Resveratrol Biosynthesis in Hairy Root Cultures of Tan and Purple Seed Coat Peanuts

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Abstract: Peanut (Arachis hypogaea) is a crop that can produce resveratrol, a compound with various biological properties, such as those that exert antioxidant, anticancer, and anti-inflammatory effects. In this study, trans-resveratrol was detected in the roots, leaves, and stems of tan and purple seed coat peanuts (Arachis hypogaea) cultivated in a growth chamber. Both cultivars showed higher levels of resveratrol in the roots than the other plant parts. Thus, both cultivars were inoculated with Agrobacterium rhizogenes, in vitro, to promote hairy root development, thereby producing enhanced levels of t-resveratrol. After 1 month of culture, hairy roots from the two cultivars showed higher levels of fresh weight than those of seedling roots. Furthermore, both cultivars contained higher t-resveratrol levels than those of their seedling roots (6.88 ± 0.21 mg/g and 28.07 ± 0.46 mg/g, respectively); however, purple seed coat peanut hairy roots contained higher t-resveratrol levels than those of tan seed coat peanut hairy roots, ranging from 70.16 to 166.76 mg/g and from 46.61 to 54.31 mg/g, respectively. The findings of this study indicate that peanut hairy roots could be a good source for t-resveratrol production due to their rapid growth, high biomass, and substantial amount of resveratrol.

Keywords: peanut; hairy root; trans-resveratrol

1. Introduction

Arachis hypogaea L., better known as peanut, is a member of the legume family and is widely disseminated across diverse regions with tropical and moderate climates from South America [1]. According to the world peanut production in 2021 reported by United States Department of Agriculture (USDA) [2], China and India account for 37% and 14% of the world production, respectively, followed by Nigeria (8%), United States (6%), Sudan (4%), Senegal (3%), Burma (3%), Argentina (3%), Tanzania (2%), and Indonesia (2%). Arachis hypogaea L. contains abundant dietary fiber, starch, albumin, and ash [3]. The roots of the plant were at one point used in folk medicine to treat diseases, such as insomnia, inflammation, and prostate enlargement, in China [4].

Resveratrol is a natural phytochemical belonging to the stilbenoid class and is mainly found in nuts and fruits, such as peanut, cranberry, pistachio, blueberry, and bilberry [5]. Its biosynthesis starts with the synthesis of p-coumaroyl-CoA from initial precursors, such as phenylalanine and tyrosine. p-Coumaroyl-CoA is then converted to resveratrol by condensing three molecules of malonyl-CoA using resveratrol synthase (RS), also called stilbene synthase [6]. The production of resveratrol is important because it possesses a variety of biological properties, such as antioxidant [7], anticancer [8], and anti-inflammatory...
It has also been reported that resveratrol can decrease the incidence of cardiovascular, Alzheimer’s disease, cancer, and show antiaging properties, because of the powerful antioxidant effects of resveratrol in peanuts [10].

Hairy root is a disease symptom that manifests as adventitious roots at the infection site caused by Agrobacterium rhizogenes. A. rhizogenes is a well-known natural genetic engineer, as it is able to transfer the T-DNA located between the TR and TL regions on the root-inducing plasmid (Ri-plasmid) to the nuclear genome of the host plant while the plant is infected [11]. A. rhizogenes-derived hairy roots maintain inexhaustible growth in comparison with the uninfected seedling roots of parent plants, without exogenous plant growth hormones [12]. A hairy root culture system processed by genetically modified A. rhizogenes has been utilized to produce transgenic plants, to investigate plant metabolic processes, and to increase production of secondary metabolite biosynthesis from plants [13].

In previous research, transformed roots with the highest contents of resveratrol (1.5 mg/g) and largest biomass (7.6 g/L) were induced from the hairy root culture of peanuts infected with Agrobacterium rhizogenes R1601 [14]. However, no research has been performed to evaluate resveratrol production in hairy roots induced from peanuts with purple seed coats. In this study, we induced hairy roots from Arachis hypogaea L. with two different seed coats, tan and purple (Figure 1), and investigated the differences in resveratrol biosynthesis and accumulation.

![Figure 1. Seed of purple (a) and tan (b) seed coat peanuts.](image-url)

**2. Results**

2.1. trans-Resveratrol HPLC Analysis in the Different Plant Parts of Tan and Purple Seed Coat Peanuts

trans-Resveratrol was detected in the roots, stems, and leaves of both cultivars, and the root and stem of the purple seed coat peanut contained higher levels of trans-resveratrol than those of the other cultivars (Table 1). In particular, roots and stems contained 7.40 and 4.07 times more trans-resveratrol than those of the tan seed coat peanut. Peanut roots may be suitable for trans-resveratrol production since the roots contained the highest level of trans-resveratrol among the plant parts. For further study, hairy roots were induced to produce excess trans-resveratrol.

| Cultivars                  | Organ | Resveratrol Content (µg/g) |
|---------------------------|-------|---------------------------|
| Tan seed coat peanut      | Root  | 7.23 ± 1.18 c           |
|                           | Stem  | 6.31 ± 1.22 c           |
|                           | Leaf  | 5.17 ± 0.03 c           |
| Purple seed coat peanut   | Root  | 53.55 ± 1.07 a          |
|                           | Stem  | 25.72 ± 4.58 b          |
|                           | Leaf  | 5.39 ± 0.07 c           |

1 A different letter indicates that mean values were significantly different at $p < 0.05$ by Duncan Multiple Range Test.
2.2. Hairy Root Induction from Tan and Purple Seed Coat Peanuts

Three hairy root lines were induced from the leaves of tan and purple seed coat peanut leaves using *A. rhizogenes* strain R1000. Detection of rol genes (A, B, C, and D) was performed to verify the transformation mediated by *A. rhizogenes* using PCR and gel electrophoresis, and all hairy root lines exhibited visible bands, while the material extracted from the uninfected seedling roots did not show bands (Figure 2). Hairy roots (2 g) and seedling roots (2 g) were cultured in SH liquid medium for 6 weeks. The fresh weight of hairy root lines of tan seed coat peanut ranged from 4.77 to 5.20 g, and the fresh weight of hairy root lines of purple seed coat peanut ranged from 5.06 to 5.41 g. The values of fresh weight in hairy root lines were significantly higher than those of the seedling roots, while the values in hairy root lines induced from tan and purple seed coat peanuts were not significantly different (Table 2).

![PCR analysis](image)

**Figure 2.** PCR analysis of *rolA* (304 bp), *rolB* (797 bp), *rolC* (550 bp), and *rolD* (1035 bp) in seedling root and hairy root of peanuts with tan seed coat and peanuts with purple seed coat. tanSR, seedling root of peanuts with tan seed; tanHR, hairy root of peanuts with tan seed coat; ppSR, seedling root of peanuts with purple seed coat; ppHR, hairy root of peanuts with purple seed coat. 1kb ladders were used in this study.

| Table 2. Fresh weight of seedling root and hairy root of tan and purple seed coat peanuts. |
|----------------------------------|---------------------------------|------------------|
| **Cultivars**                     | **Root**                        | **Fresh Weight (g)*** |
| **Tan seed coat peanut**          | Seedling root                   | 3.10 ± 0.11 c     |
|                                  | Hairy root line 1               | 5.02 ± 0.37 ab    |
|                                  | Hairy root line 2               | 4.77 ± 0.39 b     |
|                                  | Hairy root line 3               | 5.20 ± 0.14 ab    |
| **Purple seed coat peanut**       | Seedling root                   | 2.88 ± 0.41 c     |
|                                  | Hairy root line 1               | 5.22 ± 0.36 ab    |
|                                  | Hairy root line 2               | 5.06 ± 0.20 ab    |
|                                  | Hairy root line 3               | 5.41 ± 0.37 a     |

* c A different letter indicates that mean values were significantly different at p < 0.05 by Duncan Multiple Range Test.

2.3. *trans*-Resveratrol HPLC Analysis of Seedling Roots and Hairy Roots of Tan and Purple Seed Coat Peanuts

The identification and quantification of *t*-resveratrol were carried out in seedling roots and hairy roots of tan and purple seed coat peanuts (Table 3). *t*-Resveratrol levels of hairy root lines of tan seed coat peanut ranged from 46.61 to 54.31 µg/g, and the levels of hairy root lines of purple seed coat peanut ranged from 70.16 to 166.76 µg/g. The average values of *t*-resveratrol levels in hairy root lines of tan and purple seed coat peanuts were 7.19 times and 4.67 times higher than those of their seedling roots, respectively. Peanut hairy roots showed higher levels of *t*-resveratrol than seedling roots, regardless of cultivar type; however, the *t*-resveratrol levels of purple seed coat peanut hairy roots were significantly higher than those of the tan seed coat peanut hairy roots. Tan seed coat peanut hairy root line 3 and purple seed coat peanut hairy root line 1, which exhibited the highest levels of
t-resveratrol among the other hairy root lines, were selected for the expression analysis of resveratrol synthase genes.

Table 3. trans-Resveratrol analysis of seedling root and hairy root of peanuts with tan seed coat and peanuts with purple seed coat.

| Cultivars             | Root                | Resveratrol Content (µg/g) |
|-----------------------|---------------------|----------------------------|
| Tan seed coat peanut  | Seedling root       | 6.88 ± 0.21 d             |
|                       | Hairy root line 1   | 47.54 ± 4.64 de           |
|                       | Hairy root line 2   | 46.61 ± 0.64 c            |
|                       | Hairy root line 3   | 54.31 ± 6.43 d            |
| Purple seed coat peanut| Seedling root      | 28.07 ± 0.46 f           |
|                       | Hairy root line 1   | 166.76 ± 3.66 a           |
|                       | Hairy root line 2   | 70.16 ± 3.27 c            |
|                       | Hairy root line 3   | 155.94 ± 7.37 b           |

1 A different letter indicates that mean values were significantly different at \( p < 0.05 \) by Duncan Multiple Range Test.

2.4. Expression Analysis of Resveratrol Synthase Genes in Seedling Roots and Hairy Roots of Tan and Purple Seed Coat Peanuts

Among the four genes of interest (\( AhRS1 \), \( AhRS2 \), \( AhRS3 \), and \( AhRS4 \)), the expression levels of all the genes were higher in tan seed coat peanut hairy root than in its seedling root (Figure 3). Similarly, the expression levels of \( AhRS1 \), \( AhRS2 \), \( AhRS3 \), and \( AhRS4 \) were higher in purple seed coat peanut hairy root line 1 than in its seedling root. These results were consistent with those of the t-resveratrol HPLC analysis.

![Figure 3. qRT-PCR analysis of \( AhRS1 \), \( AhRS2 \), \( AhRS4 \), and \( AhRS5 \) in seedling root and hairy root of peanuts with tan seed coat and peanuts with purple seed coat. Data are represented as a normalized relative fold change to control.](image)

3. Discussion

The results obtained in this study indicate that purple seed coat peanut hairy root lines exhibited higher t-resveratrol levels than those of its seedling root, and the higher t-resveratrol production coincided with increases in the expression of \( AhRS1 \), \( AhRS2 \), \( AhRS3 \), and \( AhRS4 \). Furthermore, tan seed coat peanut hairy root lines contained higher levels of t-resveratrol than its seedling root, consistent with their expression levels of \( AhRS1 \), \( AhRS2 \), \( AhRS3 \), and \( AhRS4 \). Consistent with our results, previous studies have reported that the expression of resveratrol synthase genes in various plant species allowed its transgenic plants to biosynthesize resveratrol or to enhance resveratrol accumulation.
For example, Medicago sativa (alfalfa) transformed with AhRS produced 15 µg/g fresh weight (FW) of resveratrol [15], Oryza sativa L. (rice) transformed with AhRS1 produced 0.697 µg/g FW [16], and Rehmannia glutinosa transformed with AhRS3 produced 2.0 µg/g FW [17]. Furthermore, Lactuca sativa L. (lettuce) transformed with a stilbene synthase gene of Parthenocissus henryana accumulated 56.40 µg/g FW [18], Solanum lycopersicum (tomato) transformed with a grapevine stilbene synthase gene (vst1) accumulated 8.7 µg/g FW [19], Vitis vinifera L. (grapevine) transformed with a novel stilbene synthase gene from Chinese wild Vitis pseudoreticulata produced 2.586 µg/g FW [20], and Ziziphus jujuba Mill. (Huping jujube) transformed with a resveratrol synthase gene from Polygonum cuspidatum produced 0.45 µg/g FW [21]. While the transgenic plants mentioned in these reports contained resveratrol contents lower than 15 µg/g FW, this study showed that peanut hairy roots may be a good source for resveratrol production because of enhanced resveratrol production of up to 166 µg/g FW.

A. rhizogenes can cause transgenic hairy roots by transferring the T-DNA region to the host plant genome [22] and the rolA, rolB, and rolC genes in the T-DNA area are often involved in the induction of secondary metabolism in many plant species [23]. In particular, the previous studies reported that the expression of rolB and rolC is important for the activation of phosphorylation—the dephosphorylation process, which is a part of the signal transduction pathway that plays a main role in the activation of plant defense responses and elicitor recognition [24,25]. Thus, this study suggests that the expression of rol genes in peanut hairy roots may increase t-resveratrol contents compared with wild-type roots.

Much effort has been made to increase the resveratrol yield in bacteria [26] that in the discussion ed resveratrol contents compared with wild-type roots since rol gene co, yeast [27], algae [28], and plants [29]. Among the potential plant materials, hairy root cultures could be a very good source for the production of secondary metabolites, as these transgenic roots can produce the same specific metabolites as their mother plants, but in excessive amounts. With respect to resveratrol production in hairy roots, Halder et al., 2016 reported that peanut hairy roots produced higher levels of resveratrol compared with non-transgenic peanut roots [30], and Hoseinpanahi et al., 2020 described that hairy roots of Wild Vitis vinifera contained a higher amount of resveratrol than their natural roots [31]. Furthermore, hairy root cultures of the peanut have been reported as suitable for resveratrol production in peanut [14] and Chinese Skullcap (Scutellaria baicalensis) [32]. Additionally, hairy roots of gherkin (Cucumis anguria) [33], bitter melon (Momordica charantia) [34], and spine gourd (Momordica dioica) [35] possess higher concentrations of phenolic compounds than non-transgenic roots. Therefore, this study suggests that hairy root cultures of peanut can be a suitable method to produce t-resveratrol, since its capacity to produce t-resveratrol is much higher than that of roots, leaves, and stems of non-transgenic peanuts.

4. Materials and Methods
4.1. Plant Materials

The tan and purple seed coat peanuts were purchased from ASIA SEED Co., LTD (Seoul, Korea) and DONG WON NONG SAN SEED Co., LTD (Seoul, Korea), respectively (Figure 1). Seeds were placed on vermiculite and then incubated in a growth chamber equipped with a flux rate of 92.5 µmol s⁻¹ m⁻² at 25 °C for 4 weeks. Afterwards, leaves, stems, and roots from both cultivars were harvested using liquid nitrogen and then freeze-dried for t-resveratrol analysis. For hairy root induction, the seeds were sterilized with 70% (v/v) ethanol for 30 s and subjected to 4% (v/v) sodium hypochlorite solution (NaClO) with two drops of Tween-20 for 10 min. The seeds were then rinsed with sterile distilled water 10 times, and excess moisture on the seed surface was removed with sterilized tissue paper. The seeds were placed on petri dishes containing 25 mL of half-strength solid SH medium. The plates were incubated at 25 °C under light with an intensity of 92.5 µmol s⁻¹ m⁻² for 4 weeks.
4.2. Hairy Root Induction

Experiments for hairy root induction were conducted according to the method reported by Park et al., 2021 [36]. Wild-type Agrobacterium rhizogenes R1000 strains were incubated in 100 mL flasks containing 30 mL of LB broth at 180 × g and 28 °C for 24 h. The cultivated A. rhizogenes R1000 at the mid-log phase (A600 = 0.6) was centrifuged at 3000 × g for 15 min. The supernatant was drained and the sunken cell pellets were re-suspended in half-strength SH liquid medium. While peanut seedlings were soaked in a suspension of A. rhizogenes R1000, they were cut to a proper size. After incubation for 20 min, the explants were removed from the suspension and patted dry with sterile tissue paper. Next, the explants were transferred to a half-strength solid SH medium and incubated in the dark at 25 °C for 2 d. Thereafter, the explants were gently rinsed 10 times with sterile distilled water, dried with sterile tissue paper, and transferred onto half-strength solid SH medium with 500 mg/L of cefotaxime. After one month, hairy roots emerged from the explants and were then isolated and transferred to SH medium with 250 mg/L of cefotaxime. After confirming that the hairy roots were aseptic, 2 g of each hairy root line and seedling root were cultured in half-strength liquid SH medium in the dark at 25 °C for one month. Our previous study demonstrated a protocol for the development of hairy root of A. hypogaea [29]. The hairy and seedling roots were harvested, and their fresh weight was measured. Lastly, the hairy root samples were soaked in liquid nitrogen and then stored at −80 °C for further analyses.

4.3. Extraction of Genomic DNA and Polymerase Chain Reaction (PCR) Analysis

Genomic DNA of seedling roots and hairy roots of tan and purple seed coat peanuts was extracted using the Plant Genomic DNA Mini Kit (Geneaid Biotech Ltd., Taipei, Taiwan). The primers for identifying fragments of rolA (360 bp), rolB (900 bp), rolC (514 bp), and rolD (1035 bp) were designed in reference to a previous study [37]. The settings for thermal cycling conditions were as follows: initial denaturation at 95 °C for 10 min, 30 cycles of amplification at 95 °C for 10 s, followed by primer annealing at 55 °C for 30 s, primer extension at 72 °C for 1 min, and final extension at 72 °C for 10 min and cooling at 4 °C. Gel electrophoresis was used to verify the expected lengths (360, 900, 514, and 1035 bp) of the targeted gene sequences (rol A, B, C, and D, respectively).

4.4. Extraction of Total RNA and cDNA Synthesis

Total RNA from tan seed coat peanut seedling root and hairy root line 3 as well as purple seed coat peanut seedling root and hairy root line 1 were isolated using the RNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA). A NanoVue Plus spectrophotometer (GE Healthcare, Buckinghamshire, UK) was used to check the quality and quantity of DNA and RNA, and the quality of RNA was assessed by agarose gel electrophoresis. Subsequently, cDNA was synthesized using a first strand synthesis kit (Toyobo, Osaka, Japan) according to the manufacturer’s instructions. The cDNA was diluted twenty-fold for quantitative real time PCR (qRT-PCR).

4.5. trans-Resveratrol HPLC Analysis

The extraction and HPLC analysis of trans-resveratrol from seedling roots and hairy roots of tan and purple seed coat peanuts was performed in accordance with the method reported by Ji et al., 2019 [38]. Briefly, 100 mg of seedling and hairy roots was extracted with 2 mL of 80% aqueous methanol (v/v) and then vortexed for 1 min, followed by sonication for 1 h at 25 °C and centrifugation at 12,000 × g and 4 °C for 15 min. The supernatant was then filtered through a vial. The analysis was carried out using an NS-4000 (Futecs, Daejeon, Korea) coupled with a UV-Vis detector and a Pronto SIL® RP-C18 column (150 × 4.6 mm, 5 μm, Bischoff Chromatography, Leonberg, Germany) under controlled conditions (UV detector wavelength, 306 nm; flow rate, 1.0 mL/min; injection volume, 50 μL; and column temperature, 30 °C). The mobile phase consisted of acetonitrile and 0.2% formic acid water (25:75, v/v). t-Resveratrol concentrations were identified based on retention times and
spiking tests, followed by quantification with reference to the corresponding calibration curves.

4.6. Gene Expression Analysis

Expression analysis of Arachis hypogaea resveratrol synthase 1 (AhRS1), AhRS2, AhRS3, and AhRS4 was performed with gene-specific primers as previously reported by Zhu et al., 2014 [39], using a CFX96 Real-Time System combined with a C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA) and the 2X Real-Time PCR Master Mix kit with SFCgreen® I (BioFACT, Daejeon, Korea). The reaction was carried out using the following protocol: pre-denaturation at 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 20 s, followed by 72 °C for 15 min. The three technical and biological replicates of tan seed coat peanut seedling root, hairy root line 3, purple seed coat peanut seedling root, and hairy root line 1 were analyzed using Bio-Rad CFX Manager 2.0 (Bio-Rad).

4.7. Statistical Analysis

The significance of differences between group means was measured using Duncan’s multiple range test in SAS 9.4 (SAS Institute, Inc., Cary, NC, USA).

5. Conclusions

This is the first study to provide information on hairy root cultures potential to produce t-resveratrol using two different peanut cultivars (purple and tan seed coat). Although the roots, leaves, and stems of these peanut cultivars contained a small amount of t-resveratrol, this study suggests that the hairy roots of purple and tan seed coat peanuts can be suitable for t-resveratrol production, since these hairy roots accumulate high levels of the compound. In particular, hairy roots of purple seed coat peanuts may be the best choice for t-resveratrol production because they produce the most.

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