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Production of Stilbenoids from the Callus of *Arachis hypogaea*: a Novel Source of the Anticancer Compound Piceatannol

KUO-LUNG KU,*,† PING-SHUN CHANG,‡ YA-CHIN CHENG,‡ AND CHING-YI LIEN†

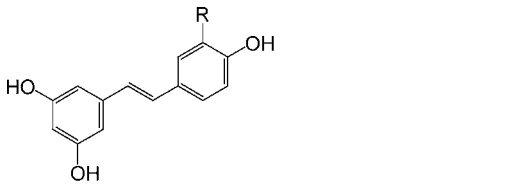
Department of Applied Chemistry and Graduate Institute of Agriculture, National Chiayi University, 300 University Road, Chiayi 60083, Taiwan

A new source to produce a significant quantity of a naturally occurring polyphenol, piceatannol, was investigated in this study. Both resveratrol and piceatannol are recognized as important ingredients in functional foods due to their beneficial health effects. However, unlike resveratrol, the piceatannol concentration in plants is very low. Thus, calluses of peanuts, an easily obtainable source, were chosen as the material to induce piceatannol production under controlled conditions. To induce resveratrol and piceatannol, calluses were exposed to the ultraviolet (UV) irradiation. Significant quantities of resveratrol and piceatannol were produced by calluses upon UV irradiation in both static and suspension culture conditions. The amounts of piceatannol and resveratrol produced in 1 g of calluses ranged from 2.17 to 5.31 μg and from 0.25 to 11.97 μg, respectively, in static culture. In suspension culture, the amounts of induced piceatannol and resveratrol were somewhat lower. The quantities of induced piceatannol and resveratrol reached a maximum at 18 h after UV irradiation treatment in static culture. In contrast, the levels of resveratrol and piceatannol remained almost constant throughout the experiments in suspension culture. The piceatannol produced by calluses in all studies was much higher than the values reported in the literature, whereas the resveratrol produced was comparable to reported values.

KEYWORDS: Piceatannol; resveratrol; ultraviolet irradiation; callus; *Arachis hypogaea*; plant tissue culture

INTRODUCTION

Piceatannol (3,4,3′,5′-tetrahydroxy-*trans*-stilbene) (**Figure 1**) is a naturally occurring polyphenol with a polyhydroxystilbene core structure. Both piceatannol and resveratrol are synthesized as phytoalexins in response to fungal attack or other environmental stress in limited plants (*1*−*8*). Resveratrol and piceatannol may have various beneficial health effects. For example, moderate intake of resveratrol or resveratrol-containing food, such as red wine, may prevent cardiovascular diseases (*9, 10*) and delay the onset of tumor cells (*11*). Recently, studies have shown that resveratrol and piceatannol play important roles in cancer prevention. Both compounds have antitumor activities toward various cancer cells (*12*−*14*). Many studies have linked the antitumor activities of resveratrol and piceatannol to their abilities to inhibit cell proliferation and arrest cells in the S phase (*12, 15*). Resveratrol, unlike piceatannol, is not a very efficient inducer of apoptosis in an ex vivo assay of primary lymphoblasts (*14*). However, it has been demonstrated that resveratrol can be converted into piceatannol by the cytochrome P450 enzyme CYP1B1. These observations not only explain the anticancer activity of resveratrol but also demonstrate that a possible dietary cancer preventative agent can be converted to a compound with known anticancer activity by an enzyme that is found in human tumors (*16*). In addition, recent studies have shown that resveratrol and piceatannol, along with other naturally occurring polyphenolic molecules, were able to increase the lifespan of yeast cells by stimulating the SIRT1 activity (*17*). As compared with resveratrol, piceatannol is a stronger antioxidant and a stronger SIRT1 stimulator (*18*).



R= H; Resveratrol; 3,5,4'-trihydroxy-*trans*-stilbene
R= OH; Piceatannol; 3,4,3',5'-tetrahydroxy-*trans*-stilbene

Figure 1. Structures of piceatannol and resveratrol.

Significant quantities of resveratrol are found in numerous sources, including red wines; however, only minute quantities of piceatannol are found in a limited number of natural sources.

* Author to whom correspondence should be addressed (telephone 886-5-271-7951; fax 886-5-271-7901; e-mail klku@mail.ncyu.edu.tw).
† Department of Applied Chemistry.
‡ Graduate Institute of Agriculture.

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Piceatannol, first isolated as an antileukemic agent from the seeds of *Euphorbia lagascae* (*8*), is also present in grape (*19*) and *Vaccinium* berries (*20*). Other plants, such as *Rheum* spp. (*21*), *Machura pomifera* (*22*), and *Senna* spp. (*23*), have also been reported to contain piceatannol in very low concentrations. Even in berries in which significant amounts of piceatannol were found, the piceatannol content was only in the nanogram range. Thus, unlike resveratrol, piceatannol is much less available due to the very low levels present in plants. Recently, studies have pointed out the importance of piceatannol in promoting human health, which makes piceatannol a highly demanded nutraceutical compound. However, it can be very expensive and time-consuming to produce large quantities of piceatannol from plants. Therefore, an inexpensive and widely available source that can provide a large quantity of piceatannol is required.

Several research groups have demonstrated the ability to produce stilbenoid compounds from cultured plant tissues in normal or induced conditions. For example, resveratrol was isolated from suspended cell cultures of grape, *Vitis vinifera* (*10*), and peanut, *Arachis hypogaea* (*24*). The resveratrol contents in grapes could be increased by ultraviolet C (UV-C) irradiation (*5*, *6*). In the callus of *A. hypogaea*, isopentenyl resveratrol was induced via UV-C irradiation (*25*). Piceatannol has never been induced in the calluses of *A. hypogaea* or other related peanut tissues (*1*, *3*, *26*−*28*). Cell culture of peanut is an ideal system to produce stilbenoid compounds, because the stilbene synthase in cultured tissues is easily induced in a controlled environment. In addition, peanuts are an inexpensive and easily obtained source to generate plant tissues for cell culture. Thus, we chose the callus of *A. hypogaea* as a study material to induce piceatannol. In this study, calluses of peanuts were exposed to UV irradiation to induce piceatannol and resveratrol. The effects of culture condition on piceatannol and resveratrol induction were also investigated using the calluses.

MATERIALS AND METHODS

Pretreatment. Calluses of peanuts used in this study were obtained from the stems of *A. hypogaea* L. cv. Tainan No. 14. To isolate tissues for callus cultures, stems of peanut plants were first cleaned with deionized water followed by 70% ethanol. The clean stems were disinfected by sonication with a 1% sodium hypochlorite solution for 3 min and then washed three times with sterile deionized water before use in callus cultures.

Culture Conditions. Calluses originated from the stems were initially grown on agar, which contained Murashige and Skoog's basal medium (*29*), 4 mg/L 1-naphthylacetic acid, and 1 mg/L 6-benzylamino purine. The medium was adjusted to pH 5.8 with either NaOH or HCl prior to the addition of agar. A disinfected stem of 3 cm in length was evenly cut into three pieces. Two pieces of this stem were implanted on slant agar medium in a test tube to produce calluses. Calluses were then transferred to a bigger agar plate every 30 days. All calluses were grown in the dark at 25 °C until they were ready for use in experiments.

For suspension cultures, 250 mL Erlenmeyer flasks containing ~50 mL of the above medium were inoculated with 1 g of calluses. These callus-containing flasks were then incubated with constant shaking (125 rpm) at 25 °C in the dark for 3 days prior to induction.

Induction and Extraction of Stilbenoid. To induce stilbenoids, 1 g aliquots of calluses from static or suspension cultures were placed into Petri dishes and exposed continuously under UV-C light for 20 min. The model TUV TL-D 30 W UV-C light of maximum wavelength 254 nm (Philips, Amsterdam, The Netherlands) was directly positioned 55 cm above the culture medium. After irradiation, Petri dishes with the calluses were incubated at 25 °C in the dark until they were sampled. All of the above procedures were carried out in a temperature-controlled aseptic laminar flow chamber. To examine the quantity of induced stilbenoids, Petri dishes were sampled at specific time intervals. The callus samples were then subjected to extraction for subsequent analyses. For each extraction, the sampled callus was dispersed and ground in 1 mL of methanol. This mixture was then filtered to collect the methanol extract. The residual callus was again extracted two more times with methanol. A total of 3 mL of combined methanol filtrate was transferred into a volumetric flask and diluted with additional methanol to 5 mL. Finally, the resulting methanol extract was directly analyzed with mass spectrometry. For HPLC analysis, the methanol extract was diluted to 20 mL with deionized water and then passed through a C18-E SPE cartridge (500 mg/3 mL) (Phenomenex, Torrance, CA). The cartridge was eluted successively with 3 mL of 10% methanol/water (v/v, 10:90) and 1 mL of 60% methanol/water (v/v, 60:40). The 60% methanol fraction was collected and subjected to HPLC analysis.

Identification of Stilbenoids Produced by Calluses. The SPE-eluted methanol solution was subjected to MS analysis by using an LCQ advantage ion trap analyzer spectrometer (Thermo Finnigan, San Jose, CA) fitted with an electrospray ionization probe. A sample was infused into the probe at a rate of 5 μL/min by using a syringe pump. The negative mode electrospray ionization was used to obtain ions for analysis. The full ESI-MS spectrum of crude methanol extract was first obtained followed by the collision-induced dissociation (CID) spectra of the selective ions. The capillary temperature and needle voltage were set to be 195 °C and 3.2 KV, respectively. The sheath gas flow was set to be 10 arbitrary units. To get a CID spectrum, the normalized ionization energy was set to 36%.

HPLC Analysis of Piceatannol. The quantity of induced piceatannol in calluses was determined by HPLC analysis. All analyses were performed using the L-7100 HPLC pump coupled with an L-7420 UV−vis detector, an L-7485 fluorescence detector (Hitachi Co. Ltd., Tokyo, Japan), and a 250 × 4.6 mm i.d. Mightsil RP-18 C-18 column (Kanto Chemical Co. Inc., Kanagawa, Japan). The excitation and emission wavelengths of the fluorescence detector were set to be 343 and 395 nm, respectively. Both UV and fluorescence chromatograms were recorded simultaneously. Only the fluorescence signals were used to calculate the piceatannol and resveratrol concentrations. The mobile phase consisted of acetonitrile and deionized water, adjusted to pH 2.1 with formic acid prior to analysis. The stepwise HPLC mobile phase conditions were as follows: initial acetonitrile composition, 20%, increased to 32% in 20 min, increased to 90% in 10 min, and then held at 90% for 5 min; injection volume, 20 μL. Representative HPLC chromatograms are shown in **Figure 4**. The piceatannol and resveratrol concentrations in this methanol extract were 0.88 and 5.01 μg/mL, respectively. The concentrations of piceatannol and resveratrol standards used in this analysis were 2.0 μg/mL. To quantify the piceatannol and resveratrol contents in calluses, a calibration curve was constructed prior to sample analysis using authentic piceatannol and resveratrol (Calbiochem, EMD Bioscience Inc., La Jolla, CA). The piceatannol and resveratrol contents in calluses were derived from the calibration curves using the authentic compounds. The linear range of quantitative analyses for piceatannol was 0.025−5.0 μg/mL. The linear range of quantitative analyses for resveratrol was 0.025−20.0 μg/mL. The corresponding correlation coefficients (R^2) were 0.9984 and 0.9999, indicating excellent correlations between peak areas and standard concentrations.

RESULTS AND DISCUSSION

It is known that peanuts produce stilbenoid compounds in response to fungal infection (*1*, *30*−*33*), UV irradiation (*25*, *34*), and other physical damage (*7*, *35*). Stilbenoids found in peanut include resveratrol, 3-isopentadienyl resveratrol, and various arachidins (*1*, *30*, *31*, *36*). Studies have shown that stilbenoids can accumulate in different parts of peanut plants in response to fungal infection (*3*), in peanut cotyledons at different stages of maturity in response to wounding (*7*), and in peanut calluses in response to UV-C (*25*). However, piceatannol, another important phytoalexin belonging to the stilbenoid family, has never been found in peanuts and related tissues.

A negative-ESI-MS spectrum of the methanol extract from UV-C-irradiated calluses was obtained. A strong signal with an *m*/*z* value of 227, which corresponds to the ionized resveratrol in negative mode, was observed. To confirm this designation,

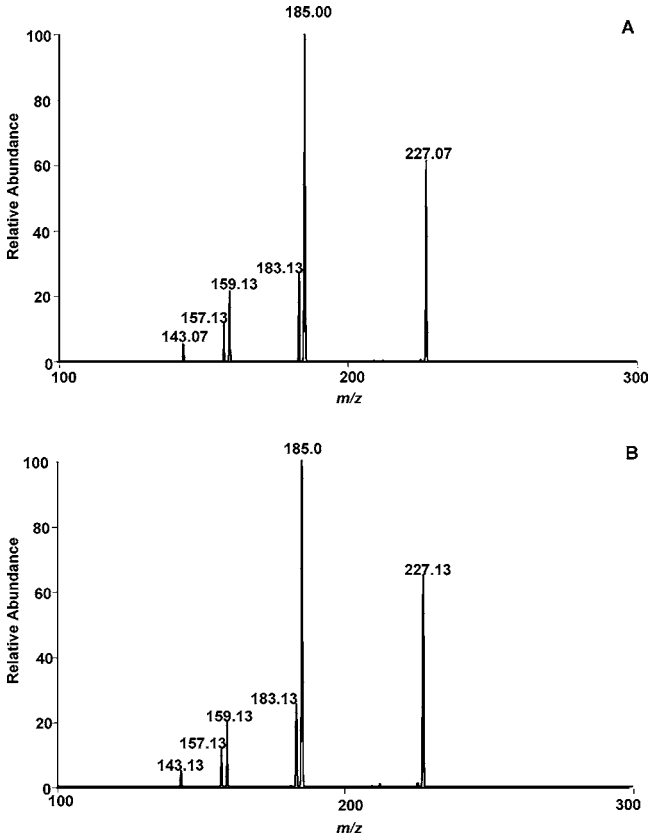

Figure 2. (**A**) CID spectrum of *m*/*z* 227 ion found in the methanolic extract of callus with UV irradiation. (**B**) CID spectrum of authentic *trans*-resveratrol.

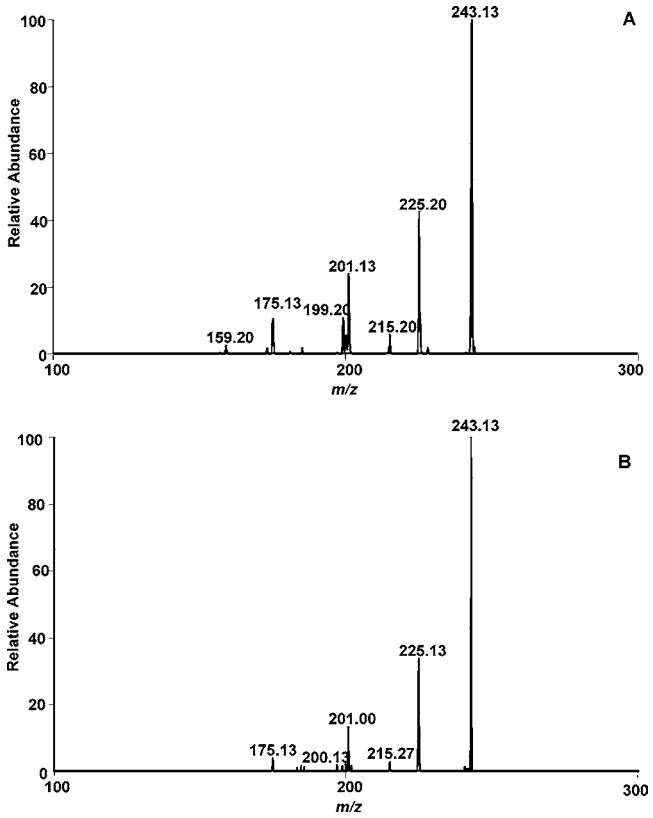

Figure 3. (**A**) CID spectrum of *m*/*z* 243 ion found in the methanolic extract of callus with UV irradiation. (**B**) CID spectrum of authentic *trans*-piceatannol.

a MS/MS analysis was carried out. The CID spectrum (**Figure 2A**) of the selected ion, *m*/*z* 227, coincided with the CID spectrum of authentic resveratrol (**Figure 2B**). A significant signal with *m*/*z* 243 (16 amu larger than that of resveratrol) was found in the total negative-ESI-MS spectrum. This difference suggested the presence of an extra oxygen atom, which, in turn, suggested the existence of an additional hydroxyl group in the structure of resveratrol. In the literature, piceatannol is one of the polyhydroxystilbene compounds with four hydroxyl groups. CID spectra of authentic piceatannol and the selected ion are shown in panels **A** and **B**, respectively, of **Figure 3**. The same fragmentation patterns observed in the two CID spectra confirm the existence of piceatannol in the methanol extracts of peanut callus.

Figure 4 shows the HPLC fluorescence chromatograms of methanol extracts from normal and UV-irradiated calluses. When the chromatograms were compared, two new peaks were observed in spectra of extracts obtained from calluses exposed to UV irradiation (**Figure 4B**), whereas no obvious peak with similar retention time was observed for the extract obtained from calluses without UV treatment (**Figure 4A**). The retention times of the new peaks were 11.5 and 17.1 min, respectively. To confirm the identities of two new peaks, piceatannol and resveratrol standards were spiked into the UV-treated calluses. Coelution of the spiked standards and the target peaks (**Figure 4C**) confirmed the identities of the 11.5 and 17.1 min peaks, indicating the presence of piceatannol and resveratrol in the UV-irradiated calluses. A mass spectrometry experiment proved that the small peak appearing at ~12 min in **Figure 4A** was not piceatannol. On the basis of the results of MS and HPLC analysis, we confirm that piceatannol as well as resveratrol can

Table 1. Contents of Piceatannol and Resveratrol during the Static Cultivation of Peanut Calluses after UV-C Irradiation

	sampling time				
	0 h	6 h	12 h	18 h	24 h
piceatannol	ND[a]	ND	2.17[a] ± 1.18[b]	5.31 ± 0.51	4.60 ± 1.46
resveratrol	ND	0.25 ± 0.16	3.31 ± 0.67	11.97 ± 0.64	1.42 ± 0.65

[a] ND, not detected. [b] Piceatannol and resveratrol contents (μg) in 1 g of callus. [c] Standard deviation calculated on three experiments.

be produced by calluses of peanuts in response to UV irradiation in a controlled environment.

As shown in **Table 1**, the amount of resveratrol produced by 1 g of UV-treated calluses ranged from 0.25 to 11.97 μg, whereas the piceatannol content ranged from 2.17 to 5.31 μg in static culture. The induced resveratrol content was comparable to reported values, but the induced piceatannol content was much higher than the reported quantities extracted from other plant sources. The irradiation power, irradiation time, and position of UV-C light used in this study concur with the induction condition reported in the literature. Cantos et al. (*5*) reported maximum resveratrol yields when UV-C irradiation power, irradiation time, and position were set to be 510 W, 30 s, and 40 cm above the grapes, respectively. When weaker irradiation is used, the irradiation time had to be much longer to produce a large quantity of resveratrol. In static culture, the increase in the level of induced piceatannol and resveratrol was time-dependent. Piceatannol was not detected until 12 h in static culture, whereas a small quantity of resveratrol was observed at 6 h. The lag period between the resveratrol and piceatannol production could indicate that resveratrol is the precursor for the synthesis of piceatannol by enzymes in plants. This

Table 2. Contents of Piceatannol and Resveratrol during the Suspension Cultivation of Peanut Calluses after UV-C Irradiation

	sampling time								
	0 h	4 h	8 h	12 h	16 h	20 h	32 h	56 h	80 h
piceatannol	0.52[a] ± 0.10[b]	0.52 ± 0.20	0.52 ± 0.27	0.38 ± 0.12	0.30 ± 0.05	0.32 ± 0.02	0.51 ± 0.11	0.43 ± 0.13	0.49 ± 0.32
resveratrol	3.93 ± 1.75	6.93 ± 3.83	3.47 ± 2.44	4.32 ± 1.24	3.14 ± 1.14	3.51 ± 1.69	4.55 ± 2.18	4.53 ± 1.01	4.12 ± 1.7

[a] Piceatannol and resveratrol contents (μg) in 1 g of callus. [b] Standard deviation calculated on four experiments.

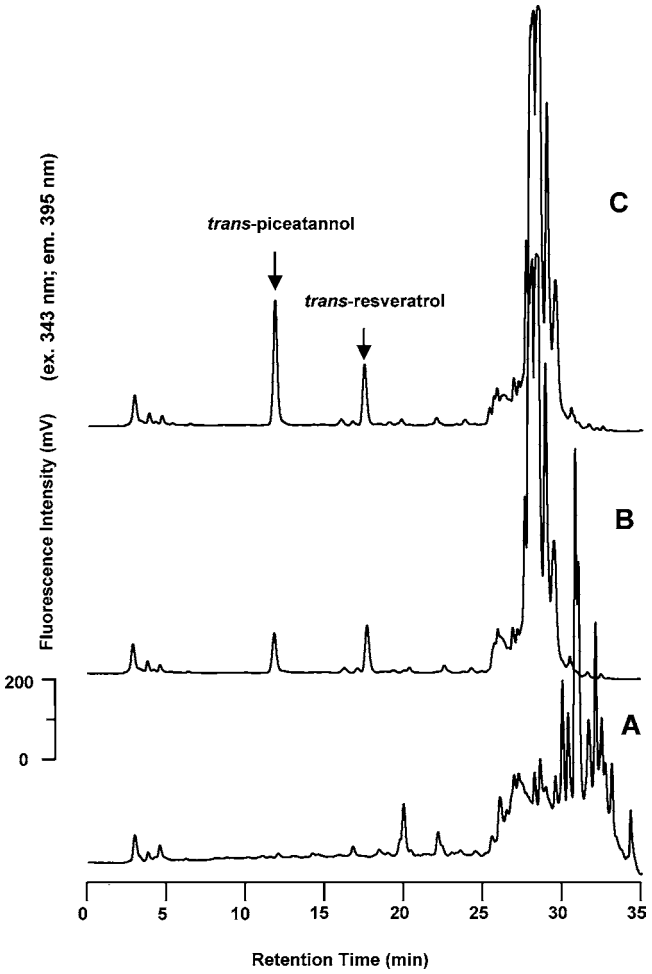

Figure 4. Fluorescence HPLC chromatograms of methanolic extracts obtained from calluses with and without UV irradiation: (**A**) without UV irradiation; (**B**) with UV irradiation; (**C**) same sample as in (**B**) spiked with resveratrol and piceatannol standards.

observation agrees with similar findings published by Fritzemeier et al. (*25*), who reported that resveratrol synthesis preceded isopentyl-resveratrol synthesis upon UV irradiation of peanut calluses.

The impact of the physical treatment on the amount of piceatannol produced by calluses of peanuts was also investigated. Because culture medium was shaken at 125 rpm in the culture condition, calluses were physically damaged. The effect of this colliding force on piceatannol induction was examined by comparing the amounts of piceatannol produced in culture conditions with and without shaking. As shown in **Table 2**, calluses received the UV irradiation and produced various quantities of resveratrol and piceatannol in almost all cases.

In suspension culture conditions, both piceatannol and resveratrol were produced by calluses at the beginning of the time-dependent experiments. The piceatannol and resveratrol quantities remained almost steady throughout the experiments and in the ranges of 0.30−0.52 and 3.14−6.93 μg/g, respectively. The lag period in piceatannol production observed in static culture conditions was not seen in suspension culture conditions. As compared with the static culture condition, calluses seemed to produce less piceatannol and resveratrol in suspension culture. The effect of UV irradiation on piceatannol and resveratrol induction in suspension appeared to be less efficient as compared with that in static culture. Because the calluses were constantly moving in suspension cultures, they may receive shorter UV irradiation than those in static cultures and produce less piceatannol and resveratrol. However, further studies are required to investigate this observation. The piceatannol quantities produced by calluses in static and suspension cultures were still much higher than the literature-reported values obtained from other plant sources.

The detailed mechanism of how piceatannol can be produced by plants is not known. In human lymphoblasts, piceatannol can be synthesized from resveratrol by one of the cytochrome P450 enzymes, CYP1B1 (*16*), the enzyme that has been detected in a wide variety of tumors. The CYP1B1 directly converts resveratrol to piceatannol, which, in turn, inhibits the growth of tumor cells. A similar enzyme in plants has not yet been discovered. However, plants have many cytochrome P450 enzymes and produce a significant quantity of resveratrol along with other polyphenol compounds in response to environmental stresses (*38*). Considering the coexistence of resveratrol and piceatannol and the case found in human lymphoblasts, it is possible that piceatannol can be synthesized by enzymes from resveratrol in UV-irradiated calluses.

On the basis of the results of this study, we conclude that a significant quantity of piceatannol can be produced by calluses of peanuts in the presence of UV-C irradiation. Considering the increasing demand for piceatannol and resveratrol and the advantages of using peanut tissues as the production source, this study provides an economical way to produce a large quantity of these biologically important antioxidants. The calluses grown in suspension are easily scaled up using a larger fermentator in a common and inexpensive medium at room temperature, which provides the basic requirement for mass production of piceatannol. However, further studies are required before commercial production of this product.

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