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Enhanced Production of Resveratrol, Piceatannol, Arachidin-1, and Arachidin-3 in Hairy Root Cultures of Peanut Co-treated with Methyl Jasmonate and Cyclodextrin

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Supporting Information

ABSTRACT: Peanut (*Arachis hypogaea*) produces stilbenoids upon exposure to abiotic and biotic stresses. Among these compounds, the prenylated stilbenoids arachidin-1 and arachidin-3 have shown diverse biological activities with potential applications in human health. These compounds exhibit higher or novel biological activities in vitro when compared to their nonprenylated analogues piceatannol and resveratrol, respectively. However, assessment of these bioactivities in vivo has been challenging because of their limited availability. In this study, hairy root cultures of peanut were induced to produce stilbenoids upon treatment with elicitors. Co-treatment with 100 μM methyl jasmonate (MeJA) and 9 g/L methyl-β-cyclodextrin (CD) led to sustained high levels of resveratrol, piceatannol, arachidin-1, and arachidin-3 in the culture medium when compared to other elicitor treatments. The average yields of arachidin-1 and arachidin-3 were 56 and 148 mg/L, respectively, after co-treatment with MeJA and CD. Furthermore, MeJA and CD had a synergistic effect on resveratrol synthase gene expression, which could explain the higher yield of resveratrol when compared to treatment with either MeJA or CD alone. Peanut hairy root cultures were shown to be a controlled and sustainable axenic system for the production of the diverse types of biologically active stilbenoids.

KEYWORDS: *peanut, Arachis hypogaea, stilbenoids, hairy roots, methyl jasmonate, cyclodextrin, resveratrol, piceatannol, arachidin-1, arachidin-3*

INTRODUCTION

Peanut (*Arachis hypogaea*), a species from the Fabaceae (Leguminosae) family, is native to South America and has been widely distributed around world as an economically important crop. This species is capable of producing stilbene derivatives, including resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) and other stilbenoids, as phytoalexins to protect itself against various pathogens.[1−3]

As the most studied member in the stilbene family, resveratrol has received tremendous interest due to its wide range of biological activities with potential impact on human health, including antioxidant, anti-inflammatory, cardioprotective, antiviral, anticancer, and antiaging properties.[4] Although many in vitro and animal studies have demonstrated significant biological effects of resveratrol, the limited oral bioavailability of this polyphenol in humans due to rapid absorption and metabolism leading to the formation of various metabolites such as resveratrol glucuronides and sulfates have questioned its biological effects in humans.[5]

Interestingly in peanut, the majority of its known stilbenoids are prenylated, harboring an isopentenyl moiety (3-methyl-1-butenyl) as in arachidin-1 and arachidin-3 (Figure 1).[6−8] Arachidin-1 and arachidin-3 have exhibited potent antioxidant and anti-inflammatory activities.[9] Moreover, arachidin-1, which contains an additional hydroxyl group when compared to arachidin-3 or resveratrol, has shown higher efficacy in inducing programmed cell death in leukemia HL-60 cells, with an approximately 4-fold lower EC_{50} than resveratrol.[10] Similarly, arachidin-1 was shown to exhibit higher anti-adipogenic activity than resveratrol and arachidin-3 by inhibiting adipocyte differentiation in 3T3-L1 cells. This anti-adipogenic activity had no toxic effect on the differentiating pre-adipocytes and therefore it has been suggested that arachidin-1 should be further explored in antiobesity strategies.[11]

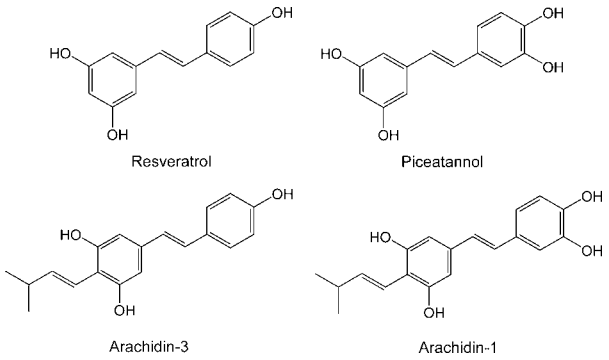

Figure 1. Chemical structures of stilbenoids identified in elicited hairy root cultures of peanut. All compounds are shown as their *trans* isomers.

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Arachidin-1 and arachidin-3 have been shown to modulate both cannabinoid receptors CB1 and CB2 in vitro. Molecular modeling studies with these compounds showed that the lipophilic side chain (3-methyl-1-butenyl group) in the structure of these stilbenoids was necessary for the high affinity attained to cannabinoid receptor CB2,[12] a therapeutic target in the treatment of neurodegenerative disorders such as Alzheimer's disease.[13] In addition, arachidin-3 and arachidin-1 are metabolized to fewer glucuronidated products than their nonprenylated analogues, resveratrol and piceatannol. This suggests that the lipophilic side chain might hinder the glucuronidation process and thereby enhance the bioavailability of arachidin-1 and arachidin-3 when compared to piceatannol or resveratrol.[12]

Despite the diverse bioactivities of peanut prenylated stilbenoids demonstrated in in vitro studies, in vivo studies have not been conducted due to their limited availability.[9,11,14,15] Furthermore, the biosynthetic pathways of these compounds have not been elucidated, which restricts potential metabolic engineering strategies for their bioproduction. To address these limitations, peanut hairy roots cultures have been developed as viable production platforms for stilbenoids.[16,17] Upon treatment with selected elicitors, the peanut hairy root cultures produce the same phytoalexins described in fungal-challenged peanut seeds. For instance, after 24 h of treatment with sodium acetate (NaOAc), peanut hairy roots secrete resveratrol, arachidin-1, and arachidin-3 into the culture medium.[16]

Different elicitors have been also used to induce the biosynthesis of stilbenoids in hairy roots of other species. For example, hydrogen peroxide (H_2O_2), a molecule in plants that mediates responses to stresses[18,19] and methyl jasmonate (MeJA), a key compound involved in defense-related signal transduction pathways in plants,[20] have been used as elicitors to induce the biosynthesis of resveratrol, piceatannol, and viniferins in hairy roots of *Vitis rotundifolia*.[21,22]

Previous studies have investigated the interaction between stilbenoids and cyclodextrins (CDs). The latter are torus-shaped oligosaccharides derived from starch that can form inclusion compounds with nonpolar molecules that fit into their hydrophobic cavity. For instance, resveratrol and CD were shown to form 1:1 inclusion complexes.[23] Interestingly, treatments with CD or CD plus MeJA were first used as an approach to increase the production of resveratrol in *Vitis vinifera* cell suspensions[24−26] and proved to be effective in enhancing the production of taxanes in cell suspension cultures of *Taxus* spp.[27] and inducing silymarin accumulation in *Silybum marianum* cell suspensions.[28]

In the current study, NaOAc, H_2O_2, MeJA, CD, and MeJA in combination with CD were tested as elicitors of stilbenoids in peanut hairy root cultures. The levels of stilbenoids in the culture medium were determined along a time course from 0 to 96 h. In addition, the effect of selected elicitor treatments on the expression of resveratrol synthase, the first committed metabolic step in stilbenoid biosynthesis, was investigated. Our results show that peanut hairy root cultures can be developed as controlled and sustainable high-level production systems for arachidin-1 and arachidin-3, two major peanut stilbenoids exhibiting a diversity of biological activities.

MATERIALS AND METHODS

Hairy Root Line and Growth Conditions. All experiments were conducted with hairy root cultures of peanut (*A. hypogaea*) cv. Hull line 3 established previously by the Medina-Bolivar laboratory.[16] Ten root tips (2−3 cm long) were inoculated into 250 mL flasks containing 50 mLof modified MS medium (MSV)[16] with 3% sucrose. The flask cultures were incubated on an orbital shaker (Innova 44R, New Brunswick Scientific) at 90 rpm and 28 °C and maintained under continuous darkness.

Elicitor Treatments. Nine-day-old hairy root cultures were treated under five different conditions to study stilbenoid production: 10.2 mM sodium acetate (NaOAc), 10 mM hydrogen peroxide (H_2O_2), 100 μM methyl jasmonate (MeJA), 9 g/L (6.87 mM) methyl-β-cyclodextrin (CD; Cavasol W7M), and 100 μM MeJA combined with 9 g/L CD. The concentration of NaOAc was established before,[16,17] whereas the concentration of MeJA was determined on the basis of previous studies with hairy root cultures of muscadine grape.[22] Preliminary studies with peanut hairy roots treated with different concentrations of H_2O_2 (Supporting Information, Figure S3) and MeJA combined with different concentrations CD (Figure S4) were used to determine the concentrations of H_2O_2 and CD used in the current study. Before elicitor treatment, the spent medium was removed and replaced with 50 mL of fresh MSV medium containing 3% sucrose and different elicitors. For the control group, the culture medium was replaced with MSV medium with 3% sucrose and 50 μL of absolute ethanol (EtOH; solvent of MeJA) (Sigma).

A 96 h time course per elicitor treatment was conducted including the following time points: 0, 0.5, 1, 3, 6, 9, 12, 15, 18, 21, 24, 30, 36, 40, 48, 72, and 96 h for the NaOAc-treated hairy roots; 0, 1, 2, 3, 6, 12, 18, 24, 48, 72, and 96 h for the H_2O_2-treated hairy roots; 0, 1, 3, 6, 12, 24, 48, 72, and 96 h for the MeJA-treated hairy roots; 0, 3, 9, 15, 24, 48, 72, and 96 h for the CD-treated hairy roots; and 0, 0.5, 1, 3, 6, 9, 12, 15, 18, 21, 24, 30, 36, 48, 60, 72, 84, and 96 h for the MeJA and CD co-treated hairy roots. All treatments were performed at 28 °C under continuous darkness with three biological replicates per time point per treatment.

Extraction and Analyses of Stilbenoids from the Culture Medium. Stilbenoids were extracted from the medium of NaOAc-, H_2O_2-, and MeJA-treated hairy roots as described before.[16] For the CD-treated and MeJA and CD-co-treated hairy roots, each medium sample (40 mL) was extracted twice with 50 mL of EtOAc in a separatory funnel. The organic phases were combined and evaporated to dryness under vacuum at 40 °C in a rotary evaporator (Büchi, rotavapor R-200). The extract was redissolved in 1.5 mL of MeOH and dried to completeness under a nitrogen stream using a Reacti-Vap III apparatus (Pierce). Extracts were redissolved in 1 mL of MeOH, filtered through a 0.2 μm nylon filter, and analyzed by reverse phase HPLC as detailed below.

HPLC analyses were performed in a Dionex Summit system equipped with a photodiode array detector. The separation was performed on a SunFire C_{18}, 5 μm, 4.6 × 250 mm column (Waters) at 40 °C with a flow rate at 1.0 mL/min. The mobile phase consisted of 2% formic acid in water (A) and MeOH (B). The column was initially equilibrated with 100% A for 1 min. Then a linear gradient was performed from 60% A and 40% B to 65% A and 35% B (1− 20 min), followed by a linear gradient from 65% A and 35% B to 100% B isocratic elution in 5 min (20−25 min). Reference compounds included commercially available *trans*-resveratrol (Biophysica) and *trans*-piceatannol (Axxora). *trans*-Arachidin-1 and *trans*-arachidin-3 standards were purified from elicited peanut seeds as described.[16] Dilutions of the standards were made in MeOH to obtain calibration curves for quantitative analysis. Calibration curves were established using absorbance at 320 nm for resveratrol and piceatannol and at 340 nm for arachidin-1 and arachidin-3.

Resveratrol Feeding Experiment. The spent media of 9-day-old hairy root cultures were removed and replaced with 50 mL of fresh MSV medium containing 3% sucrose. Resveratrol was fed into the hairy root culture at a concentration of 60 μM. A second experimental group included 60 μM resveratrol with 9 g/L CD. All cultures were maintained at 28 °C and 90 rpm under continuous darkness. A 1 mL aliquot was collected from each treatment at the following time points: 0, 0.5, 1, 3, 6, 12, and 24 h. Each aliquot was mixed thoroughly with 1 mL of EtOAc, and then the EtOAc phase was collected, dried under a

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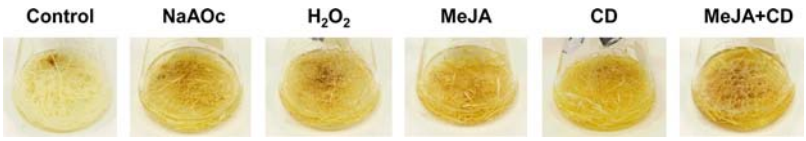

Figure 2. Phenotype of peanut cv. Hull hairy root line 3 after 48 h of treatment with different elicitors: sodium acetate (NaOAc, 10.2 mM); hydrogen peroxide (H_2O_2, 10 mM); methyl jasmonate (MeJA, 100 μM); methyl-β-cyclodextrin (CD, 9 g/L); and 100 μM MeJA with 9 g/L CD (MeJA+CD). Control represents nonelicited culture.

nitrogen stream, and dissolved in 500 μL of MeOH for HPLC analysis as described above. Three biological replicates were included per treatment.

RNA Extraction and Quantitative Real-Time PCR. Nine-day-old hairy root cultures were treated with 100 μM MeJA, 100 μM MeJA with additional 9 g/L CD, and 9 g/L CD alone to study the effect among these treatments on the expression of a previously described peanut resveratrol synthase gene, *JC1* (GenBank accession no. EU418492).[29] A 48 h time course per treatment was conducted including the following time points: 0, 0.5, 3, 9, 15, 24, and 48 h. Three biological replicates were performed at each time point per treatment.

Total RNA was isolated from 15 mg of lyophilized peanut hairy roots using TRIzol reagent (Life Technologies), according to the manufacturer's instructions. RNA concentration was estimated from absorbance readings at 260 nm, and the purity of the total RNA was estimated from the ratio of absorbance readings at 260 and 280 nm using a NanoDrop 800 spectrophotometer (Thermo Scientific). One microgram of total RNA was reversed transcribed in a 20 μL reaction mixture containing oligo (dt) primers using the iScript Select cDNA Synthesis Kit (Bio-Rad) following the manufacturer's instructions. The reaction mixture was incubated at 42 °C for 90 min, followed by an incubation at 85 °C for 5 min, after which the samples were cooled on ice or kept at −20 °C for later use. Quantitative real-time PCR (qPCR) reactions were carried out using iQ SYBR Green Supermix (Bio-Rad). The PCR reaction mixtures comprised 2× qPCR mix (5 μL), forward primer (0.4 μL, final concentration 0.8 μM), reverse primer (0.4 μL, final concentration 0.8 μM), water (0.2 μL), and 18 ng of cDNA template (4 μL) in a final volume of 10 μL. For target gene *JC1*, forward primer 5′-TATGTATTTAACAGAAGAAATAC-3′ and reverse primer 5′-AGTTGCAGCCTCTTTTCCAACT-3′ were used as specific primers as described previously.[29] Three reference genes, *ACT7* (encoding actin 7), *EFα1* (encoding elongation factor α1) and *TBP2* (encoding TATA box binding protein), were selected previously[30] and used to normalize the expression of resveratrol synthase in peanut hairy roots. All of the reactions were prepared using an automated pipetting system (epMotion 5075; Eppendorf) on Hard-Shell Thin-Wall 384-Well Skirted PCR plates (Bio-Rad). Technical replicates were run in triplicate, and the amplification program was followed as described previously.[30]

Statistical Analysis. Each treatment included three biological replicates. Two-way ANOVA with multiple-comparisons tests was conducted for the data in Figures 5 and 6 and analyzed with GraphPad Prism 6, version 6.02. Tukey's Studentized range (HSD) tests were done for the data in Tables S1−S6 of the Supporting Information and analyzed with SAS (Statistical Analysis Systems) statistics program version 9.2.

RESULTS AND DISCUSSION

Response of Peanut Hairy Root Cultures to Different Elicitors. A previously established hairy root culture of peanut cv. Hull line 3 was selected for elicitation studies on the basis of its vigorous growth in liquid MSV medium.[16] Accordingly, the best growth stage for producing the maximum level of arachidin-1 and arachidin-3 in the culture medium after 24 h of treatment with 10.2 mM NaOAc was 9-day-old hairy root cultures.[16] In preliminary studies, cultures at day 9 (mid-exponential growth stage as determined before[16]) showed the most reproducible levels of the different stilbenoids analyzed, independent of the elicitor used, when compared to 6-, 12-, and 15-day-old cultures. Therefore, we used 9-day-old cultures to compare the effects of the five treatments, namely, NaOAc, H_2O_2, MeJA, CD, and MeJA with CD, on production of stilbenoids along a time course from 0 to 96 h. In contrast to the white root tissue observed in nontreated control cultures, the peanut hairy root tissue showed a dark yellow color 48 h after elicitor treatment, the cultures treated with CD or co-treated with MeJA and CD being the ones with the darkest color (Figure 2). Because the hairy roots of peanut secrete most of stilbenoids into culture medium,[16] a slight yellow color that is the characteristic color of stilbenoids found in peanut root mucilage[8] was also observed in the medium of hairy root culture treated with NaOAc, H_2O_2, and MeJA alone. Strikingly, the medium of the cultures treated with CD or MeJA with CD turned a strong and bright yellow color (Figure S1), suggesting higher levels of arachidin-1, arachidin-3, and other stilbenoids in these cultures.

Effect of Different Elicitor Treatments on the Yield of Stilbenoids. The stilbenoids resveratrol, arachidin-1, and arachidin-3 have been described in fungal-challenged seeds[11,31] and elicited hairy roots of peanut.[16,17] Indeed, these stilbenoids accumulated in the culture medium of peanut hairy roots lines from different cultivars (i.e., Andru II and Hull) upon treatment with NaOAc as elicitor.[16,17] Interestingly, the prenylated stilbenoids arachidin-1 and arachidin-3 were not described in elicitor-treated peanut callus. Nonetheless, the nonprenylated analogue of arachidin-1, that is, piceatannol, was reported in these callus cultures upon elicitor treatment.[32−34]

Although resveratrol, arachidin-1, and arachidin-3 were described in peanut hairy root cultures upon 24 h of treatment with NaOAc,[16] detailed studies comparing distinct elicitor treatments over different periods have not been reported. Therefore, in this study a 96 h time course of accumulation of stilbenoids in the hairy root culture medium was conducted for each of the five elicitor treatments (NaOAc, H_2O_2, MeJA, CD, and MeJA with CD). In addition to the three stilbenoids described before (resveratrol, arachidin-1, and arachidin-3), piceatannol was also included in the study because it was previously described in callus cultures of peanut.[32−34] To study the potential molar contribution of the nonprenylated stilbenoids (resveratrol and piceatannol) to the formation of their prenylated analogues (arachidin-3 and arachidin-1), the yield of stilbenoids in culture medium was expressed as nanomoles and normalized per gram dry weight of hairy root tissue (nmol/g DW; Tables S1−S5). The levels of stilbenoids in the MeJA and CD co-treatment group are also reported in milligrams per liter of medium (Table S6). It is worth mentioning that in preliminary studies only trace levels of these stilbenoids were detected in the root tissue upon elicitor treatment; therefore, we report here the levels of stilbenoids in the culture medium.

The accumulation of resveratrol and arachidin-3 in the medium started at 3−6 h post elicitor treatment, whereas

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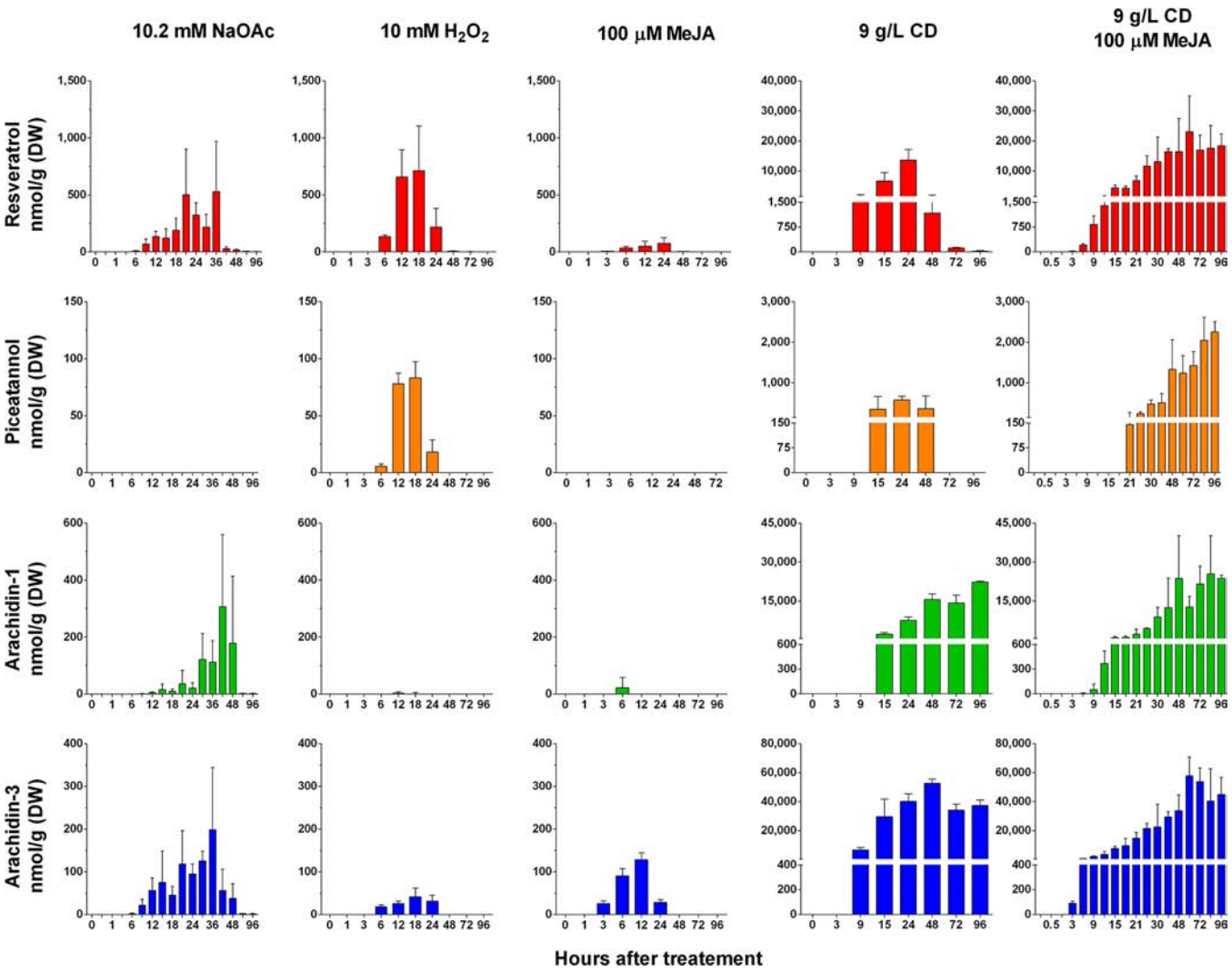

Figure 3. Time course of stilbenoid yield in the medium of peanut hairy roots culture after treatment with different elicitors. Values are the average of three replicates, and error bars represent standard deviation. Statistical analyses are shown in Tables S1−S5. DW, hairy root dry weight.

arachidin-1 was detected 3 h later. Among the five treatments, MeJA combined with CD led to the highest levels of resveratrol in the culture medium (Figure 3). No significant differences were observed in the levels of resveratrol between 24 and 96 h (Table S5), suggesting that resveratrol levels reached a plateau with an average yield of 16716 nmol/g DW. These levels corresponded to an average yield of 40.29 mg/L (24− 96 h time points, Table S6). Most interestingly, the levels of resveratrol remained at these high levels until the end of the time course (96 h). On the other hand, when the cultures were treated with NaOAc, H_2O_2, MeJA, or CD, the levels of resveratrol declined significantly toward the end of the time course (Figure 3 and Tables S1−S4). Remarkably, the yield of resveratrol in the elicited peanut hairy roots was much higher than that obtained from abiotic or biotic stimulated peanut callus[32−34] and sliced and fungi-challenged peanut kernels.[9,14,35] However, the highest yield of resveratrol (~17 μmol/g DW) in peanut hairy root cultures upon co-treatment with 9 g/L (6.87 mM) CD and 100 μM MeJA was still 94 times lower than the 1600 μmol/g DW concentration of resveratrol found in grape cell suspension cultures co-treated with 50 mM dimethyl-β-cyclodextrin (DIMEB) and 100 μM MeJA for >160 h.[25] This increased yield of resveratrol could be due to a higher concentration or different type of methylated cyclodextrin used in the grape study. In preliminary studies with peanut hairy root cultures, 15 mM was the highest concentration of CD used in combination with 100 μM MeJA (Figure S4). Higher concentrations of CD could be tested in peanut hairy root cultures to determine their effect on resveratrol production. Moreover, besides resveratrol, the stilbenoids piceatannol, arachidin-1, and arachidin-3 are also produced by peanut hairy roots, and these compounds may use resveratrol as a biosynthetic precursor. Additionally, the difference in the yield of resveratrol between the grape cell suspension and peanut hairy root cultures could be attributed to the distinct genetic and production capability of each of these systems.

Piceatannol was detected only in the hairy root cultures treated with H_2O_2, CD or MeJA with CD (Figure 3). Similarly to resveratrol, piceatannol levels remained high toward the end of the time course only in the cultures co-treated with MeJA and CD. Statistical analyses showed that the levels of this stilbenoid reached a maximum between 72 and 96 h with an average yield of 1909.92 nmol/g DW (Table S5). In the case of the CD-treated group, the highest yield was 582.13 at 24 h, which was significantly different from the 15 and 48 h elicitor treatments (Table S4), whereas, in the H_2O_2-treated group,

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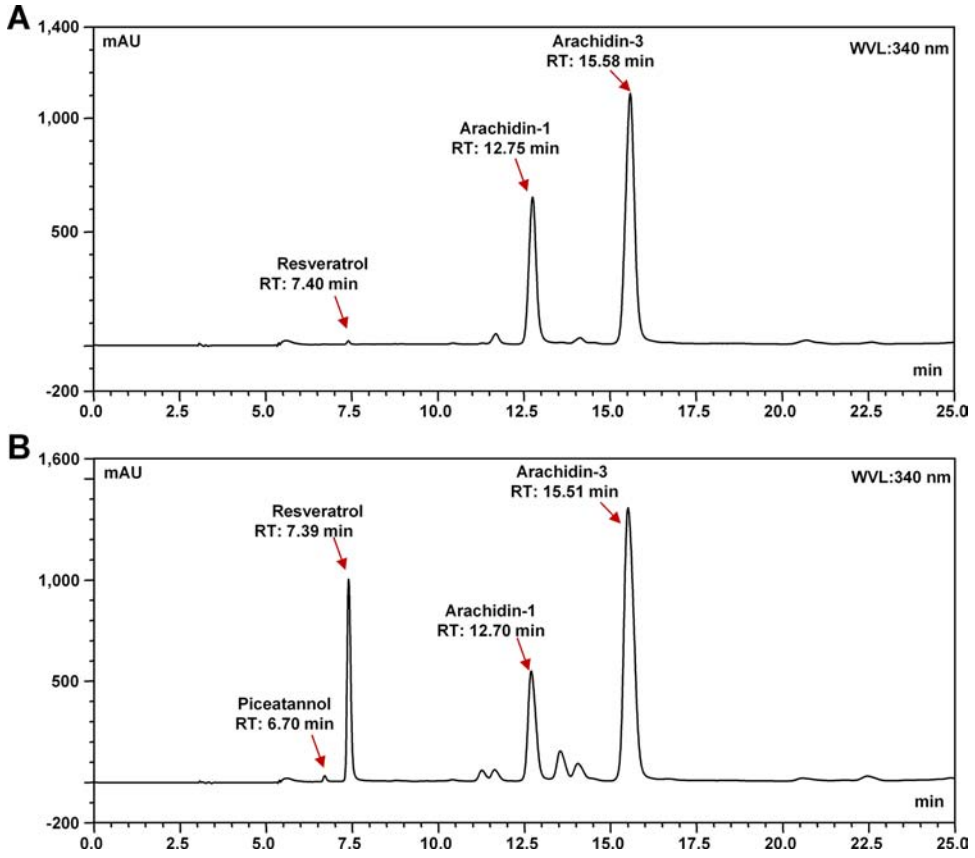

Figure 4. HPLC chromatogram (UV 340 nm) of ethyl acetate extract from the medium of peanut hairy root culture: (A) cultures were treated for 48 h treatment with 9 g/L methyl-β-cyclodextrin (CD); (B) cultures were co-treated for 48 with 100 μM methyl jasmonate and 9 g/L CD.

piceatannol yields reached their maximum at an average of 80.65 nmol/g DW (Table S2). Altogether, yields of piceatannol in the MeJA with CD-co-treated group were ~3-fold higher than in the CD-treated cultures and ~24-fold higher than in the H_2O_2-treated cultures. In the CD-treated and MeJA and CD-co-treated groups, piceatannol started to accumulate later than resveratrol. Similarly, piceatannol was also detected later than resveratrol in peanut callus exposed to fungi and chemicals elicitors,[34] suggesting that piceatannol was synthesized from resveratrol, which contains one fewer hydroxyl group. Meanwhile, in the H_2O_2-treated hairy root cultures, the accumulation of piceatannol was quite similar to the accumulation pattern of resveratrol. To date, the enzyme involved in piceatannol synthesis in peanut has not been described, although the biosynthesis of resveratrol has been elucidated in plants.[3] Interestingly, a cytochrome P450 enzyme capable of converting resveratrol to piceatannol has been identified in a human cancer cell line.[36]

The highest concentrations of arachidin-1 were obtained in the CD-treated and MeJA and CD-co-treated cultures. Under either treatment, the levels of arachidin-1 remained high until the end of the time course (96 h; Figure 3). In the CD-treated cultures, the 96 h treatment showed the highest yield of arachidin-1 (22316.80 nmol/g DW), and this time point was significantly higher than all other time points in the time course (Table S4), whereas in the MeJA and CD-co-treated cultures, the concentration of arachidin-1 reached a plateau at 30 h. The average yield of arachidin-1 from 30 to 96 h was 18396.70 nmol/g DW (Table S4). This yield was equivalent to 56 mg/L (30−96 h time points, Table S6). Arachidin-1 levels declined significantly after 48 h in the NaOAc-treated cultures. In these cultures, arachidin-1 levels were highest between 30 and 48 h with an average yield of 179.73 nmol/g DW. Very low amounts of arachidin-1 were detected at a few time points of the MeJA- or H_2O_2-treated cultures (Tables S2 and S3). Overall, the levels of arachidin-1 in the CD-treated and MeJA with CD-co-treated cultures were ~124- and ~102-fold higher than that in the NaOAc-treated cultures, respectively.

Similarly to arachidin-1, the highest yields of arachidin-3 were obtained in the cultures treated with CD or co-treated with MeJA with CD (Figure 3). In the CD-treated cultures, the highest levels of arachidin-3 were found from 24 to 48 h of treatment with an average yield of 46423.27 nmol/g DW (Table S4), whereas in the MeJA and CD-co-treated cultures, the levels of arachidin-3 reached a plateau after 48 h, because no significant differences in arachidin-3 yields among the different time points between 48 and 96 h were observed (Table S5). During this time period of 48−96 h the average yield of arachidin-3 was 46160.99 nmol/g DW. This yield represented ~148 mg/L of arachidin-3 (48−96 h time points, Table S6). In the NaOAc-treated cultures the levels arachidin-1 declined significantly after 48 h, whereas in the H_2O_2- and MeJA-treated cultures a significant decrease was observed after 24 h. The average maximum yield in the NaOAc-treated cultures was 122.78 nmol/g DW, which represented time points of 15 and 21−36 h. Overall, the yield in either the CD- or MeJA with CD-treated cultures was ~377-fold higher than that in the NaOAc-treated cultures.

As far as we know, arachidin-1 and arachidin-3 have been described only in peanut. Nonetheless, other prenylated

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stilbenoids have been reported in a small number of plant species.[37−39] The maximum yields of these compounds in aerated and sliced peanut seeds have been 1586.53 nmol/g DW (495.7 μg/g DW) and 8158.11 nmol/g DW (2414.8 μg/g DW) peanut kernel for arachidin-1 and arachidin-3, respectively.[9] Herein we show that the hairy root cultures of peanut can be designed to produce much higher levels of arachidin-1 and arachidin-3. The accumulation of arachidin-1 was found 3 h later than that of resveratrol and arachidin-3 in any of the different treatments, much earlier than that of piceatannol, the nonprenylated analogue of arachidin-1. Whereas the highest yields of arachidin-1 and arachidin-3 were similar between the CD and MeJA with CD treatments, the levels of resveratrol and piceatannol were always higher under the MeJA with CD co-treatment. These differences could be appreciated by comparing the HPLC chromatograms of extracts from CD-treated versus MeJA and CD-co-treated groups (Figure 4). Therefore, the latter elicitation co-treatment is a preferred strategy to produce higher levels of diverse types of stilbenoids in peanut hairy root cultures.

The location and formation of phytoalexins induced by infection with soil fungal strains in peanut kernels were studied previously. Resveratrol could be found in deeper layers of the kernel slices, which were close to healthier tissue, whereas arachidin-1, arachidin-3, and other stilbenoids were detected near the infected area, suggesting that resveratrol might be the precursor of other stilbenoids in peanut.[40] In the MeJA and CD-co-treated group, the molar ratio of resveratrol to its prenylated analogue arachidin-3 was between 0.4 and 0.5, whereas that of piceatannol to its prenylated analogue arachidin-1 was only between 0.08 and 0.09. Meanwhile, piceatannol was not detected in the medium of the cultures upon treatment with NaOAc or MeJA alone, whereas arachidin-1 was found in these cultures. These results suggest that the main stilbenoids responding to the elicitor treatments are resveratrol, arachidin-3, and arachidin-1 in peanut hairy root cultures.

Effect of Methyl-β-cyclodextrin on the Levels of Resveratrol in the Medium of Peanut Hairy Root Cultures. The degradation of the stilbene-type phytoalexins resveratrol and pterostilbene have been reported during the interaction of grapevines with *Botrytis cinerea*,[41,42] whereas the catabolism of stilbenoids in non-pathogen-challenged plant tissues has not been studied. After treatment with NaOAc, H_2O_2, or MeJA alone, resveratrol and other stilbenoids accumulated in the medium of hairy root cultures of peanut and reached their maximum levels between 12 and 36 h. Thereafter, their concentrations decreased to barely detectable levels after 72 h of treatment (Figure 3). A similar decrease in resveratrol and piceatannol levels was reported when peanut callus was challenged with a fungal spore suspension as biotic elicitor[33] and UV irradiation as abiotic stress,[35] suggesting that peanut might have the ability to degrade these stilbene-type phytoalexins inside the root tissue or secrete specific enzymes to metabolize them after a temporal exposure to stress. However, the levels of resveratrol, piceatannol, arachidin-1, and arachidin-3 in the hairy root cultures co-treated with MeJA and CD remained high even up to 96 h. Interestingly, the levels of arachidin-1 and arachidin-3 also remained high in the medium over time in CD-treated peanut hairy root cultures, whereas resveratrol and piceatannol levels were not sustained over time. In grape cell suspension cultures co-treated with MeJA and CD, resveratrol levels were maintained at high levels in the medium up to 120 h, and this level was much higher than that of cultures treated with MeJA alone. In the latter cultures, no significant amounts of resveratrol were detected in the spent medium after 24 h.[25] These results could be due to CD's capability of forming inclusion complexes with stilbene compounds such as resveratrol,[23,43] piceatannol,[44] and pinosylvin,[45] which might protect stilbenoids from uptake or degradation by the host as suggested before.[23,24]

To study the effect of CD on the levels of resveratrol in the culture medium of peanut hairy roots, resveratrol alone or together with CD (9 g/L) was added to the peanut hairy root culture, and the concentration of resveratrol in the medium was determined at different time points. The 60 μM concentration of resveratrol used in this experiment represented the average level of this stilbenoid found in the culture medium upon co-treatment with MeJA and CD. In the control group containing resveratrol alone, the concentration of this stilbenoid declined dramatically to 27.04 ± 0.83 μM after 0.5 h and to 1.74 ± 1.29 μM after 1 h. These represented reductions of about 47 and 97% of the initial resveratrol concentration, respectively. Resveratrol was not detected after 3 h of incubation (Figure 5).

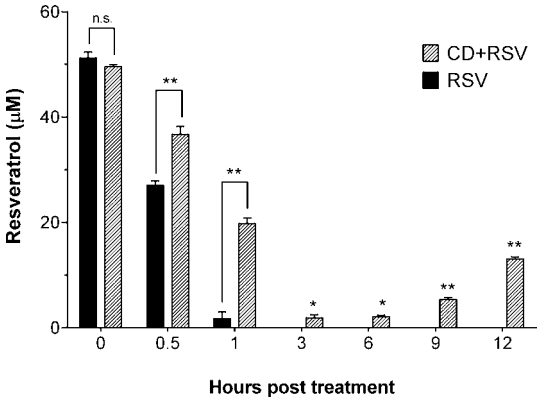

Figure 5. Effect of methyl-β-cyclodextrin (CD) on the levels of resveratrol (RSV) in the medium of hairy root cultures of peanut. Nine-day-old peanut hairy roots were co-inoculated with 60 μM RSV, and the concentration of RSV that remained in the medium was determined by HPLC. RSV, resveratrol alone; CD+RSV, RSV plus 9 g/L CD. Values are the average of three replicates, and error bars represent standard deviation. (*) $p < 0.05$; (**) $p < 0.001$; (n.s.) not significant by two-way ANOVA with Sidak's and Bonferroni's multiple-comparisons test.

Notably, resveratrol was stable in fresh culture medium and in culture medium from elicited cultures after 48 h of treatment with MeJA, in which no stilbenoid was detected (Figure S2). This suggests that peanut hairy roots do not secrete any enzymes that might metabolize resveratrol into the medium. However, resveratrol could have been converted into other metabolites by cell wall bound enzymes. Indeed, a cell wall bound peroxidase has been shown to dimerize resveratrol in grapevine cell suspension cultures.[46]

In the resveratrol and CD coculture group, the concentrations of resveratrol in the medium after 1 and 3 h of incubation were 19.78 ± 1.04 and 1.88 ± 0.61 μM, respectively. The rate at which the levels of resveratrol were reduced in the medium was statistically significantly lower than in control group (Figure 5). CD−stilbenoid inclusion complexes could promote an active biosynthesis and extracellular accumulation of stilbenoids by diminishing the usual feedback inhibition and/

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or toxicity expected when a secondary metabolite is present at high levels in the cytoplasm, such as resveratrol in cell suspensions of *Vitis vinifera*[26] and taxol in cell suspensions of *Taxus baccata*.[47] Moreover, after 9 h of treatment with CD and resveratrol, the concentration of resveratrol rose to 5.35 ± 0.39 μM. Interestingly, arachidin-3 was also detected in medium (Table S4), confirming that CD also induces the biosynthesis of stilbenoids in peanut hairy root cultures as shown in Figure 3. Similarly, resveratrol biosynthesis was induced when grapevine cell suspension cultures were treated with CD alone, indicating that CD acts as elicitor.[23,24] Our results show that CD can stabilize resveratrol levels in the medium of peanut hairy root cultures, leading to several hundred-fold increases in the levels of these compounds in culture medium upon co-treatment with MeJA and CD.

Expression of Resveratrol Synthase Gene (*RS*) in Peanut Hairy Roots after Elicitor Treatment. MeJA and CD were reported to have a synergistic effect on the expression of the stilbene pathway in grape cell suspension cultures.[25] To this end, we studied the effect of CD and MeJA on the expression of resveratrol synthase (*RS*), a key gene involved in stilbenoid biosynthesis. In our previous study, a resveratrol synthase gene from peanut was cloned and functionally characterized in planta via transient expression in *Nicotiana benthamiana*. Therefore, the expression of this gene was studied by quantitative PCR in hairy roots of peanut treated with MeJA, CD, or MeJA combined with CD (Figures 6 and S5).

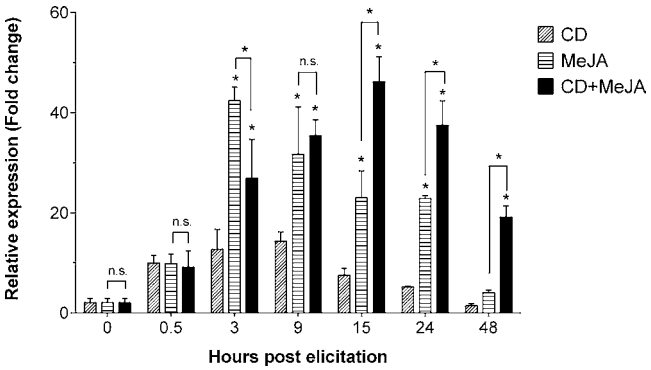

Figure 6. Relative expression of resveratrol synthase gene (*RS*) in peanut hairy roots after treatment with different elicitors. Nine-day-old peanut hairy roots were treated with 100 μM methyl jasmonate (MeJA), 100 μM methyl jasmonate with 9 g/L methyl-β-cyclodextrin (CD+MeJA), and 9 g/L methyl-β-cyclodextrin alone (CD). Relative expression of *RS* was normalized using *ACT7*, *EFα1*, and *TBP2* as reference genes. Values are the average of three replicates, and error bars represent standard deviation. Statistical analysis was performed by two-way ANOVA with Tukey's and Bonferroni's multiple-comparisons test. The asterisks above bars represent significant difference when compared to CD treatment group (*, $p < 0.001$; n.s., not significant), and the asterisks above the connecting line represent significant difference between MeJA and CD+MeJA groups (*, $p < 0.001$; n.s., not significant).

In the CD group, *RS* expression showed a peak at 9 h post treatment followed by a slight decrease during the next 6 h (Figure S5). When compared to the other two treatments, CD alone showed the lowest induction. After 3 h of treatment with MeJA alone, *RS* expression increased rapidly to (42.4 ± 2.7)-fold higher than the minimum *RS* expression value in the time course. This level of expression was significantly higher than the CD or CD with MeJA groups (Figure 6). Then the *RS* expression dropped quickly to (23.0 ± 5.4)-fold change at 15 h post treatment and finally to (4.0 ± 0.5)-fold change at 48 h post treatment, which was close to the *RS* expression at 0 h time point (Figures 6 and S5). This early induction of *RS* expression was also observed in peanut hairy roots treated with the elicitor NaOAc.[48]

In the hairy root cultures co-treated with CD and MeJA, the expression level of the *RS* increased gently to the highest point at 15 h post treatment with (46.2 ± 5.0)-fold change. Then it was followed by a slow decline, after it reached the peak. *RS* expression was maintained at (19.2 ± 1.2)-fold increase at 48 h post treatment, which was significantly higher that that of the CD or MeJA groups. These observations correlated to the highest levels of resveratrol observed in the cultures co-treated with MeJA and CD relative to cultures treated with CD alone (Figure 3). The relative expression level of resveratrol synthase (stilbene synthase) and central phenylpropanoid genes, phenylalanine ammonia-lyase, cinnamate 4-hydroxylase, and 4-coumarate CoA ligase, were also quantified through a time course in grapevine cell suspension culture incubated with MeJA, CD, or the combined treatment with MeJA and CD.[25] Compared to treatments with MeJA or CD alone, a sustainable expression of resveratrol synthase and phenylpropanoid-related genes was also observed in the MeJA and CD co-treated group. These results suggest that in grapevine cell suspension culture and peanut hairy root culture, MeJA and CD have a synergistic effect on stilbene biosynthesis pathway gene expression, which could be correlated with the increased yield of stilbenoids in the medium. Interestingly, a recent study has shown than CD is also capable of encapsulating MeJA to form MeJA−CD complexes with a 1:1 ratio in stoichiometry.[49] Further studies are necessary to evaluate a potential role of CD on the stability of MeJA in peanut hairy root cultures.

In conclusion, although prenylated stilbenoids isolated from treated peanut kernels show a diverse bioactivities in vitro, their production using these biological systems is limited. To address this issue, hairy root cultures of peanut co-treated with MeJA and CD have been developed as a controlled and sustainable system for the high-level production of the prenylated stilbenoids arachidin-1 and arachidin-3. Furthermore, peanut hairy root cultures also provide a viable platform to identify regulatory genes and key enzymatic steps in stilbenoid biosynthesis beyond resveratrol formation. For instance, these genes encode for enzymes involved in prenylation and hydroxylation reactions, which introduce the various functional groups that contribute to the structural and functional diversity of stilbenoids in peanut.

■ ASSOCIATED CONTENT

Supporting Information

Figures S1−S5 and Tables S1−S6. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

CD, methyl-β-cyclodextrin; EtOAc, ethyl acetate; EtOH, ethanol; H_2O_2, hydrogen peroxide; HPLC, high-performance liquid chromatography; MeJA, methyl jasmonate; MeOH, methanol; NaOAc, sodium acetate; *RS*, resveratrol synthase; RT, retention time

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