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Production and secretion of resveratrol in hairy root cultures of peanut

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Abstract

Resveratrol and its derivatives are natural stilbenes associated with many health benefits that include those conferred by their antioxidant and anticancer properties. While stilbenes can be recovered as an extract from a selected number of plants, these products are not suitable for many applications in the food/pharmaceutical sectors due to high levels of impurities as well as the overall low concentration of resveratrol and its derivatives in the extract. To deliver a highly defined and enriched resveratrol product, hairy root cultures of peanut (*Arachis hypogaea*) were established and tested as a bioproduction system for resveratrol and associated derivatives. Analyses by HPTLC and GC–MS of ethyl acetate extracts showed that a single 24 h sodium acetate elicitation resulted in a 60-fold induction and secretion of *trans*-resveratrol into the medium of peanut hairy root cultures. *trans*-Resveratrol accumulated to levels of 98 μg/mg of the dried extract from the medium representing 99% of the total resveratrol produced. Other stilbenes, including *trans*-pterostilbene, were also detected in the medium. Our results demonstrate the capacity of hairy root cultures as an effective bioprocessing system for valued nutraceuticals like resveratrol and resveratrol derivatives. In being able to effectively induce and recover high levels of resveratrol and associated derivatives from the media fraction, hairy roots may offer a scalable and continuous product recovery platform for naturally-derived, high quality, enriched nutraceuticals.

Keywords: Peanut; *Arachis hypogaea*; Leguminosae; Resveratrol; Pterostilbene; Stilbenes; Hairy roots; Elicitation

1. Introduction

trans-Resveratrol **1** (*trans*-3,5,4′-trihydroxystilbene, Fig. 1), and its many derivatives (Larronde et al., 2005; Rimando and Barney, 2005), are naturally occurring phytoalexins produced in a select number of plant species. These plant polyphenols have received considerable interest based upon a number of associated health benefits (Baur and Sinclair, 2006; Delmas et al., 2006). Most notably, the significant levels of resveratrol **1** in red wine have been credited to the phenomenon known as "the French Paradox", wherein low incidence of heart disease is observed among a population with a relatively high saturated fat diet and moderate wine consumption (Frankel et al., 1993; Siemann and Creasy, 1992). Over the past two decades, numerous health benefits impacting cardiovascular disease, various cancers, atherosclerosis and aging have been linked with resveratrol **1** (reviewed; Baur and Sinclair, 2006; Roupe et al., 2006). While resveratrol **1** is

Abbreviations: B5, Gamborg's B5 medium with 2% sucrose (Gamborg et al., 1968); bp, base pairs; cv., cultivar; GC–MS, gas chromatography–mass spectrometry; HPTLC, high performance thin layer chromatography; PCR, polymerase chain reaction; *Rf*, retardation factor; TL-DNA, left T-DNA; TR-DNA, right T-DNA.

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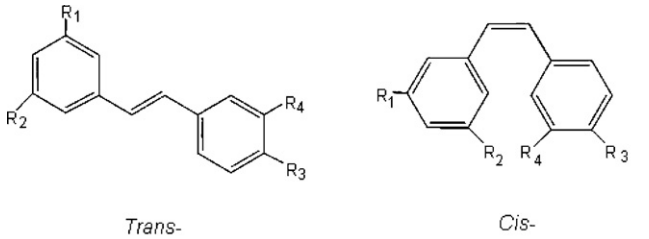

	R_1	R_2	R_3	R_4
Resveratrol 1	OH	OH	OH	H
Pterostilbene 2	OCH_3	OCH_3	OH	H
Resveratrol trimethyl ether 3	OCH_3	OCH_3	OCH_3	H
Piceatannol 4	OH	OH	OH	OH
Piceid (polydatin) 5	O-Glucose	OH	OH	H

Fig. 1. Chemical structure of *trans*- and *cis*-isomers of resveratrol and related stilbenes.

one of the better known and well studied phytochemicals, several other resveratrol derivatives have also been shown to have similar and/or additional health benefits. Included among these derivatives is the methylated resveratrol compound, pterostilbene **2** (Fig. 1), that has demonstrated *in vivo* effects for reducing cholesterol levels (Rimando et al., 2005).

A number of taxonomically unrelated plant families have been reported to produce marked levels of resveratrol **1** including: grapes, peanuts, several types of berries, some pine trees and most recently tomato fruit skin (Ragab et al., 2006). Resveratrol **1** is a phytoalexin, a class of antibiotic compounds produced as part of the plant's defense system and believed to be the major active stilbene that confers pathogen resistance in these plants (Dixon, 2001). The terminal enzyme in the production of resveratrol **1** is resveratrol synthase, which condenses *p*-coumaroyl-coenzyme A and three malonyl-coenzyme A molecules to form resveratrol **1** (Schöppner and Kindl, 1984). This enzyme is highly regulated by elicitors and general plant defense compounds in an effort to protect the plant. Resveratrol **1** exists as both the *trans*- and *cis*-isomers with numerous reports suggesting *trans*-resveratrol to be the most bioactive form of this molecule (Roupe et al., 2006). *trans*-Resveratrol **1** can readily be converted to *cis*-resveratrol when exposed to UV light and is unstable when exposed to high pH conditions.

The use of complementary or alternative beneficial products for human health is increasing worldwide with their continued popularity in Europe and Asia and a dramatic upward trend of their use in the United States (Frost and Sullivan, 2005). Currently resveratrol **1** is primarily marketed as an herbal or dietary supplement in the form of pills, capsules, powders, and extracts from raw botanical sources (i.e. grape seeds/skins; Japanese knotweed *Polygonum cuspidatum*), with more recent applications beginning to incorporate this popular phytochemical into fortified food/beverage products (i.e. Old Orchard Beverage Company, Sparta, MI). While stilbenes are cost-effectively recovered from these raw materials and will continue to serve this market, these relatively crude sources of resveratrol **1** often lack the consistency and purity required for many applications in the food/pharmaceutical sectors. Furthermore, more natural product consumers and nutrition practitioners are demanding higher quality supplements that are scientifically tested and better defined in their product content (validated by third party quality assurance testing) in a desire to mitigate ineffective and/or erratic responses with these supplements.

The majority of resveratrol-containing dietary supplements are composed of unknown/unidentified botanical components wherein resveratrol **1** and resveratrol derivatives only make up a small fraction of the product. While chemically-synthesized resveratrol **1** may address this issue, natural sources often contain derivatives, co-factors and other phytonutrients that provide added or synergistic benefits to the nutraceutical product and are often preferred by the consumer (Wallace, 1998). Recent studies showing anti-aging benefits of resveratrol **1** (Baur et al., 2006) further accelerate interest in a natural, food-grade, source of enriched resveratrol/resveratrol derivatives that delivers a more defined and consistent product composition and ensures a stable supply chain, several biotic production strategies targeting recombinant plants, yeast and bacteria have been advanced (Becker et al., 2003; Paiva and Hipskind, 2005; Watts et al., 2006). While these approaches potentially offer a more consistent, concentrated resveratrol **1** source, widespread use of these strategies have not been adopted due in part to natural product consumers' negative perception of genetically modified organisms and issues with associated production efficiency/costs. Grape cell suspension cultures for resveratrol **1** production avoid some of these recombinant issues and provide potential production of a suite of resveratrol compounds (Bru et al., 2006; Liu et al., 2003), however, cell suspension production systems have reported issues of genetic instability and losses of secondary metabolite production following elicitation (Gossens et al., 2003) or repetitive subculturing (Chattopadhyay et al., 2002).

To this end, plant hairy roots offer a novel and sustainable tissue-based system that preserves the multiple specialized cell types believed important in maintaining better consistency in the synthesis of bioactive secondary molecules. Tissue-based systems more accurately reflect the metabolic phenotype and performance of the host plant in comparison to plant cell cultures and further the potential of producing various combinations of valued products from a single production line (Guillon et al., 2006a,b Sevon and Oksman-Caldentey, 2002). Recent advances with large scale production have successfully produced ginseng roots in a 10,000 l bioreactor establishing the feasibility of the root system to accommodate industrial processes (Sivakumar et al., 2006).

Here we report the development of peanut hairy root lines for sustained and reproducible production of a naturally-derived source of resveratrol and resveratrol derivatives. These phytochemicals, can be readily recovered from the culture medium in relatively enriched form, detected and quantitated. The use of plant elicitors to enhance secretion of resveratrol **1** and other resveratrol derivatives support our efforts in exploring the commercial potential of this scalable bioprocessing system for valued botanical compounds with nutraceutical properties such as resveratrol and its associated derivatives.

2. Results and discussion

2.1. Establishment of peanut hairy root lines

With numerous studies demonstrating that peanut is among a divergent group of plants with endogenously high levels of resveratrol **1** (Chen et al., 2002; Liu et al., 2003; Sanders et al., 2000; Sobolev and Cole, 1999), we targeted a runner peanut cultivar for establishing hairy root lines as a sustainable bioproduction platform in the delivery of a well-defined and enriched resveratrol **1** product. In initial experiments with 21-day old seedlings of cv. Andru II, a minimum of one explant of each type (stem, leaf, petiole, hypocotyl and cotyledon) successfully initiated hairy roots. The root initials were observed at the infection sites as early as 14 days after inoculation (Fig. 2a). The peanut hairy roots had an average root thickness of 1 mm and were highly branched with no apparent root hairs, and showed plagiotropic growth when cultured on solid B5 medium (Fig. 2b).

Previously, Akasaka et al. (1998) reported the formation of hairy roots on peanut epicotyls cultured *in planta* and thus isolated root cultures were not established. Our peanut hairy roots (Fig. 2b) showed a similar phenotype that includes no root hairs along the main root and higher lateral branching than wild-type roots (Fig. 2c). The absence of root hairs has been observed in other *Agrobacterium rhizogenes*-derived roots from legumes such as alfalfa (Medina-Bolivar, unpublished) as well as species from other families including *Trichosanthes kirilowii* (Savary and Flores, 1994). Whole intact seedlings inoculated on tissues held above the medium surface were less successful and slower to respond. Likewise, host explants of smaller size or mass (i.e. petioles and young expanding leaf blades) yielded fewer root initials and roots from these explants generally exhibited an overall slower growth rate.

Due to the fact that various plants can exhibit differential susceptibility to a given *Agrobacterium* strain, several different strains for generating peanut hairy root lines were tested. Of the two *A. rhizogenes* strains tested (R1000 and ATCC 15834), the agropine type strain, ATCC 15834, was successful in providing the most consistent hairy root responses. *A. tumefaciens* EHA105 containing the pRYG plasmid harboring the *rol* genes (Komarnytsky et al., 2004) was successful in the initiation of hairy roots; however, these roots did not provide sustained proliferation in liquid cultures. Based on these observations, the ATCC 15834 strain was used in all successive experiments. Improvement in overall infection events and recovery of

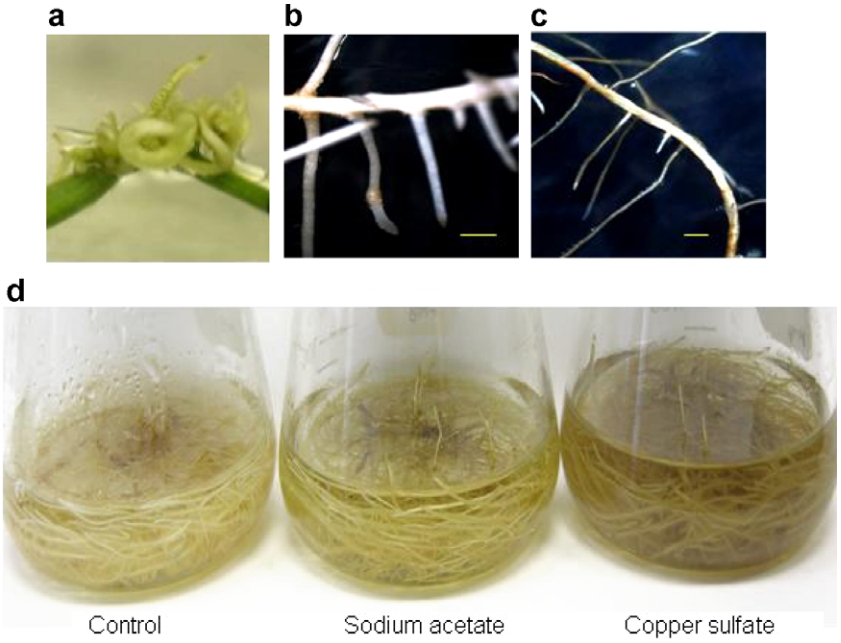

Fig. 2. (a) Hairy roots of peanut cv. Andru II initiated from stem explants. (b) Hairy roots of peanut cv. Andru II line 2 cultured in B5 medium. Scale bar, 5 mm. (c) Roots (wild-type) from peanut seedlings cultured in B5 medium. Scale bar, 5 mm. (d) Elicitation of hairy root cultures of peanut cv. Andru II. Twelve-day cultures were either non-elicited (control) or elicited for 24 h with 10.2 mM NaOAc or 600 μM $CuSO_4$.

sustainable hairy root tissues were obtained with the use of younger explants (15-day old seedlings). Isolated cotyledonary node tissue was more reliable for hairy root production than stem, petiole or hypocotyl explants and consistently responded with larger more prolific roots at the inoculation site (Table 1). Isolation of independent hairy root lines on B5 medium containing cefotaxime was performed to eliminate excess Agrobacteria associated with the tissues. Hairy root lines were tested for vigor and those lines failing to sustain vigorous growth were discarded.

2.2. PCR analysis of hairy roots

In order to assess the genetic status of the hairy roots, we used a PCR-based analysis that targeted the *A. rhizogenes rolC*, *aux1* and *virD2* genes. The *rolC* and *aux1* genes, located on independent T-DNAs (TL-DNA and TR-DNA, respectively) of the Ri plasmid of *A. rhizogenes* strain 15834, are diagnostic for T-DNA integration into the host genome. The *virD2* gene, located outside the T-DNA, is diagnostic for the presence of any remaining Agrobacteria in the root tissue. Three peanut hairy roots lines (2, 3 and 5) showing the highest growth rates in liquid cultures as well as line J-pRYG exhibiting substandard growth were analyzed. While both *rolC* and *aux1* genes were detected in hairy root lines 2, 3 and 5, only the *rolC* gene was identified in line J-pRYG (Fig. 3). The coexistence of the *rolC* and *aux1* genes indicated that hairy root lines 2, 3 and 5 successfully integrated both the TL-DNA (Schmlling et al., 1988; Slightom et al., 1986) and the TR-DNA (Camilleri and Jouanin, 1991) of the pRi15834 plasmid. While initiation of hairy roots in peanut explants was also achieved in the absence of *aux* genes (i.e. line J-pRYG), these roots did not sustain growth in successive subculturing. Similar responses were observed with other lines of peanut developed with the pRYG vector (data not shown), suggesting gene products of both the TL-DNA and TR-DNA are needed to establish high biomass yielding peanut hairy roots. This observation is consistent with the studies of Komarnytsky et al. (2004), where it was reported that indole-3-acetic acid supplementation was needed to support the growth of tobacco hairy roots established with the pRYG vector that only contains *rol* genes. More studies with different hairy root lines from different plants species will be needed to confirm the contribution of *aux* genes in promoting hairy root growth. No peanut hairy roots lines analyzed carried the *virD2* gene (Fig. 3) indicating the absence of *A. rhizogenes* ATCC 15834 contamination in these cultures. High biomass accumulation, sustainable growth following several subculture events and T-DNA integration were used as the selection criteria in assigning peanut hairy roots lines 2, 3, and 5 of cv. Andru II as the lead production lines for further analysis.

Table 1
Response of peanut explants to inoculation with *Agrobacterium rhizogenes* 15834

Seedling number	Petiole	Stem	Cotyledonary node	Hypocotyl
1	HR	Callus	HR prolific	No response
2	Callus	Callus	HR	No response
3	HR	Callus	HR	HR
4	HR	Callus	HR	Callus
5	Callus	HR	HR prolific	No response
6	HR	HR	HR	Callus
7	Callus	No response	HR prolific	Callus
8	HR	HR + callus	HR prolific	HR
9	HR	Callus	HR prolific	Callus
10	HR prolific	HR + callus	HR	HR

Observations were made after 3 weeks. HR = Hairy root.

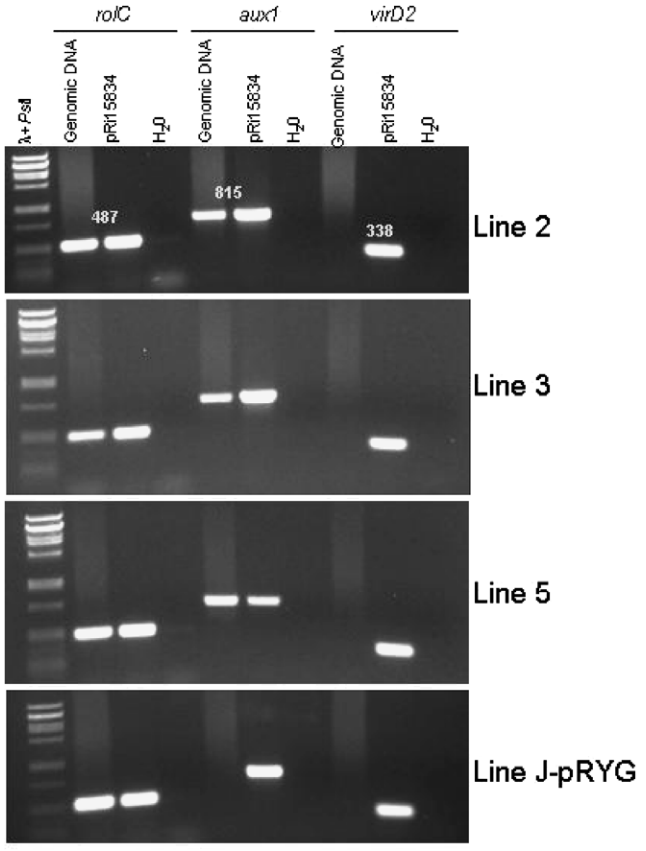

Fig. 3. Analyses by PCR of hairy root lines. Genomic DNA was isolated from hairy root lines 2, 3, 5 and J-pRYG. Analyses were performed with primers targeting the *rolC*, *aux1* and *virD2* genes. Plasmid pRi15834 DNA was used as positive control.

2.3. Elicitation of resveratrol **1**

As many plant stressors and environmental elicitors have been shown to radically induce the expression of polyphenolic compounds, we evaluated the effect of five elicitors (biotic and abiotic) on resveratrol **1** production. The elicitors tested in this preliminary screen included cellulase (1 μg/ml), chitosan (10 mg/l), laminarin (1 mg/ml), sodium

acetate (10.2 mM) and copper sulfate (600 μM); concentrations previously shown to be effective at inducing phytoalexins in other cell and tissue culture systems (Aziz et al., 2003; Cheng et al., 2006; Medina-Bolivar, unpublished). As shown in Fig. 2d, within 24 h following the addition of either sodium acetate or copper sulfate to the culture medium, the whitish phenotype of non-elicited peanut hairy roots exhibited a distinct yellow or brown coloration, respectively; an expected phenotypic response concomitant with the presence of phenolic compounds. Chitosan-induced cultures also exhibited a light yellowish color while, no color difference was observed in laminarin- or cellulase-treated cultures (data not shown).

With an overall goal of producing an easily recoverable enriched fraction of resveratrol, the culture medium was initially evaluated for resveratrol **1** product due to its much lower chemical complexity relative to that of root tissue fractions. To rapidly assess the various hairy root lines for their respective ability to induce and secrete resveratrol **1** in response to our test elicitors, we used a thin layer chromatography (TLC) method that leverages the fluorescent nature of resveratrol **1** and its derivatives. Ethyl acetate was used as a solvent to recover resveratrol **1** from the medium. This solvent allowed for partitioning of the culture media into two phases with the organic phase enriched in resveratrol and other related stilbenes.

Both sodium acetate and chitosan, elicited hairy roots to produce compounds recovered from the culture media with *Rf* values comparable to that of a resveratrol and pterostilbene (Fig. 4a). These compounds showed the expected blue fluorescence characteristic of resveratrol **1** and other related stilbenes visible at λ 365 nm (Fig. 4a) or as dark grayish spots when detected at λ 254 nm (not shown). Because chitosan is standardly solubilized in acetic acid, the ability of this solvent to induce fluorescent compounds in this system was tested. As shown in Fig. 4a, acetic acid

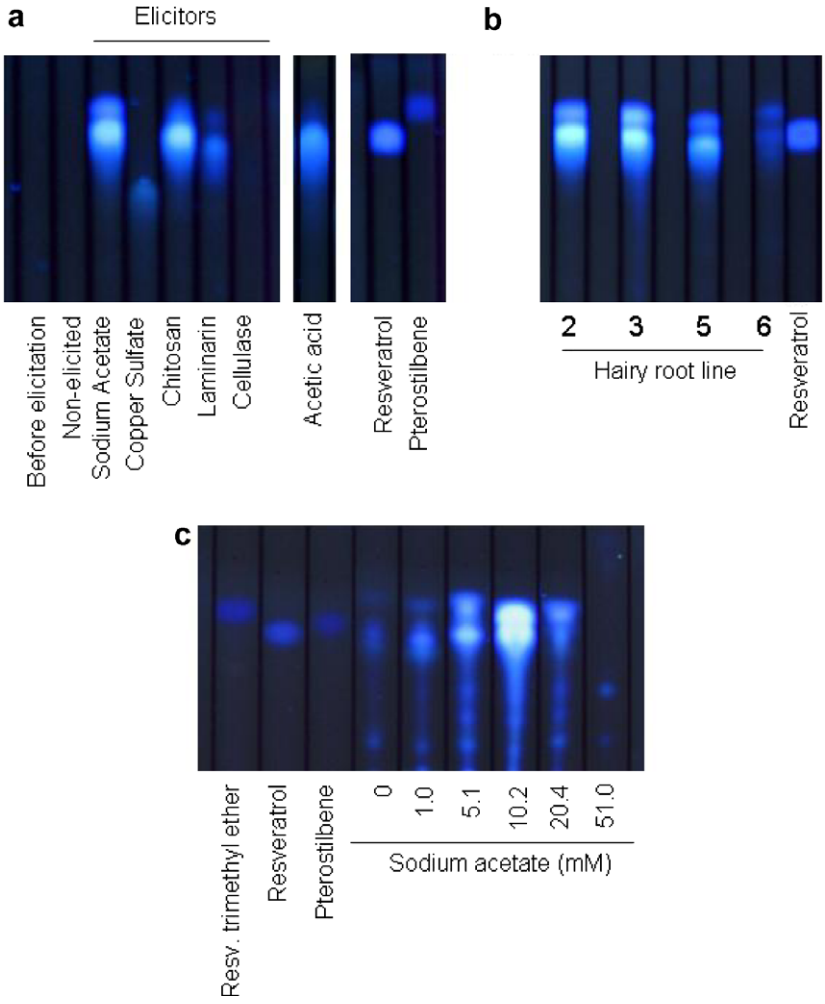

Fig. 4. (a) TLC of ethyl acetate extracts from the media of hairy root line 2 treated with different elicitors. Twelve-day cultures were elicited for 24 h with either 10.2 mM NaOAc, 600 μM $CuSO_4$, 10 mg/l chitosan, 1 mg/ml laminarin or 1 μg/ml cellulase. Acetic acid (1 mM) was used as solvent control for chitosan. Resveratrol **1** (10 μg); pterostilbene **2** (10 μg). (b) TLC of ethyl acetate extracts from the media of different hairy root lines of peanut. Twelve-day cultures were elicited for 24 h with 10.2 mM NaOAc. Resveratrol **1** (10 μg). (c) Effect of NaOAc elicitation. HPTLC of ethyl acetate extracts from the media of hairy root line 2 treated for 24 h with NaOAc (0 to 51 mM). Resveratrol trimethyl ether **3** (2 μg); resveratrol **1** (2 μg); pterostilbene **2** (6 μg). Thirty-five microgram of extract were loaded per lane.

alone (1 mM; concentration used for chitosan) induced the production of fluorescent products; however, the intensity of fluorescence was higher in the presence of chitosan suggesting both chitosan and acetic acid are additive in inducing the production of these compounds. While fluorescent compounds by TLC were elicited with laminarin (Fig. 4a), reduced intensity of TLC products suggests lower expression of these compounds with this elicitor. Interestingly, copper sulfate induced a fluorescent compound with an *Rf* distinct from that of resveratrol **1**, which was not observed with any other elicitors. No fluorescent compounds were observed for cellulase-elicited hairy roots.

Based on these initial observations, sodium acetate was chosen as the elicitor in all further studies. In addition to exhibiting marked induction of a product correlating with the *Rf* of *trans*-resveratrol **1** (Fig. 4a), sodium acetate is cost-effective in consideration of the commercial scale-up of this system. Furthermore, we observed reproducible elicitation profiles in several, independently-generated hairy root lines. Line 2 which exhibited the highest response to sodium acetate elicitation, was selected for further investigation (Fig. 4b). A dose–response shown in Fig. 4c, indicated 10.2 mM sodium acetate had the most effective response in producing compounds with *Rf* values in the range of resveratrol **1**, pterostilbene **2** and the fully methylated resveratrol analog, resveratrol trimethyl ether **3**.

A detailed growth curve and medium conductivity analysis of line 2 indicated that the culture was in an early exponential growth phase at the time of elicitation on day 12 (Fig. 5b and c). Because the effectiveness of an elicitor is likely impacted by the developmental stage of the culture (Pitta-Alvarez and Giulietti, 1999), we evaluated the sodium acetate response of the peanut hairy root culture at various time points across their growth cycle ranging from 12 through 21 days (Fig. 5a). Non-elicited root cultures of corresponding age served as controls. While, no fluorescent compounds with the corresponding *Rf* of resveratrol **1** were found by TLC in the medium of non-elicited 12- or 15-day cultures, products in this *Rf* range were detected in the medium from 18- to 21-day cultures suggesting that culture age impacts the production/secretion of these fluorescent compounds independent of elicitation. Moreover, the production and secretion of these compounds could be significantly induced throughout the exponential growth of the roots cultures. Elicitation profiles appeared to be biphasic with initial product induction observed early in exponential growth (12 days) and a second induction peak in late exponential growth (21 days). At 21 days, the amount of the observed secreted fluorescent compounds may represent a combination of both endogenously secreted and sodium acetate-elicited compounds.

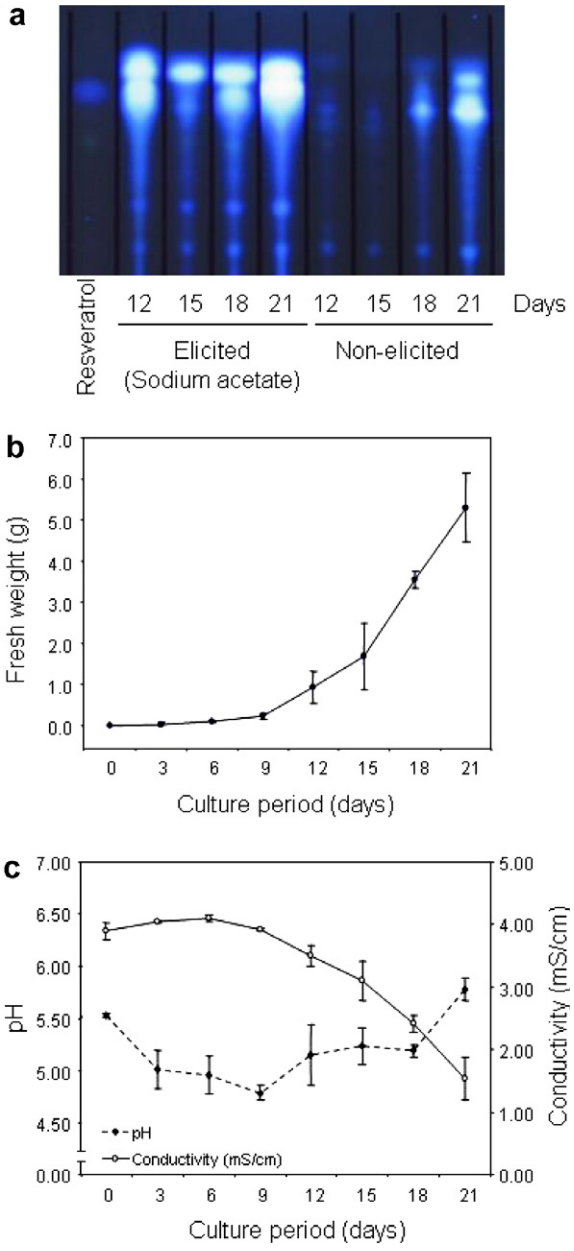

Fig. 5. (a) HPTLC of ethyl acetate extracts from the medium of hairy root line 2 treated with 10.2 NaOAc at different stages of growth. Resveratrol **1** (2 μg). Thirty-five microgram of extract were loaded per lane. (b) Growth curve of peanut hairy root line 2 in liquid B5 medium. (c) Measurements of medium conductivity and pH at different stages of growth. Determinations were recorded every 3 days.

2.4. Gas chromatography–mass spectrometry analyses of elicited and non-elicited hairy root culture

To validate our TLC/HPTLC results, a GC–MS approach was used that selectively targeted the detection of resveratrol **1** and pterostibene **2** in the medium and root tissue of 12-day non-elicited and sodium acetate elicited hairy root cultures (line 2). Selected monitoring ions for these compounds were used as previously described (Rimando et al., 2004) and discrimination between *cis* and *trans*-isomers was achieved by comparison of the retention time and mass spectra of authentic *cis* and *trans* standards (Figs. 6 and 7). Quantitative data of resveratrol **1** in ethyl acetate extracts prepared from the medium and root tissue are shown in Table 2. Under non-elicited conditions the levels of *trans*-resveratrol **1** ranged from 692 to

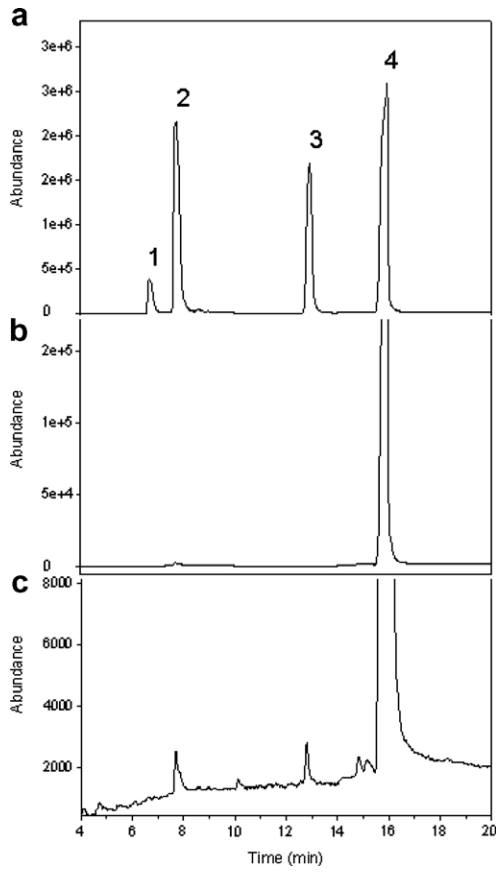

Fig. 6. (a) GC–MS trace of mixture of *cis*-pterostilbene **2** (peak 1, 6.6 min; $[M]^+$-TMS *m/z* 328), *cis*-resveratrol **1** (peak 2, 7.7 min; $[M]^+$-TMS *m/z* 444), *trans*-pterostilbene (peak 3, 13.0 min; $[M]^+$-TMS *m/z* 328) and *trans*-resveratrol **1** (peak 4, 16.0 min; $[M]^+$-TMS *m/z* 444); (b) GC–MS trace of ethyl acetate extract from the medium of NaOAc-elicited culture showing peak of *trans*-resveratrol **1**; (c) reconstructed ion chromatogram from the GC–MS analysis of the medium of NaOAc-elicited culture showing the peaks of *cis*- and *trans*-resveratrol **1**, and of *trans*-pterostilbene **2** (*cis*-pterostilbene was not found).

1813 ng/mg extract dry weight (DW). These levels were on the average 80-fold higher than *cis*-resveratrol **1** (8–31 ng/mg extract DW) levels in non-elicited hairy root medium extracts. While no fluorescent chemicals with *Rfs* corresponding to resveratrol **1** or pterostilbene **2** were detected by HPTLC in the 12-day non-elicited samples, the enhanced sensitivity of GC–MS did detect small amounts of these compounds (Tables 2 and 3).

In the interest of scaling the production capacity of this hairy root system for high quality, natural resveratrol **1** production and comparing different bioreactor platforms, an effective, universally-applicable quantitative method for resveratrol **1** recovery will be required. Therefore, we have calculated resveratrol **1** recoveries based on the dry weight (DW) of the ethyl acetate extract of the culture media. The labscale recovery for *trans*-resveratrol **1** ranged from 50 to 98 μg/mg extract DW, reflecting an approximate 60-fold increase above the levels of resveratrol **1** detected in the non-elicited culture extracts (Table 2). Although *cis*-resveratrol was detected in both non-elicited and elicited cultures, the levels in the elicited cultures were relatively low ranging from 47 to 399 ng/mg media extract to an average of 10 ng/mg root tissue extract. This preferential production of the *trans*- over the *cis*-isomer is quite significant, as *trans*-resveratrol is known to be more active and thus the desired form for a resveratrol product (Roupe et al., 2006).

Estimating that 6 mg of culture medium ethyl acetate extract (DW) is obtained from a gram of root tissue DW, the current production rate of this system ranges between 300 and 588 μg of *trans*-resveratrol **1** per gram DW of root tissue. The levels of *trans*-resveratrol **1** remaining in the root tissue were only 0.2–1.1 μg/mg extract DW (Table 2), suggesting that approximately 99% of the total *trans*-resveratrol **1** produced in hairy roots is effectively secreted into the culture medium with sodium acetate elicitation. We are currently testing a low cost and easy-to-use bioreactor to grow hairy roots of several plant species and recover ~60 g DW root biomass per liter for a 2 weeks culture (Nopo-Olazabal and Medina-Bolivar, unpublished). Estimates at the current production capacity of this bioreactor, recover 18–35 mg of secreted *trans*-resveratrol **1** per liter of medium with a single elicitation treatment; production/recovery rates will likely improve with further optimization of this system.

Previously, peanut plants grown either hydroponically or in the field for 120 days were reported to produce resveratrol **1** at levels of 74 and 114 μg/g whole root DW, respectively (Liu et al., 2003). Notably, in addition to a 3–5 fold increase in product recovery after only a single elicitation of a 12-day culture, this hairy root bioproduction system has the added advantage that resveratrol **1** and its derivatives are secreted and can be recovered from the low complexity media versus a whole root extract. By avoiding the recovery of these metabolites from root tissue, the secreted fraction provides a product that is more enriched in the desired compounds and has increased purity over commercially available resveratrol-containing products extracted from botanical tissue (i.e. peanut roots, grape skin/seeds, and *Polygonum* root).

Because resveratrol **1** is primarily recovered from the root medium, a multiple elicitation regime of the culture could significantly increase the total yield of resveratrol generated by this system. This concept of a semi-continuous operation and product recovery through multiple elicitations has been demonstrated for the production of tanshinones in *Salvia miltiorrhiza* hairy roots (Yan et al., 2005). While this study focused on resveratrol products from singly elicited, early log-phase growing hairy roots, we have shown that these cultures can be elicited at multiple stages of exponential growth (Fig. 5). In addition, ongoing studies suggest that the cultures can be re-elicited several times to enhance product recovery (data not shown).

In light of previous studies demonstrating antidiabetic (Manickam et al., 1997), cholesterol-reducing (Rimando et al., 2005) and cancer-preventive (Tolomeo et al., 2005)

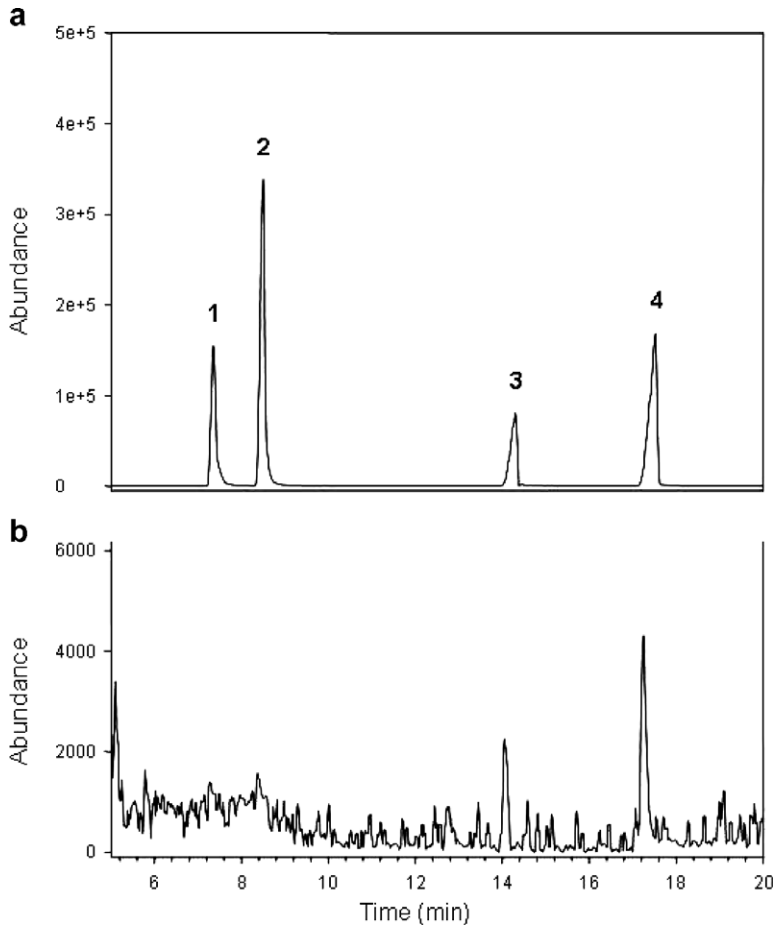

Fig. 7. GC–MS trace of (a) mixture of *cis*-pterostilbene **2** (peak 1, 7.3 min; $[M]^+$-TMS *m*/*z* 328), *cis*-resveratrol **1** (peak 2, 8.2 min; $[M]^+$-TMS *m*/*z* 444), *trans*-pterostilbene **2** (peak 3, 14.1 min; $[M]^+$-TMS *m*/*z* 328) and *trans*-resveratrol **1** (peak 4, 17.3 min; $[M]^+$-TMS *m*/*z* 444); (b) reconstructed ion chromatogram from GC–MS analysis of root tissue extract from NaOAc-elicited culture showing peaks of *cis*- and *trans*-resveratrol, and of *trans*-pterostilbene (*cis*-pterostilbene was not found).

Table 2
Quantitation of resveratrol in ethyl acetate extracts of the medium and root tissue of non-elicited or sodium acetate-elicited hairy root cultures of peanut, line 2

	Medium		Root tissue	
	trans-Resveratrol	*cis*-Resveratrol	*trans*-Resveratrol	*cis*-Resveratrol
Elicited				
A	97,992	387	253	10
B	85,282	399	1134	ND
C	50,339	47	–	–
Non-elicited				
D	1416	31	210	ND
E	1813	8	587	3
F	692	9	–	–

Amounts are given in ng per mg extract.
Each sample (A–F) corresponds to the extract of an individual culture. The value for each sample is the mean of two determinations. ND, not detected; –, not analyzed.

Table 3
Quantitation of pterostilbene in ethyl acetate extracts of the medium and root tissue of non-elicited or sodium acetate-elicited hairy root cultures of peanut, line 2

	Medium		Root tissue	
	trans-Pterostilbene	*cis*-Pterostilbene	*trans*-Pterostilbene	*cis*-Pterostilbene
Elicited				
A	237	ND	136	ND
B	126	ND	44	ND
C	61	ND	–	–
Non-elicited				
D	22	ND	ND	ND
E	33	ND	ND	ND
F	140	ND	–	–

Amounts are given in ng per mg extract.
Each sample (A–F) corresponds to the extract of an individual culture. The value for each sample is the mean of two determinations. ND, not detected; –, not analyzed.

properties of pterostilbene **2**, we were interested in a quantitative assessment of this related, potentially bioactive stilbene. GC–MS analysis of elicited hairy root culture media samples reported levels of *trans*-pterostilbene **2** that varied between 61 and 237 ng/mg extract DW, reflecting an approximately 2-fold increase in elicited over non-elicited

media fractions (Table 3). While sodium acetate elicitation of hairy roots induced resveratrol **1** levels 60-fold, pterostilbene **2** levels were only minimally increased (2-fold). Additional abiotic or biotic elicitors may be more effective at preferentially inducing pterostibene **2** and will be addressed in future studies. The HPTLC analyses indicate the presence of other induced compounds of different polarity than resveratrol **1** and pterostilbene **2**. Studies are currently being performed to confirm the identity of these compounds.

These quantitative analyses clearly demonstrate the potential production capacity of peanut hairy root cultures for *trans*-resveratrol **1** and other health beneficial stilbenes, such as pterostilbene **2**, together accounting for close to 10% of the extract recovered from the media fraction following a single elicitation of the culture. While resveratrol **1** has been the subject of countless studies, many of the other stilbene derivatives have shown equivalent or enhanced activity and bioavailability (Baur and Sinclair, 2006). Preliminary results evaluating the antioxidant activity of this hairy root extract suggest other components present in this enriched extract enhance this beneficial activity (Lorence and Medina-Bolivar, data not shown). Ongoing studies evaluating isolated fractions of the elicited peanut hairy root extracts on various cancer cell lines may provide valuable insight in dissecting the biological activities attributed to the individual components of this defined extract. Furthermore, this study has focused exclusively on the analysis of ethyl acetate fractions from the culture medium. As other more polar resveratrol derivates, such as piceid 5 (Fig. 1), may in fact be present in the aqueous fraction, we will evaluate extracts of these more polar fractions to determine the potential presence and recovery of additional inducible stilbenes in the peanut hairy root cultures.

Studies have established acetate as an effective elicitor of secondary metabolite production in a number of hydroponically grown plants and roots (Pitta-Alvarez and Giulietti, 1999; Poulev et al., 2003). Sodium acetate clearly elicited the production and secretion of valued resveratrol **1** and resveratrol derivatives from peanut hairy roots cultures. However, this abiotic stimulus may also provide an important precursor to the system that contributes to the overall production of these secondary metabolites. Studies evaluating tropane alkaloid production support a precursor role of acetate (Pitta-Alvarez and Giulietti, 1999). In addition to promoting the release of alkaloids into the culture medium, acetate was suggested to be used as a precursor and enter the tropane alkaloid pathway via acetoacetyl-CoA. In fact, malonyl-CoA, one of the main precursors of resveratrol **1**, also utilizes acetate as precursor in its biosynthesis and therefore acetate may play a precursor role in addition to elicitor of resveratrol **1**. Changes in membrane permeability and pH resulting from acetate application were suggested to the induced release of tropane alkaloids and may also contribute to the increased stilbene levels recovered in this study.

3. Concluding remarks

The hairy root culture platform is a unique bioproduction system for generating well-defined, highly-enriched fractions of resveratrol **1** and other beneficial stilbene compounds. In capturing the spatio-temporal organization of the source plant, this tissue-based culture system may better preserve the natural metabolic processes as they occur in nature. Maintenance of tissue integrity likely supports the distinctive genetic and biosynthetic stability of hairy roots and enables their fast growth for generating the requisite biomass. The issue of genetic stability of hairy roots is a major advantage in that *in vitro* cell suspension culture systems are subject to high somaclonal variation that leads to their reduced capacity in responding to elicitation and subsequent reduction in the production of desired metabolites. In addition to demonstrating stable hairy root phenotype in peanut, we established the ability of these cultures to retain metabolic responses endogenous to their host plant that are capable of producing notable levels of resveratrol **1** and related derivatives.

While crude botanicals (i.e. grape seed extracts, knotweed root extracts) and synthetics are the leading commercialized sources of resveratrol, hairy roots may fill the need in this market for a more consistent, controlled natural source of resveratrol as well as other valued resveratrol-derivatives. We demonstrated that 99% of the total resveratrol **1** produced in peanut hairy roots is secreted into the medium. This in effect provides a more defined and enriched source of resveratrol- and other resveratrol derivatives with putative nutraceutical benefits. Continuous progress and advances in root bioreactor technology have led to recent demonstrations of large-scale production of anticancer compounds using the hairy root system (ROOTec; http://www.rootec.com) and industrial production of ginseng roots in 10,000 l bioreactors (Sivakumar et al., 2006; Wink et al., 2005). Studies are in progress to determine maximum recovery of resveratrol/resveratrol derivatives from peanut hairy roots including examining select bioreactor strategies as well as testing different extraction procedures.

Continued efforts that leverage hairy roots as a distinctive production strategy for the commercial production of resveratrol **1** will also contribute to the advancement of this environmentally-friendly, resource-sparing technology for other valued natural products for which native plant stocks may be limited.

4. Experimental

4.1. Establishment of hairy root cultures

Seeds of peanuts (*Arachis hypogaea*) cv. Andru II (kindly provided by Dr. Daniel Gorbet, University of Florida) were surface sterilized as follows. Seeds were presoaked for 2 min in sterile water containing 0.003% Ivory™

detergent; immersed for 15 min in sterilization solution (50% Clorox™, 0.003% Ivory™ detergent) and rinsed in sterile water. To minimize Chlorox™ damage to the embryo, the testa was aseptically removed and seeds were further rinsed in two changes of sterile water over a 15 min period. Disinfected seeds were placed individually on plates containing B5 medium (Gamborg et al., 1968) to allow emergence of the radicle and to screen for viable healthy seedlings. Germinated seedlings were transferred to Magenta™ boxes containing B5 medium and allowed to grow in continuous light at 28 °C for 15–21 days. Explants used for hairy root production were harvested following expansion of two true leaves.

Cotyledon, hypocotyl, leaf blade, stem, petiole and cotyledonary-node explants were evaluated for their responsiveness to *Agrobacterium* infection. Three strains of *Agrobacterium* were tested: *A. rhizogenes* ATCC 15834, *A. rhizogenes* R1000 and *A. tumefaciens* EHA105 harboring pRYG plasmid (containing *rolA*, *rolB* and *rolC* genes, Komarnytsky et al., 2004). The latter plasmid was kindly provided by Dr. Ilya Raskin (Rutgers University, NJ) and mobilized into *A. tumefaciens* by freeze-thaw method (Holsters et al., 1978). Intact seedlings were inoculated with Agrobacteria at the stem, petiole, or cotyledonary node. All other isolated explants were inoculated via epidermal incisions as described before (Medina-Bolivar and Cramer, 2004; Medina-Bolivar et al., 2003). Inoculated intact seedlings and explants were maintained in B5 medium plates under constant light at 28 °C. Hairy roots that developed at the inoculation site were harvested and transferred to B5 medium plates containing 600 mg/l cefotaxime, and maintained at 28 °C under continuous darkness. Roots were subcultured twice in this medium prior to transfer on antibiotic-free medium. Liquid hairy root cultures were established by inoculating ten 1-cm root tips into 250 ml flasks containing 50 ml of B5 medium. Roots were subcultured every 2 weeks and maintained on an orbital shaker at 90 rpm, 28 °C and continuous darkness. A growth curve was performed for hairy roots of line 2 cultured in liquid as described above. Fresh weight of roots and conductivity and pH of the medium were determined every 3 days until day 21 of culture. Before weighing the roots, excess medium was removed with paper towels. The conductivity of the medium was measured using a SevenEasy (Mettler Toledo, USA) conductivity meter and the pH determined with an Accumet® Basic AB15 (Fisher Scientific, USA) pH meter.

4.2. PCR analysis

The *A. rhizogenes* transferred genes *rolC* and *aux1* were used as targets for PCR analyses in peanut hairy roots, whereas *virD2* gene was used to detect for the presence of contaminating Agrobacteria in the tissue. Genomic DNA from four hairy roots lines (2, 3, 5 and J-pRYG) of cv. Andru II were obtained using the DNeasy® Plant Mini kit (Qiagen, USA). A primer pair of 5′-TGTGACAAGCAGCGATGAGC-3′ and 5′-GATTGCAAACTTGCACTCGC-3′ (Bonhomme et al., 2000) was used to amplify a 487-bp fragment of the *rolC* gene (Slightom et al., 1986) and a second pair of primers, 5′-CCAAGCTTGTCAGAAAACTTCAGGG-3′ and 5′-CCGGATCCAATACCCAGCGCTTT-3′ was designed to amplify a 815-bp fragment of the *aux1* gene (GenBank accession No. DQ782955). In addition, primers (5′-ATGCCCGATCGAGCTCAAGT-3′ and 5′-CCTGACCCAAACATCTCGGCT-3′), amplifying a fragment of 338 bp (Haas et al., 1995) were used for detecting the *virD2* gene. The reactions were performed with puReTaq Ready-To-Go PCR beads (Amersham Biosciences, USA) containing 180 ng plant genomic DNA (or 10 ng Ri plasmid DNA) and 0.3 μM of each primer. Conditions for *rolC* amplification were as follow: initial denaturation at 95 °C for 3 min, 30 cycles of amplification (95 °C 30 s, 49 °C 30 s and 72 °C 1 min) and 10 min extension at 72 °C; for *aux1*, initial denaturation at 95 °C for 3 min, 30 cycles of amplification (95 °C 30 s, 58 °C 30 s and 72 °C 1 min) and 72 °C for 10 min; for *virD2*, initial denaturation at 95 °C for 3 min, followed by 30 cycles of amplification (95 °C 30 s, 56 °C 30 s and 72 °C 45 s) and 10 min at 72 °C. PCR amplicons were visualized after electrophoresis in 0.7% agarose gels.

4.3. Elicitor treatments

In a preliminary experiment to identify the most appropriate elicitor for stilbene induction, 12-day peanut hairy root cultures were incubated for 24 h with different elicitors. The elicitors tested were 10.2 mM sodium acetate, 1 mg/ml laminarin, 600 μM copper sulfate, 1 μg/ml cellulase and 10 mg/l chitosan. Except for chitosan which was dissolved in acetic acid, all other elicitors were dissolved in water and filter sterilized before added to the medium. Acetic acid at a final concentration of 1 mM was used as a solvent control for chitosan. Prior to elicitation, the 12-day culture media was removed and replaced with fresh B5 medium containing the elicitor. Control cultures were replaced with B5 medium witout elicitor. After 24 h, the media was collected and frozen at −20 °C. In addition, the root tissue was collected, fresh weight measured and then the tissue was rapidly frozen in liquid nitrogen prior to storage at −80 °C. After this initial screening of elicitors, sodium acetate at 10.2 mM was selected and used in all the successive elicitation experiments. To investigate the effect of different doses of sodium acetate, 12-day cultures were elicited with B5 medium containing sodium acetate at concentrations from 0 to 51 mM. The medium was collected after 24 h and processed as described above.

4.4. Effect of age of culture on elicitor response

Hairy root cultures at 12, 15, 18 or 21 days where elicited with 10.2 mM sodium acetate. Before elicitation the medium from the 12, 15, 18 or 21 day cultures was removed, and the pH and conductivity were measured

and then the medium was then frozen at −20 °C. Fresh B5 medium (control) or with 10.2 mM sodium acetate (elicited) was added to the cultures and then collected after 24 h. The pH and conductivity were measured and then the medium was frozen at −20 °C.

4.5. Extraction of resveratrol and derivatives from culture medium and root tissue

Media samples (50 ml) were thawed and partitioned with 30 ml ethyl acetate in a separatory funnel. The organic phase was recovered and dried to completeness under nitrogen stream using a RapidVap N_2 evaporation system (Labconco, USA) at 40 °C and 40% rotor speed. These dried samples were weighed and individually resuspended in etOAc (50 μl) for further analysis.

Root tissue (frozen at −80 °C) was lyophillized in a FreeZone 4.5 freeze drying system (Labconco) and then dried tissue (50 mg) were homogenized with etOAc (3 ml) in a glass homogenizer, followed by vortexing for 20 s. The homogenate was filtered through Whatman 1 filter paper and then the filtrate was dried to completeness under nitrogen stream as above. These dried samples were weighed and resuspended in EtOAc (30 μl) for further analysis.

4.6. Qualitative TLC and HPTLC analysis of the medium

Aliquots of the extracts and pure, authentic standards of *trans*-resveratrol **1** (Sigma, USA) and *trans*-pterostilbene **2** (Sigma) were spotted on 10×10 (HPTLC) or 20×20 (TLC) silica gel 60 CF_{254} plates (Merck). Linear ascending development of the samples was carried out using a mobile phase of EtOAc:AcOH:H_2O (17:1:2) as described by Nepote et al., 2004. Plates were air dried and visualized under UV light (λ 254 and λ 365 nm) using a Chromato-Vue® C-75 (UVP, USA) dark cabinet operated by Camera-Windows-Canon PowerShot G6 software version 5.0.0.15.

4.7. Quantitative analysis of resveratrol and pterostilbene by GC–MS

Dried medium or root tissue extracts (0.1 mg) were derivatized with 500 μl of a mixture of *bis* (trimethylsilyl)trifluoroacetamide:dimethyl formamide (1:1), heated at 70 °C for 40 min. After cooling to room temperature, samples were analyzed by gas chromatography/mass spectrometry (GC–MS) on a JEOL GCMate II system (JEOL USA Inc., USA). The GC temperature program was as follows: initial temperature 190 °C, then increased to 239 °C at a rate of 20 °C/min and held at this temp for 3 min, then increased to 242 °C at a rate of 0.2 °C/min and held at this temp for 4 min, then finally increased to 300 °C at a rate of 40 °C/min and held at this temperature for 0.4 min (total run time 26 min). The GC capillary column used was DB-5 (0.25 mm i.d., 0.25 mm film thickness, 30 m length; Agilent Technologies, USA). The carrier gas was ultra high purity helium (nexAir, USA), 1 ml/min flow rate. The inlet (splitless), GC interface, and ion chamber temperatures were 250 °C, 250 °C, and 230 °C, respectively. The injection volume was 2 μl.

Analyses of *cis*- and *trans*-resveratrol **1** and *cis*- and *trans*-pterostilbene **2** were carried out in a selected ion monitoring mode (retention times 7.7, 15.7, 6.6. and 12.8 min, respectively; Fig. 6). *cis*- and *trans*-resveratrol **1** were monitored for *m*/*z* 444 (and 429, 207, 147 as qualifier ions). *cis*- and *trans*-pterostilbene **2** were monitored for *m*/*z* 328 (and 313, 296, 156 as qualifier ions). Quantitation was performed using external standards of a commercial sample of *trans*-resveratrol **1** (Sigma) and a synthetic sample of *trans*-pterostilbene **2** (synthesized from methylation of resveratrol). The *cis*-isomers were obtained by UV-irradiation (λ 306 nm) of an ethanolic solution the *trans* isomers (1 mg/ml) for 24 h.

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