Investigation of Microbial Elicitation of *trans*-Resveratrol and *trans*-Piceatannol in Peanut Callus Led to the Application of Chitin as a Potential Elicitor

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It is well-known that the invasion of microbes such as fungi in some plants, including peanut, can induce the biosynthesis of stilbenoids such as *trans*-resveratrol and *trans*-piceatannol. However, in a recent study it was found that not all kinds of microorganisms possessed such potential. The Gram-negative bacterium *Pseudochrobactrum asaccharolyticum* isolated from the peanut callus failed to act as an elicitor. After systematic investigation, the different inductive effects between fungi and Gram-negative bacteria were attributed to the chitin content of the cell wall. Results showed significantly more *trans*-resveratrol and *trans*-piceatannol was induced by fungi (8.92–16.35 and 2.15–7.01 μg/g of fresh calluses, respectively) than by bacteria (1.77–2.72 and 0.16–0.52 μg/g of fresh calluses, respectively), regardless of species and viability. Such great differences prompted the direct utilization of chitin, the distinctive component of fungal cell wall, as an elicitor. The results that *trans*-resveratrol induced by chitin was about two-thirds the amount induced by sterilized fungi, whereas *trans*-piceatannol (2.55 $\pm$ 0.60 μg/g) was close to that by sterilized fungi, revealed chitin is not only an important fungal constituent responsible for the induction of *trans*-resveratrol and *trans*-piceatannol but also an efficient elicitor by itself. These findings suggested sterilized fungi and chitin can be used as a safe and fast elicitor, as far as the risk of viable microbes is concerned, to induce *trans*-resveratrol and *trans*-piceatannol in the well-controlled peanut tissue culture.

KEYWORDS: Fungi; sterilization; *trans*-resveratrol; *trans*-piceatannol; peanut callus; chitin

INTRODUCTION

A good number of stilbenoids have been discovered in plants acting as phytoalexins in response to biotic or abiotic challenge (*1*−*5*). Among these secondary metabolites, *trans*-resveratrol and *trans*-piceatannol have been extensively studied and are well-known to possess health-promoting biological activities (*6*−*12*). Not only was the distribution of these two stilbenoids in grape, peanut, and their products (*13*−*17*) reported, but also their elicitation by abiotic stresses such as UV irradiation, ultrasound exposure, and chemical treatment (**Table 1**) and biotic stresses (*24*−*27*) has been widely studied. Here we raised the following questions: Can all kinds of microbes act as elicitors? Is any constituent of microbes responsible for the induction of *trans*-resveratrol and *trans*-piceatannol?

In fact, during routine subculture in our laboratory, some of the peanut calluses were found to secrete a pale yellow gluey fluid and withered after 2−3 weeks. Unexpectedly, only traces of *trans*-resveratrol and *trans*-piceatannol were detected in these latent-infected peanut tissues. On the other hand, the discovery that *trans*-resveratrol and *trans*-piceatannol were effectively induced by plant pathogenic fungi led to the hypothesis that chitin was the key constituent contributing to the induction of stilbenoids in peanut calluses. In addition, a chitin oligosaccharide elicitor-binding protein named CEBiP was isolated from the plasma membrane of suspension-cultured rice cells and related to the biosynthesis of phytoalexins (*28*). Although significant induction of *trans*-resveratrol and *trans*-piceatannol has been achieved by artificial fungal infection (*24*−*27*), simultaneous production of fungal toxins should be a safety concern. Therefore, from the safety point of view, this work was aimed to utilize the sterilized fungi and chitin as elicitors to produce *trans*-resveratrol- and *trans*-piceatannol-containing peanut callus for direct food use.

MATERIALS AND METHODS

Callus Culture. Peanut calluses were obtained from the seeds of *Arachis hypogaea* L. cv. Tainan No. 14 (Tainan District Agricultural Research and Extension Station, Taiwan) and prepared according to the procedure described by Ku et al. (*20*) with modification. Briefly, deshelled seeds were rinsed with deionized water, disinfected by sonication in 1% sodium perchlorite solution containing 2 drops of Tween 20 for 2 min, and washed with sterile deionized water. The clean seeds were grown in Murashige and Skoog's basal medium for 1 week. Then 0.5 cm of the hypocotyls was aseptically cut and cultured in MS medium containing 3 mg/L 1-naphthylacetic acid and 1 mg/L 6-benzylaminopurine at 28 °C in the dark for 3 weeks prior to induction of stilbenoids. Calluses were subcultured every 4 weeks.

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Table 1. Maximum Yields of *trans*-Resveratrol and *trans*-Piceatannol Obtained after Abiotic Stimulation

compound	treatment	max yield	ref
trans-resveratrol	grapevine leaves under UV-C irradiation for 15 min plus 48 h of induction	750 μg/g of fw	18
	grapes under UV-C irradiation for 30 s plus 3 days of incubation	∼3 mg/200 g of fw, 115 μg/g of skin	19
	grapes under UV-C irradiation for 60 s followed by 5 days of storage	2315.9 ± 190.4 μg/100 g of berries, both *cis* and *trans* forms	16
	peanut calluses under UV-C light for 20 min followed by incubation for 18 h	11.97 ± 0.64 μg/g of fw	20
	sliced peanut kernels exposed to ultrasound for 4 min and then incubated for 36 h	3.96 ± 0.96 μg/g of dry weight	17
	grapes treated with (1) ozone (3.88 g/h, 5 h) followed by storage (22 °C, 95% RH, 2 days) or (2) UV-C irradiation for 1 min plus storage for 1−2 days	∼1400 μg/100 g of fw	21
	20 h of aerobic incubation of germinating peanut slices	147.3 ± 14.0 μg/g of dry weight	22
	grapes under cumulative treatment of 10 mM methyl jasmonate spray	0.34 μg/g of berries	23
trans-piceatannol	grapes under UV-C irradiation for 60 s followed by 3 days of storage	173.4 ± 15.6 μg/100 g of fw	19
	peanut calluses under UV-C light for 20 min followed by incubation for 18 h	5.31 ± 0.51 μg/g of fw	20

Treatment of Calluses. *Bacteria.* Two strains of Gram-negative, plant pathogenic bacteria were chosen to examine whether similar elicitation will be found in peanut calluses. *Xanthomonas campestris* pv. *citri* XW24, isolated from leaves with citrus canker, was a kind gift from Dr. Li-Yuan Wang of Chiayi Agricultural Experiment Station, Taiwan Agricultural Research Institute. Another Gram-negative strain isolated from the pale yellow excretions on the surface of aseptically cultured peanut seeds in our laboratory and named AHX TN14 was later identified as *Pseudochrobactrum asaccharolyticum.* The PCR primers used to amplify 16S rRNA gene fragments were 27F-AGAGTTTGATCMTGGCTCAG and 1492R-TACGGYTACCTTGTTACGACTT. Bacteria were grown on a nutrient agar plate at 25 °C for 2 days and then suspended with sterile deionized water. Sterilized bacteria were prepared by autoclaving the suspension at 121 °C for 30 min. One gram of callus was treated with 1 mL of viable and sterilized bacteria (0.006 g of dry wt/mL) separately and incubated at 28 °C in the dark for 12 h.

Fungi. Botryodiplodia theobromae LBBT HC6-1 isolated from lima bean and *Botrytis cinerea* FCBC TN1 isolated from strawberry were maintained in our laboratory. These fungi were used in this study because LBBT HC6-1 is known as a general plant pathogen and FCBC TN1 has been shown to be effective on *trans*-resveratrol elicitation in grapevine (*24*). A mass of mycelia was equally cut by a puncher (5 mm diameter) and cultured on potato dextrose agar (PDA) lined with cellophane paper at 25 °C for 3 days (LBBT HC6-1) or 14 days (FCBC TN1). The paper with each fungal mycelium (ca. 0.002 g of dry weight) was cut into 2 × 2 cm dimension, autoclaved (121 °C, 30 min) if sterilization was required, and placed on 1 g of callus for induction of stilbenoids. The mycelia (5 mm diameter) of spore-forming fungus *B. theobromae* LBBT HC6-1 were alternatively cultured on potato agar (PDA excluding dextrose) slant medium at 25 °C for 10 days for spore formation. One milliliter of the spore suspension (10^5 conidia/mL) was similarly used for induction of stilbenoids as the bacteria.

Chitin. Chitin (Sigma C7170) was pulverized and passed through a 0.22 mm sieve. The chitin powder weighed, and 0.1, 0.2, or 0.4 g was individually suspended in 10 mL of deionized water and autoclaved at 121 °C for 30 min. Similarly, 1 g of callus was treated with 1 mL of cooled chitin suspension at different levels and incubated at 28 °C in the dark for 12 h, because results of preliminary experiments showed the production of *trans*-resveratrol in peanut calluses under biotic or abiotic elicitation reached the maximum after 12 h at induction.

Extraction of Stilbenoids and HPLC Analysis. About 1 g of callus was ground in 1 mL of methanol and filtered with Whatman no. 2 filter paper. The residue was extracted with another 1 mL of fresh methanol twice. The filtrate was combined, adjusted to 5 mL with methanol, and filtered through a 0.22 μm disk filter prior to HPLC analysis.

Quantitative determination of *trans*-resveratrol and *trans*-piceatannol in methanol extracts was performed by RP-HPLC as previously described by Ku et al. (*20*). All analyses were performed using an L-7100 pump coupled with an L-7485 fluorescence detector (Hitachi, Tokyo, Japan) and a Mightysil C-18 column (250 × 4.6 mm, 5 μm, Kanto Chemical Co., Kanagawa, Japan). Fluorescence of the target compounds was monitored at 343 (excitation) and 395 nm (emission). Linear gradient elution composed of solvent A (1% formic acid) and solvent B (1% formic acid

in acetonitrile) was as follows: 20% B increased to 32% B within 20 min, further increased to 90% within 10 min, and then held at 90% for 5 min. The injection volume was 20 μL. Authentic compounds and LC grade reagents were all purchased from Merck (Darmstadt, Germany).

Statistical Analysis. Data were analyzed by analysis of variance using Duncan's multiple-range test. Differences were considered to be significant at $P < 0.05$ (SAS program Windows version 6.2). All values are presented as mean ± SD.

RESULTS AND DISCUSSION

Effects of Different Microorganisms on the Production of Stilbenoids in Peanut Calluses. In our regular callus maintenance practice, we found the calluses originated from some particular seeds, although treated aseptically, became brown after subseeding and withered eventually. Meanwhile, there was pale yellow excretion found not only on the surface of the calluses but also on the surface of aseptically germinated seeds originally used to generate the calluses. Incubation of the pale yellow excretions surprisingly resulted in discovery of bacterial growth, later identified as *P. asaccharolyticum.* Such latent infection indeed introduced stress so that phytoalexins *trans*-resveratrol and *trans*-piceatannol, normally not detectable in healthy sprouts, were synthesized during aseptic germination, but still limited to trace amounts (1.22 ± 0.45 μg of *trans*-resveratrol and 0.46 ± 0.19 μg of *trans*-piceatannol per g of fresh sprout, $n = 3$). This finding indicated that bacteria may not be as effective as fungi for stilbenoid elicitation in peanut callus.

To systematically compare the inductive effects of fungi and bacteria on phytoalexin synthesis, two strains of each microbe were selected to treat the calluses. Obviously, more *trans*-resveratrol was induced by fungi (8.92−16.35 μg/g) than by bacteria (1.77−2.72 μg/g), regardless of species and viability (**Table 2**). The amount of *trans*-piceatannol induced by fungi (2.15−7.01 μg/g) was also significantly greater than that by bacteria (0.16−0.52 μg/g).

Our results were similar to the resveratrol production in grapevine leaves elicited by a soil bacterium belonging to the *Bacillus* genus (170 × 10^6 conidia/mL) alone or together with gray mold disease-causing fungus *Botrytis cinerea* (2.4 × 10^5 conidia/mL) (*24*). Resveratrol concentrations in bacteria-infected leaves of *Vitis vinifera* and *Vitis rupestris* were 0−6.07 and 1.98−12.93 μg/g, respectively, with maxima shown on the third day after infection. The elicitation of resveratrol by the fungus was stronger than that by the soil bacterium in *V. vinifera*, similar to the microbial effects we found in peanut calluses. However, the Gram-positive *Bacillus* strain revealed stronger eliciting effect on *V. rupestris*. These results suggested the degree of resveratrol elicitation may be influenced by both plant and elicitor species. Because the *Bacillus* species is not a known pathogen to peanuts, whether peanut will respond to the *Bacillus* species to produce phytoalexins remains to be understood.

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Table 2. Production of *trans*-Resveratrol and *trans*-Piceatannol in Peanut Callus Inoculated with Viable or Sterilized Microorganisms for 12 h[a]

microorganism[b]	*trans*-resveratrol (μg/g of fresh weight)	*trans*-piceatannol (μg/g of fresh weight)
control	ND	ND
fungus 1, viable (*n* = 6)	15.52 A	7.01 A
sterilized (*n* = 6)	8.92 AB	2.15 BC
fungus 2, viable (*n* = 7)	15.91 A	2.25 BC
sterilized (*n* = 7)	16.35 A	3.74 B
bacterium 1, viable (*n* = 7)	1.85 B	0.33 C
sterilized (*n* = 4)	2.13 B	0.39 C
bacterium 2, viable (*n* = 6)	1.77 B	0.52 C
sterilized (*n* = 4)	2.72 B	0.16 C

[a] Means bearing different letters in the same column are significantly different as determined by Duncan's multiple-range test at $P < 0.05$. ND, not detectable. [b] Fungus 1, *Botryodiplodia theobromae* LBBT HC6-1; fungus 2, *Botrytis cinerea* FCBC TN1; bacterium 1, AHX TN14; bacterium 2, *Xanthomonas campestris* pv. *citri* XW 24. Bacteria and fungi (mycelia) sterilized by autoclaving were not able to grow during 1 week of incubation.
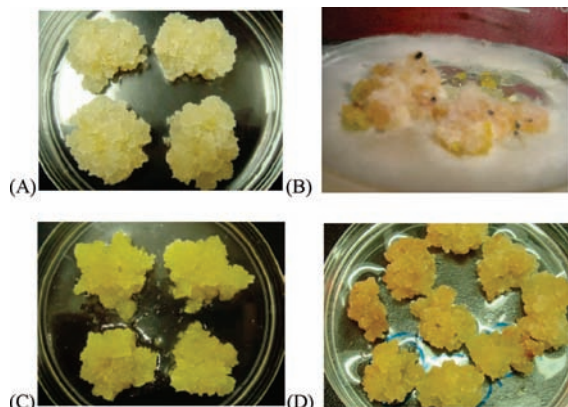
Figure 1. Appearance of peanut calluses after induction with viable or sterilized *Botryodiplodia theobromae* LBBT HC6-1: (**A**) blank, 12 h; (**B**) viable, 12 h; (**C**) sterilized, 12 h; (**D**) sterilized, 18 h.

The color of peanut calluses was changed from white (**Figure 1A**) to bright yellow (**Figure 1C**) with the accumulation of stilbenoids induced by sterilized *B. theobromae* LBBT HC6-1 for 12 h. Meanwhile, the infected calluses at 12 h (**Figure 1B**) were surrounded by fungal mycelia and showed locally darker spots, which appeared on calluses after ≥18 h of incubation with sterilized fungi (**Figure 1D**) and led to withering eventually. The HPLC−fluorescence chromatograms of methanol extracts of normal and sterilized *B. cinerea* FCBC TN1-treated calluses are shown in **Figure 2**. The peaks of *trans*-piceatannol and *trans*-resveratrol were confirmed by spiking with the authentic compounds. In addition, at a retention time of about 30 min there were significant fluorescence signals including *trans*-arachidin-1 as compared to the description of Sobolev et al. (*5*).

Factors Affecting Stilbenoid Production. *Form of Fungi.* The inductive effects of fungal spores and mycelia on stilbenoid production of peanut calluses were compared. Although sterilized mycelium induced less *trans*-resveratrol (8.92 ± 7.80 μg/g of fresh weight), there was generally no significant difference in *trans*-resveratrol production between spore and mycelium treatment (**Table 3**). Similarly, the induction of *trans*-piceatannol by spores was close to that by mycelia with the most *trans*-piceatannol (7.02 ± 5.33 μg/g of fresh weight) induced by viable mycelia.
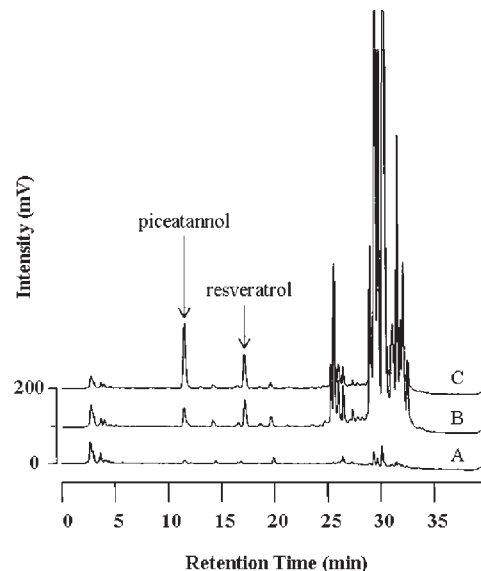
Figure 2. HPLC−fluorescence chromatograms of methanol extracts of (**A**) normal callus and (**B**) sterilized *Botrytis cinerea* FCBC TN1-treated callus. (**C**) The peaks of *trans*-piceatannol and *trans*-resveratrol were confirmed by spike method.

Table 3. Production of *trans*-Resveratrol and *trans*-Piceatannol in Peanut Callus after Inoculation with Different Forms of *Botryodiplodia theobromae* LBBT HC6-1 for 12 h[a]

treatment[b]	*trans*-resveratrol (μg/g offresh weight)	*trans*-piceatannol (μg/g of fresh weight)
control	ND	ND
spore, viable (*n* = 6)	11.18 A	4.10 AB
sterilized (*n* = 9)	15.25 A	1.26 B
mycelium, viable (*n* = 6)	15.52 A	7.02 A
sterilized (*n* = 6)	8.92 A	2.15 B

[a] Means bearing different letters in the same column are significantly different as determined by Duncan's multiple-range test at $P < 0.05$. ND, not detectable. [b] Spores and mycelia were sterilized by heating in boiling water for 15 min and autoclaving, respectively. Neither killed spores nor fungi were able to grow during 1 week of incubation.

Results in **Table 3** suggest that viability rather than the form of fungi was the important factor affecting stilbenoid production in peanut calluses.

Viable spores of *Aspergillus caelatus* were utilized to infect peanut seeds at the ratio of 10^7 spores/6 g of seeds, and the overall *trans*-stilbenoid production after 24 h of incubation was estimated at 2.1 mg/g of seed (mb) (*5*). However, in the case of callus, the optimal application ratio for *trans*-resveratrol and *trans*-piceatannol production was found to be 10^5 spores, viable or sterilized, per gram of callus.

Viability of Fungi. **Table 3** shows that the productions of *trans*-resveratrol induced by viable and sterilized *B. theobromae* LBBT HC6-1 mycelia were 15.52 ± 9.16 and 8.92 ± 7.80 μg/g of fresh callus, respectively, but significantly more *trans*-piceatannol was induced by viable (7.02 ± 5.33 μg/g μg/g) than by sterilized mycelia (2.15 ± 1.43 μg/g). In contrast to mycelia, the viability of spores played a less important role, because there was slightly more *trans*-piceatannol induced by viable spores (4.10 ± 1.75 μg/g) than by sterilized ones (1.26 ± 0.52 μg/g) and no difference in *trans*-resveratrol production.

Because *trans*-piceatannol, 3,4,3′,5′-tetrahydroxy-*trans*-stilbene, has one more hydroxyl group on the 3′-position than *trans*-resveratrol

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Table 4. Contents of *trans*-Resveratrol and *trans*-Piceatannol in Peanut Callus Stimulated by Chitin at Different Levels for 12 h[a]

chitin (g/g of callus)	*trans*-resveratrol (μg/g of fresh weight)	*trans*-piceatannol (μg/g of fresh weight)
0	ND	ND
0.01	7.67 A	0.68 B
0.02	5.86 A	0.99 B
0.04	10.43 A	2.55 A

[a] Means bearing different letters in the same column are significantly different as determined by Duncan's multiple-range test at $P < 0.05$ ($n = 3$). ND, not detectable.

and the time course obtained in our laboratory (*20*) showed *trans*-piceatannol was synthesized later than *trans*-resveratrol, it is believed that *trans*-resveratrol is easily induced by contact of fungal cell wall, whereas the unknown key enzyme for *trans*-piceatannol synthesis needs further stimulation, such as invasion of the viable spore and mycelia. Sobolev (*27*) studied the distribution of induced phytoalexins in peanut kernels upon infection of *Aspergillus* strains including the aflatoxin-generating *A. flavus* and *A. parasiticus*. In the first 24 h of incubation, only *trans*-resveratrol was found in tissues far away from the infected area, whereas five other more complicated phytoalexins, *trans*-arachidin-1, *trans*-arachidin-2, *trans*-arachidin-3, *trans*-3-isopentadienyl-4,3′,5′-trihydroxystilbene, and SB-1, were found only in areas close to the infected surface. With increasing incubation time, more phytoalexins along with *trans*-resveratrol appeared in the remote area. This also implied that *trans*-resveratrol is synthesized prior to other stilbenoids.

Chitin. On the basis of previous findings and the distinct difference in cell wall compositions of fungi and bacteria (*29−33*), chitin, rich in fungal cell wall, was suspected to be the key component contributing to the elicitation of stilbenoids by sterilized fungi. Due to the insolubility of chitin and technical restriction, chitin suspension was prepared at three ratios, that is, 0.01, 0.02, and 0.04 g/g of callus. Results in **Table 4** show *trans*-resveratrol induced by chitin at 0.04 g/g of callus was about two-thirds the amount induced by sterilized fungi, whereas *trans*-piceatannol induction by chitin (2.55 ± 0.60 μg/g) was close to that by sterilized fungi *B. theobromae* LBBT HC6-1 (2.15 ± 1.43 μg/g) and *B. cinerea* FCBC TN1 (3.74 ± 1.07 μg/g).

The recognition of chitin and chitin fragments for defense signaling has been elucidated in some plant cells other than peanut. Liswidowati et al. (*34*) found specific proteins including stilbene synthase (STS) and phenylalanine ammonia-lyase (PAL) were preferably synthesized when grapevine cells were cultured in the presence of fragments of *B. cinerea* cell wall or of a glucan prepared from the same cell wall, indicating the defense system of grapevine cells upon fungal attack led to induction of pathogen-related (PR) proteins which may possess glucanase and chitinase activities to destroy fungi or catalyze phytoalexin synthesis. Recent research showed some plants synthesize chitinases to destroy the invasive pathogenic fungi and to produce chitin fragments (chitin oligosaccharides) initiating signaling pathways (*35*). A chitin oligosaccharide elicitor-binding protein named CEBiP was isolated from the plasma membrane of suspension-cultured rice cells and related to the biosynthesis of phytoalexins, because knockdown of the CEBiP gene resulted in the cancellation of up-regulation of genes encoding enzymes such as PAL and caffeoyl-CoA 3-*O*-methyltransferase (*28*, *36*). Our findings suggest peanut calluses may have a similar chitin signaling system.

Reactive oxygen species (ROS) were simultaneously induced by chitin oligosaccharide elicitor in suspension-cultured rice cells (*28*). The amount of H_2O_2 induced by *N*-acetylchito-octaose $(GlcNAc)_8$ was 5 times more than that induced by chitosan octasaccharide, indicating degree of acetylation may affect binding and signaling. Therefore, it is suggested that the less expensive, highly acetylated, and water-insoluble chitin is more effective than water-soluble chitosan for elicitation of phytoalexins in peanut calluses as the signaling may be enhanced.

Nowadays, resveratrol can be chemically synthesized with 71% overall yield (*37*, *38*). Metabolical engineering of resveratrol biosynthesis has also been achieved in plants, microbes, and animals (*39*). However, the overall yield of *trans*-piceatannol synthesized via (*E*)-selective Wittig−Horner reaction was reported as only 40% (*40*). Piceatannol is more expensive because of scarcity. Our work provided a safe and fast method to simultaneously produce *trans*-resveratrol and *trans*-piceatannol using well-controlled peanut tissue culture.

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