyl)ethyl] chromone (2), 6-methoxy-2-[2-(4′-methoxyphenyl)ethyl] chromone (3) and 6-methoxy-2-(2-phenylethyl) chromone (4), were detected by LC–MS in the cell suspension culture of *A. sinensis* elicited with crude extracts of *M. flavolivens*. Three hundred and seventy eight, 196 and 31 μg g^{-1} DW of 2-(2-phenylethyl) chromones were obtained in the cell cultures induced by water extracts, 50 and 95% ethanol extracts of *M. flavolivens*, respectively. The results show that water-soluble materials in the crude extracts are the main components inducing the production of 2-(2-phenylethyl) chromones in the cell cultures.

Abbreviations: NAA – 1-naphthalene acetic acid; BA – benzyladenine; DW – dry weight; FW – fresh weight; HPLC – high performance liquid chromatography; LC–MS – liquid chromatography–mass spectrum; MS – Murashige and Skoog's medium

Introduction

Aquilaria sinensis (Lour.) Gilg. (Thymelaeceae), a large evergreen tree, distributed in Hainan, Guangdong, Guangxi, and Fujian provinces of China, is the main plant species for the production of agarwood used as traditional Chinese drugs since 16th century (Liu, 1999). Agarwood (also called Chen Xiang in Chinese; gaharu, jinkoh, or eaglewood, depending on the region) is the most highly valuable resinous wood used as incenses, perfumes, traditional medicines and other products in the world market (Takagi et al., 1982; Chakrabarty et al., 1994; Ng et al., 1997; Liu, 1999). Agarwood cannot generate in the normal wood tissues but may form in the cut or burned tissues in *Aquilaria* spp. Cutting and burning damage in wood tissue of tree is probably the way of infecting fungi naturally *in vivo*. Recently, several fungi which are responsible for the formation of agarwood have been isolated and identified (Jalaluddin, 1977; Qi, 1995). It was suggested that agarwood is a pathological product formed as the result of a fungal disease in wood tissue of intact

tree of *Aquilaria* spp. (Qi, 1995). But the mechanism of agarwood formation in tree of *Aquilaria* is still unclear.

2-(2-Phenylethyl) chromones, which are a major group of constituents in agarwood, are believed to be relevant to the quality of agarwood (Shimada et al., 1986) and may have antiallergic activity (Yang, 1998). Recently, more than 30 of 2-(2-phenylethyl) chromones have been isolated and identified in *Aquilaria* spp. (Qi, 1995). We discovered that 6,7-dimethoxy-2-(2-phenylethyl) chromone (1) and 6-methoxy-2-[2-(4′-methoxyphenyl)ethyl] chromone (3) were the major compounds in infected wood tissues of *A. sinensis in vivo* and these compounds increased with the time of fungal infection (Qi et al., 2000). 2-(2-Phenylethyl) chromones can therefore be a marker for the studies on the formation of agarwood in *Aquilaria* spp.

In this paper, we report the production of 2-(2-phenylethyl) chromone compounds induced by crude extract of *Melanotus flavolivens* (B. etc) Sing. in cell suspension cultures derived from the root tissue of *A. sinensis*. This would be a new system to study the mechanism of agarwood formation in *Aquilaria*. It is the first time that the synthesis of chromone compounds in cultured cell is reported.

Materials and methods

Materials

Seeds of *Aquilaria sinensis* (Lour) Gilg were collected from South China Botanical Garden, Guangzhou.

Melanotus flavolivens (B. etc) Sing was purchased from the Institute of Microbiology, Chinese Academy of Sciences.

2-(2-Phenylethyl) chromone compounds (Figure 1) were prepared from agarwood by the method of previous report (Lin and Qi, 2000).

Cell suspension culture and treatments

Coat-peeled off seeds were surface-sterilized using mercuric chloride (0.1% v/v) for 10–15 min, and washed with sterile distilled water 3–4 times. The surface-sterilized seeds were then placed on solid MS (Murashige and Skoog, 1962) basal media for germination. Callus cultures were initiated from the root tissues of a 2-week-old plant grown *in vitro* and were cultured in media containing MS salts and vitamins supplemented with NAA (1.1 μM), BA (2.2 μM) and sucrose (15 g l^{-1}). The cultures were maintained under dark condition at 26 ± 2 °C and subcultured every 3 weeks.

Cell suspension cultures were established from the callus cultures, and were maintained in 500 ml Erlenmeyer flasks with 150 ml of liquid MS medium consisting of NAA (10.7 μM), BA (2.2 μM) and sucrose (30 g l^{-1}) on a rotary shaker at 110 rpm at 26 ± 2 °C. All the cultures for cell growth and chromone production were under the same culture condition and subcultured every 10 days.

Initial inoculums of 100 g l^{-1} (FW) of cells were used for all experiments. Cell suspension cultures grown in 500 ml Erlenmeyer flasks with 150 ml of liquid MS medium were elicited by adding sterilized fungal extracts in day 7 and continuously cultured to day 24. Equal volume of water, 50% ethanol or 95% ethanol solvents used in the experimentations was set as controls. For the quenching cell, the cell suspension cultures were treated in 121 °C for 10 min before elicitation by adding sterilized fungal extracts. The cells were harvested to detect cell weight and 2-(2-phenylethyl) chromones every 3 days. Freeze-dried cells were cocultured with fungal extracts in the medium mentioned above and harvested in 1 and 3 days.

Preparation of fungal extracts

Fungal cultures of *M. flavolivens* were produced in shake-flasks with liquid potato medium at

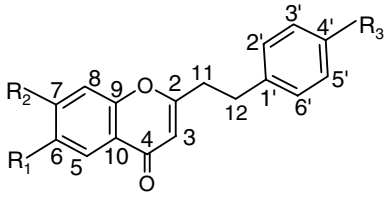

Figure 1. Chemical structure of 2-(2-Phenylethyl) chromones. Note: 6,7-dimethoxy-2-(2-phenylethyl) chromone (1): R_1 = R_2 = OCH_3; R_3 = H; 6,7-dimethoxy-2-[2-(4′-methoxyphenyl)ethyl] chromone (2): R_1 = R_2 = R_3 = OCH_3; 6-methoxy-2-[2-(4′-methoxyphenyl)ethyl] chromone (3): R_1 = R_3 = OCH_3; R_2 = H; 6-methoxy-2-(2-phenylethyl) chromone (4): R_1 = OCH_3, R_2 = R_3 = H.

100 rpm at 26 ± 2 °C for 2 weeks, and mycelia were harvested for the fungal extract preparation. Mycelia were dried in oven at 60 °C and extracted by water and ethanol (50 and 95%, v/v), respectively. The extracts were dried in the rotary distillatory at 50–80 °C.

Estimation of 2-(2-phenylethyl) chromones

The cells were dried in oven at 60 °C and mashed in mortar. The dry cell powder (0.5–1 g) was extracted twice with 20–40 ml anhydrous ethanol at room temperature overnight. After combining and concentrating the extracts in the rotary distillatory at 40 °C, 5–10 ml Na_2CO_3 (5% v/v) was added to the residues and shook the sample violently for 20 s. It was then extracted with methylene chloride, washed with water for 3 times and then dried in anhydrous sodium sulfate (Na_2SO_4). The methylene chloride was collected under 40 °C and the residue was dissolved in 1 ml of methanol. The samples were prepared for HPLC analysis after filtration with a 0.2-μm membrane. A 20 μl sample was used for HPLC analysis.

Analytical HPLC was performed on HP 1100 system controller equipped with the UV absorbance detector. Oven temperature was 35 °C. Reversed-phase column used was a 250 mm × 4.6 mm (i.d.), 5 μm Kromeril-C18 phase and the mobile phase consisted of methanol–tetrahydrofuran–water (50:5:45) with a flow rate of 0.8 ml min^{-1}. The injection volume was 20 μl. 2-(2-Phenylethyl) chromones were quantified at 217 nm. Samples were compared with the standard on the basis of retention time and peak area of 2-(2-phenylethyl) chromones. The total amount of 2-(2-phenylethyl) chromones was the sum of chromone 1, 2, 3 and 4 in each treatment respectively. Standard errors were calculated from three replicates.

The LC–MS system consisted of a high performance liquid chromatography (PE 200) interfaced to API-2000 mass spectrometer equipped with a Turboion Spray (ABI Co USA). Positive-ion ESI was performed using an ion source voltage of 5.0 kV, a declustering potential 83 V, a focusing potential 350 V and an entrance potential 10 V. Nebulization was aided with a coaxial nitrogen sheath gas provided at a pressure of 25 psi. Desolvation was aided using a counter current nitrogen flow set at a pressure of 30 psi and a capillary temperature of 150 °C. The column used was a 150 mm × 3.9 mm (i.d.), 4 μm Nova-Pak C18 phase (Waters) and the mobile phase consisted of methanol–water (60:40) with 0.7 ml min^{-1} flow rate. The injection volume was 10 μl. The mass spectrum monitored ions in the range m/z 100–500 amu. The LC eluant was injected into an API-2000 mass spectrometor at a flow rate of 0.7 ml min^{-1}. The mass spectrum parameters were optimized with 2-(2-phenylethyl) chromones as the reference.

Results and discussion

In the cell suspension cultures, treated with 4 mg l^{-1} crude extracts of *M. flavolivens*, four 2-(2-phenylethyl) chromones were detected by HPLC and identified by mass spectrum in comparison with standard compounds. The production of 6,7-dimethoxy-2-(2-phenylethyl) chromone (1) is more sensitive to the treatment of crude extracts of *M. flavolivens* comparing with others. 155.9 μg g^{-1} DW of Chromone 1 is detected in the cell cultures treated with water crude extracts, but only 1.1 μg g^{-1} DW with 95% ethanol crude extracts (Figure 2). Similar results were obtained in the wood tissues of intact tree of *A. sinensis* infected with *M. flavolivens* for 5 months. In that case, the concentration of chomone (1) and 6-methoxy-2-[2-(4′-methoxyphenyl)ethyl] chromone (3) was 203.6 and 54.9 μg g^{-1}, respectively (Qi et al., 2000). Neither wood tissues in intact tree nor cell suspension cultures could produce any chromone compounds without the treatment of fungi or fungal extracts. According to our results, the extracts of *M. flavolivens* or the fungi itself may induce the *de novo* synthesis of 2-(2-phenylethyl) chromones in the cultured cells or intact tree of *A. sinensis*.

The cell suspension cultures of *A. sinensis* reached cell stationary phase on day 15 of cultivation. When the sterilized crude fungal extracts of *M. flavolivens* were added to the medium at day 7, the cells stopped growing and the 2-(2-phenylethyl) chromones generated rapidly. After 15 days of cultivation, the concentration of 2-(2-phenylethyl) chromone in the cells decreased (Figure 3). But we failed to detect any 2-(2-phenylethyl) chromones in the quenched and freeze-dried cells. It is clear that the fungal extracts both elicit the biosynthesis of 2-(2-phenylethyl) chromones and inhibit the cell growth in

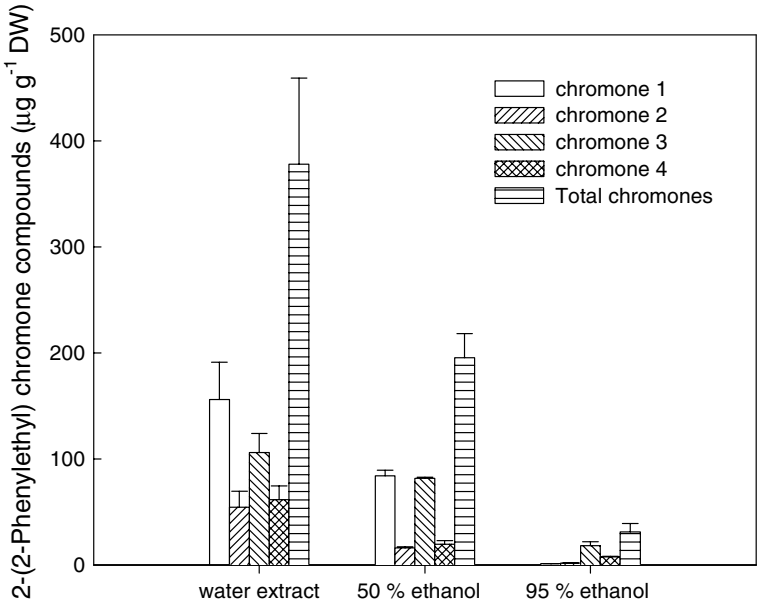

Figure 2. Effect of 4 mg ml^{-1} different solvent crude extracts of *Melanotus flavolivens* (B. etc) Sing on the production of 2-(2-phenylethyl) chomone compounds in cell suspension cultures of *Aquilaria sinensis* (Lour.) Sing (values are mean ± SD, $n = 3$).

the cell suspension culture of *A. sinensis*. Similar results were reported recently (Zhang et al., 2001; Yuan et al., 2002). Chen and Chen (2000) suggested that the decrease of biomass accumulation was due to the defense response to reduce primary metabolism of cell. Our results indicate that crude extracst of *M. flavolivens* caused the defense responses and changed primary as well as secondary metabolisms in the cell. These responses and changes in metabolism are similar

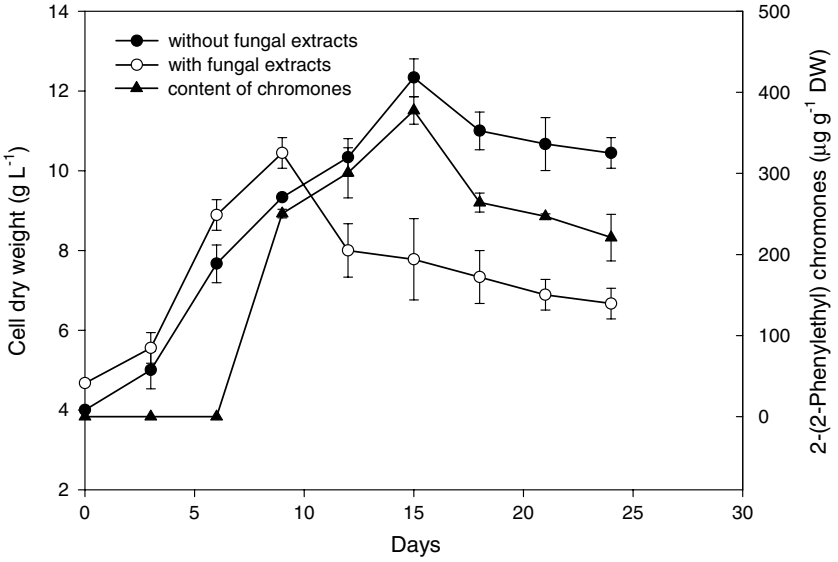

Figure 3. Cell growth and production of 2-(2-phenylethyl) chromones in cell suspension cultures derived from root shoot of *Aquilaria sinensis* (Lour.) Sing. (values are mean ± SD, $n = 3$). All cells were cultured in 500 ml Erlenmeyer flask with 150 ml liquid MS medium consisting NAA (10.7 μM), BA (2.2 μM) and sucrose (30 g l^{-1}) in dark condition. Cell suspension cultures were elicited with or without 4 mg ml^{-1} water crude extract of *Melanotus flavolivens* in day 7 of cultivation. For quenching cells, the cell suspension cultures were treated in 121 °C for 10 min and then elicited with sterilized fungal extracts in day 7 of cultivation.

to the results of both *in vitro* and *in vivo* tests in *A. sinensis* (Yu and Liang, 1980).

Elicitors are defined as molecules that stimulate defense or stress-induced responses in plants (Van Etten et al., 1994). Fungal elicitors mainly derive from the cell walls of fungal pathogens (Szabo et al., 1999). In our experiments, the 95% ethanol extracts barely induced the production of 2-(2-phenylethyl) chromone in the cell suspension culture while the water extracts exerted a significant induction (Figure 2). The total amount of 2-(2-phenylethyl) chromones is 378, 196 and 31 μg g^{-1} DW in the cell cultures induced by water extracts, 50% ethanol extracts and 95% ethanol extracts, respectively. The results show that the main constituents to elicit the production of 2-(2-phenylethyl) chromones in the cell cultures are water-soluble materials in crude extracts of *M. flavolivens*.

The basic chemical structure of 2-(2-phenylethyl) chromones and flavonoids is similar. Although flavonoid compounds distribute widely in plants, 2-(2-phenylethyl) chromones have been obtained only from a few plant species: *Eremophila georgei* (Picker et al., 1976) (Myoporaceae), *Bothriochloa ischaemum* (Wang et al., 2001) (Gramineae) and *Aquilaria* spp. (Thymelaeaceae) (Yang, 1998). It is known flavonoid derived from chalcone compound which is synthesized from a molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA by chalcone synthase (Shirley, 1996). However, there has been no report about the biosynthesis of 2-(2-phenylethyl) chromone yet.

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