



Total phenolics, resveratrol content and antioxidant activity of seeds and calluses of pinto peanut (*Arachis pinto* Krapov. & W.C. Greg.)

Isabela Brandão de Sousa-Machado¹ · Tayanne Felipe¹ · Renata Garcia¹ · Georgia Pacheco¹ · Davyson Moreira² · Elisabeth Mansur¹

Received: 19 October 2017 / Accepted: 3 June 2018 / Published online: 26 June 2018
© Springer Nature B.V. 2018

Abstract

Arachis pinto is a peanut species native to Brazil, which is cultivated in many countries for animal forage, soil cover, landscaping, and recovery of degraded areas. Tissue culture studies for this species have been focused in plant production, whereas works on in vitro secondary metabolites production are scarce. The goal of the present work was to establish callus cultures from different seed explants of *A. pinto*, aiming at evaluating the potential for metabolites production and antioxidant activity. Embryonic axes, leaflets, and cotyledons were cultured on solidified MS medium supplemented with picloram (PIC), 2,4-dichlorophenoxyacetic acid (2,4-D), thidiazuron (TDZ) or different combinations of 6-benzyladenine (BA) and α -naphthaleneacetic acid (NAA), under light or dark conditions. Friable calluses with a high biomass (4.3 ± 0.3 g FW per callus) were obtained from embryonic leaflets cultured on medium supplemented with 17.6 μ M BA plus 5.4 μ M NAA, in the dark. Cotyledons and embryonic axes cultured in the presence of 4.4 μ M BA combined with 10.8 μ M NAA formed heterogeneous calluses with a compact base and a large friable surface. *Trans*-resveratrol and other stilbenes that were not found in seeds were detected in callus extracts, especially those originated from cotyledons, although these materials showed lower total phenolic contents (TPC) when compared with seeds with and without testa, as well as cotyledons. Extracts from seeds with testa and from calluses derived from cotyledons and embryonic axes showed the highest EC₅₀ in DPPH assays. No correlation between TPC, *trans*-resveratrol and antioxidant activity was observed.

Keywords Phytoalexin · Stilbenoids · Auxin · Cytokinin · Seed explants · Callogenesis

Introduction

The genus *Arachis* L. (Fabaceae) is native to South America, with 81 described species (Krapovickas and Gregory 1994; Santana and Valls 2015), and Brazil is considered a major genetic diversity center. The most economically important

species of the genus, groundnut (*A. hypogaea* L.), is the fourth oilseed crop around the world, producing seeds with high nutritional value (Silva et al. 2010), whereas some other species have gained agronomic importance more recently.

Among these species, pinto peanut (*Arachis pinto* Krapov. & W.C. Gregory) is increasingly valued as forage due to its characteristics of perennial crowns, ability to spread via stolons, high digestibility and nutritional value (Ladeira et al. 2002), adaptability to different types of soil, shading tolerance, cold tolerance, and good fire resistance (Bresolin et al. 2008; Adjolohoun et al. 2013). Several studies demonstrated an increased efficiency of animal production per hectare of pasture when using *A. pinto*, which ultimately reduces the need of deforestation of new areas for this purpose. This species is also used for soil cover, as ornamental, and for the recovery of degraded areas (Carvalho and Quesenberry 2009). In the last 30 years, several *A. pinto* cultivars were released around the world, mainly in Australia and Latin America. In Brazil, at least four cultivars were

Communicated by Sergio J. Ochatt.

✉ Isabela Brandão de Sousa-Machado
bela_biouerj@hotmail.com

Elisabeth Mansur
elisabeth.mansur@gmail.com

¹ Instituto de Biologia Roberto Alcântara Gomes, Núcleo de Biotecnologia Vegetal, Rua São Francisco Xavier 524 PHLC sala 505, Maracanã, Universidade do Estado do Rio de Janeiro, Rio de Janeiro 20550-013, Brazil

² Instituto de Tecnologia em Fármacos, Fundação Oswaldo Cruz, Avenida Brasil 4036, Maré, Rio de Janeiro 21040-361, Brazil

developed, namely Amarillo MG100, Alqueire-1, Belmonte, and BRS Mandobi. Amarillo MG100, the first cultivar produced in the country and the most currently commercialized, is characterized by high nutritional value, persistence under grazing, and good seed production.

Plant tissue culture is an important tool for micropropagation, conservation, genetic transformation, and in vitro production of secondary metabolites. *Arachis* species have been largely studied in this context, especially the groundnut. Protocols for plant regeneration of different genotypes of *A. hypogaea* were established through organogenesis and somatic embryogenesis (Iqbal et al. 2011; Matand et al. 2013). On the other hand, wild species of the genus were studied to a lesser extent with this approach. Organogenesis has been obtained with the use of explants from in vivo and in vitro plants, seedlings and seeds, while embryogenesis was reported using explants from in vivo plants, seedlings and seeds (Pacheco et al. 2009; Poëaim et al. 2015). In vitro regeneration of *A. pintoi* was achieved through organogenesis from seedling leaflets (Pittman et al. 1983), mature leaflets (Burtnik and Mroginski 1985; Rey et al. 2000), and seed explants (Pacheco et al. 2007). Somatic embryogenesis was obtained from mature leaflets (Rey et al. 2000), leaves from in vitro plants, and seed explants (Rey and Mroginski 2006). Plant development through meristematic amplification was achieved from in vitro plant explants (Rey and Mroginski 2003), and embryo axes (Pacheco et al. 2007).

The production of bioactive metabolites has been also studied in some *Arachis* species, including phenolic acids and phytosterols, which possess antioxidant, antibacterial, antifungal, anti-inflammatory and antitumor activities (Lopes et al. 2011). In vitro production of stilbenoids by calluses (Ku et al. 2005; Yang et al. 2010a, b) and root cultures of *A. hypogaea* has also been reported by some authors (Medina-Bolívar et al. 2007; Kim et al. 2008; Condori et al. 2010; Halder and Jha 2016). In addition, recent studies have detected low amounts of *trans*-resveratrol in leaves of some species of the genus (Lopes et al. 2013). We have previously reported the allelopathic activity of seed extracts from different *A. hypogaea* cultivars as well as antioxidant properties of *A. repens* leaf extracts, which contain *trans*-resveratrol and other phenolic compounds (Garcia et al. 2016; Casimiro et al. 2017). *Trans*-resveratrol (3,5,4-trihydroxy stilbene) is synthesized by a number of plant species, and has been associated to therapeutic effects on inflammation, and viral infections, as well as antioxidant and neuroprotective activities. Moreover, it is capable of minimizing the risk of cardiovascular disease and various types of cancer, the two most common causes of death around the world (Mallikarjuna et al. 2016; Aune et al. 2017).

Considering the potential of in vitro systems for producing bioactive compounds, the goal of this work was to perform a comparative analysis of the content of *trans*-resveratrol and

phenolic compounds, as well as the antioxidant potential of extracts from calluses and seeds of *A. pintoi*.

Materials and methods

Plant material and culture conditions

Fruits of *A. pintoi* cultivar Amarillo MG100 were purchased from Realpecuária Sementes Nacionais e Importadas, São Paulo, Brazil. Basal media consisted of MS (Murashige and Skoog 1962) salts containing MS vitamins plus 3% (w/v) sucrose, and solidified with 0.7% (w/v) agar (Merck). The pH of all media was adjusted to 5.8, and plant growth regulators (PGR) were added before autoclaving for 15 min at 121 °C. Cultures were maintained in a growth chamber at 25 ± 2 °C, in the dark or under a 16 h photoperiod, using a total irradiance of $46 \mu\text{mol m}^{-2} \text{s}^{-1}$ provided by cool-white fluorescent lamps.

Callus induction

Seeds were dehulled from the fruits, and surface-sterilized with 70% (v/v) alcohol for 1 min followed by 1.5% (w/v) sodium hypochlorite containing 0.02% (v/v) Tween 80 for 45 min under agitation, and washed three times with sterile deionized water.

Seed explants (cotyledons, immature leaflets and embryonic axes without leaflets) were inoculated on MS medium supplemented with different concentrations of picloram (PIC) at 4.1 to 41.4 μM , 2,4-dichlorophenoxyacetic acid (2,4-D) at 4.52 to 45.2 μM or thidiazuron (TDZ) at 4.54 to 45.4 μM . The balance of cytokinins and auxins was evaluated using different combinations of 6-benzyladenine (BA) and α -naphthaleneacetic acid (NAA). Low ratios BA/NAA consisted of concentrations of 0.44–4.4 μM BA plus 8.8–17.6 μM NAA. High ratios BA/NAA comprised 8.8–17.6 μM BA plus 0.54–5.4 μM NAA. Cultures were maintained in the presence or absence of light for 60 days. Four flasks ($8 \times 7 \text{ cm}^2$) closed with polypropylene caps, each containing three explants, were used per treatment.

Callogenesis rates, expressed as the percentage of responsive explants, were determined for each treatment. Biomass accumulation of friable calluses was estimated by the evaluation of fresh (FW) and dry (DW) weights, after 30 days of culture. Dry weights were determined after drying the material at 50 °C to constant weight.

Extract preparation

Seeds (with and without testa), cotyledons and calluses derived from cotyledons, immature leaflets and embryonic axes without leaflets were dried at 50 °C for 48 h and

grounded in mortar. Samples were defatted with *n*-hexane (50 mL/g DW) in an ultrasonic bath for 30 min. The solid residue was extracted with 80% ethanol (50 mL/g DW) in ultrasonic bath for 30 min. Extracts were filtered, and solvent was evaporated to dryness. Samples were stored at $-20\text{ }^{\circ}\text{C}$ until analysis.

HPLC-DAD-UV analysis

Qualitative *trans*-resveratrol HPLC-DAD-UV analysis were carried out using a Shimadzu® Nexera XR equipped with a SPD20A UV-Vis DAD, CBM20A controller, DGU20A degasser, LC20AD binary pumps, CTO20A oven and SILA20A autosampler. Extracts were solubilized in methanol (10 mg/mL), and filtered with Milipore 0.45 μm filter. Qualitative analysis were performed using a Thermo C18 analytical column (250 mm \times 4.6 mm i.d. \times 5 μm particle size), maintained at $50\text{ }^{\circ}\text{C}$, with sample injection volume of 20 μL . Solvent system consisted of ultrapure aqueous acetic acid solution, pH 3, (solvent A) and acetonitrile (solvent B) with gradient elution starting at 95% of A and 5% of B to 5% of A and 95% of B in 80 min and more 10 min in initial condition in order to system re-equilibration, in a flow rate at 1.0 mL/min. The UV absorption was monitored at 254 and 306 nm. The chromatograms were handled using Shimadzu LabSolutions software.

Trans-resveratrol quantification was done in the same equipment with Supelco Ascentis® Phenyl analytical column (250 mm \times 4.6 mm i.d. \times 5 μm particle size), injection volume of 20 μL and flow rate of 1.0 mL/min. Solvent system consisted ultrapure aqueous acetic acid solution, pH 3, (solvent A), acetonitrile (solvent B) and methanol (solvent C). Gradient elution started at 90% of A, 2% of B and 8% of C; changed linearly to 80% of A, 12% of B and 8% of C, in 3 min; changed to 5% of A and 95% of B in 3 to 9 min; changed to initial conditions and kept for more 2 min. *Trans*-resveratrol was detected by comparison of retention time and UV spectrum with the standard (Sigma). This method was validated according to the following parameters: (a) selectivity, (b) linearity, (b) intra-day and inter-day precision, (c) accuracy, (d) recovery, (e) robustness and (f) limits of detection and quantification (Moreira et al. submitted).

Determination of total phenolics

Total phenolic content was determined using the Folin–Ciocalteu colorimetric method with modifications (Holland et al. 2011). Briefly, 90 μL of extracts resuspended in methanol at 0.5 mg/mL were incubated with 180 μL of 10% (v/v) Folin–Ciocalteu solution plus 730 μL of 100 mM Na_2CO_3 solution for 2 h in the dark. Quantification was performed on the basis of the standard curve of gallic acid at 740 nm, using a spectrophotometer UV-Vis BioMate 3S (Thermo

Scientific). Total phenolic content was expressed in mg gallic acid equivalents (GAE) per g DW. All tests were done in triplicate.

Determination of antioxidant activity

Antioxidant activity analysis was carried out using the 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) free radical scavenging method as described by Brand-Williams et al. (1995). Briefly, 25 μL of extracts diluted in 100% methanol (0.1–40 mg/mL) were incubated with 975 μL of methanol solution of DPPH (0.6 mM) for 1 h in the dark. Absorbance was measured at 515 nm (UV-Vis BioMate 3 S – Thermo Scientific). The scavenging rate was calculated according to the equation:

Scavenging rate (%) = $(A_0 - A_1)/A_0 \times 100$, where A_0 is the absorbance of the control without extracts and A_1 is the absorbance with the sample.

The effective concentration that scavenged 50% of the free radicals (EC_{50}) was calculated for each sample by non-linear regression from a graph of % DPPH activity versus sample concentration (mg/mL). All tests were done in triplicate.

Statistical analysis

The experiments were repeated three times, using groups of 10 explants. Data were subjected to analysis of variance (ANOVA), and means were compared with the Tukey–Kramer Test at 0.05% significance level, using the software Graph Pad InStat version 3.0 (Graph Pad Software Inc., San Diego, CA, USA).

Results

Callus induction

Different types of calluses were produced according to explant type, growth regulator, and light conditions. In media supplemented with 2,4-D, cotyledons and embryonic axes originated heterogeneous calluses, mostly compact with a small friable surface (Fig. 1a). Development of shoots and roots occurred at low frequencies, both in the presence and absence of light. Cotyledon explants reached 40% of callogenesis in response to 13.56 μM 2,4-D and incubation in the dark (Table 1). Embryonic leaflets were unresponsive to 2,4-D.

Mucilaginous calluses were formed from all explants in media supplemented with PIC (Fig. 1b), at frequencies between 5 and 90%. Globular regions were formed mainly in the dark. Best results for cotyledons and embryonic

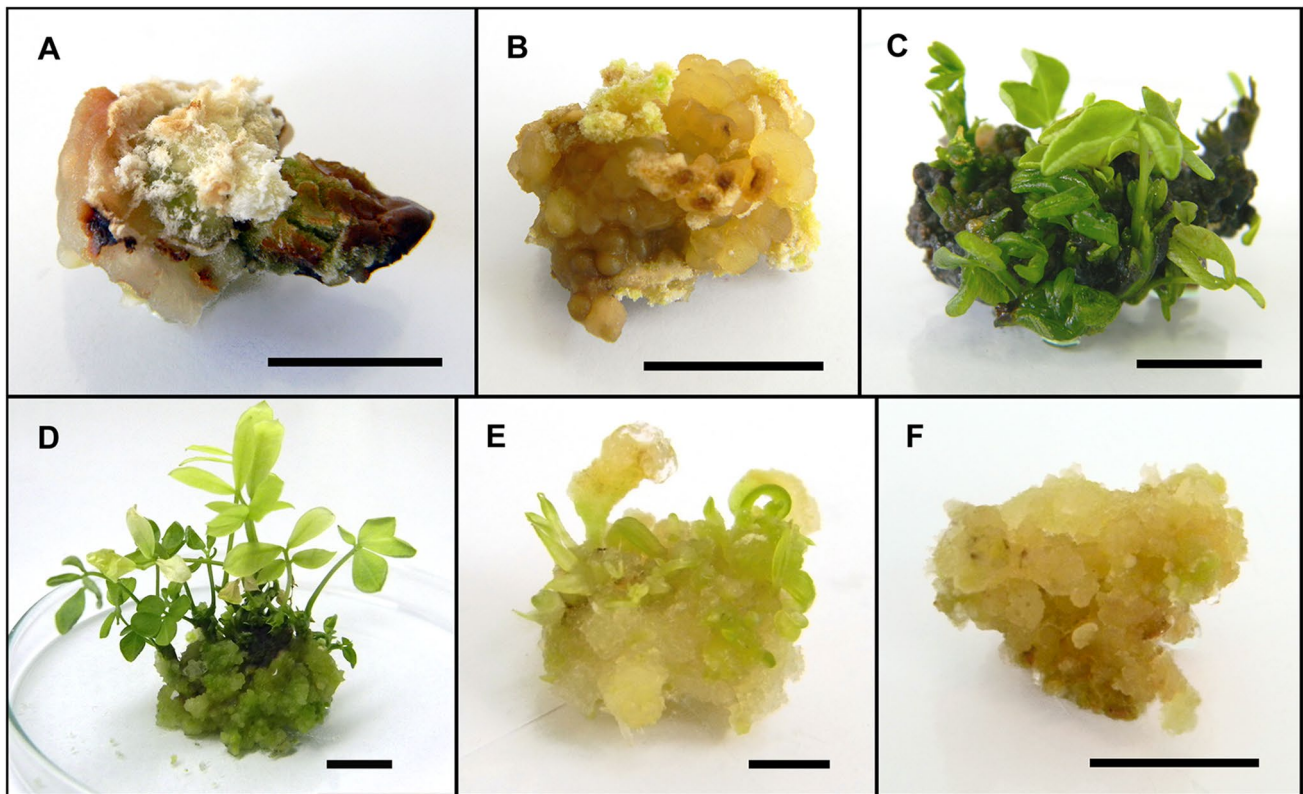


Fig. 1 Callus formation from *A. pintoii* seed explants cultured on MS medium supplemented with different growth regulators for 60 days, in the presence or absence of light. **a** Heterogeneous callus obtained from cotyledons cultured on medium containing 22.6 μM 2,4-D, in the presence of light, **b** Mucilaginous callus obtained from cotyledons in response to 12.4 μM PIC, in the dark, **c** compact organogenic callus derived from embryonic axes cultured in the presence of 4.5 μM

TDZ, in the presence of light, **d** heterogeneous organogenic callus derived from embryonic axes in response to 13.2 μM BA and 2.7 μM NAA, in the presence of light, **e** heterogeneous callus derived from embryonic axes in response to 4.4 μM BA and 10.8 μM NAA, in the absence of light, **f** Friable callus obtained from immature leaflets cultured on medium supplemented with 17.6 μM BA and 5.4 μM NAA, and maintained in the dark. Bars = 1 cm

axes were obtained in the dark in response to 12.42 μM or 28.98 μM PIC, respectively (Table 1).

Cotyledons and embryonic axes cultured on media containing TDZ originated compact and heterogeneous calluses in the absence of light. Higher TDZ concentrations induced the formation of organogenic calluses in the presence of light, but with oxidation of the original explants, which became dark on the surface (Fig. 1c). Embryonic leaflets produced only non-morphogenic heterogeneous calluses in both light and dark conditions. Highest callogenesis frequencies from all explants were observed in response to 45.4 μM TDZ (Table 1).

Combinations of BA and NAA induced different frequencies of callus according to explant, light conditions and BA/NAA ratio (Table 2). Cotyledons and embryonic axes gave rise mainly to heterogeneous calluses, containing friable and compact regions that originated shoots in response to almost all combinations, especially those containing high ratios of BA/NAA (Fig. 1d). Media

containing low BA/NAA ratios, especially 4.4 μM BA in combination with 10.8–21.6 μM NAA, induced the formation of increased friable areas, which could be easily separated (Fig. 1e). When 0.44 μM BA was used in association with the same NAA concentrations, cotyledons showed the development of adventitious roots with minor callus formation. Immature leaflets originated only non-morphogenic friable calluses, except in response to low ratios BA/NAA (0.44–2.2 μM BA plus 10.8–21.6 μM NAA), which resulted in the formation of heterogeneous calluses. Friable calluses were obtained from embryonic leaflets in response to high BA/NAA ratio (17.6 μM BA plus 0.54–5.4 μM NAA) and incubation in the dark (Fig. 1f).

The friable regions of calluses derived from embryonic axes and cotyledons showed high biomass accumulation in response to the combination of 4.4 μM BA and 10.8 μM NAA (0.251 ± 0.03 and 0.209 ± 0.02 g DW, respectively). On the other hand, calluses derived from immature leaflets

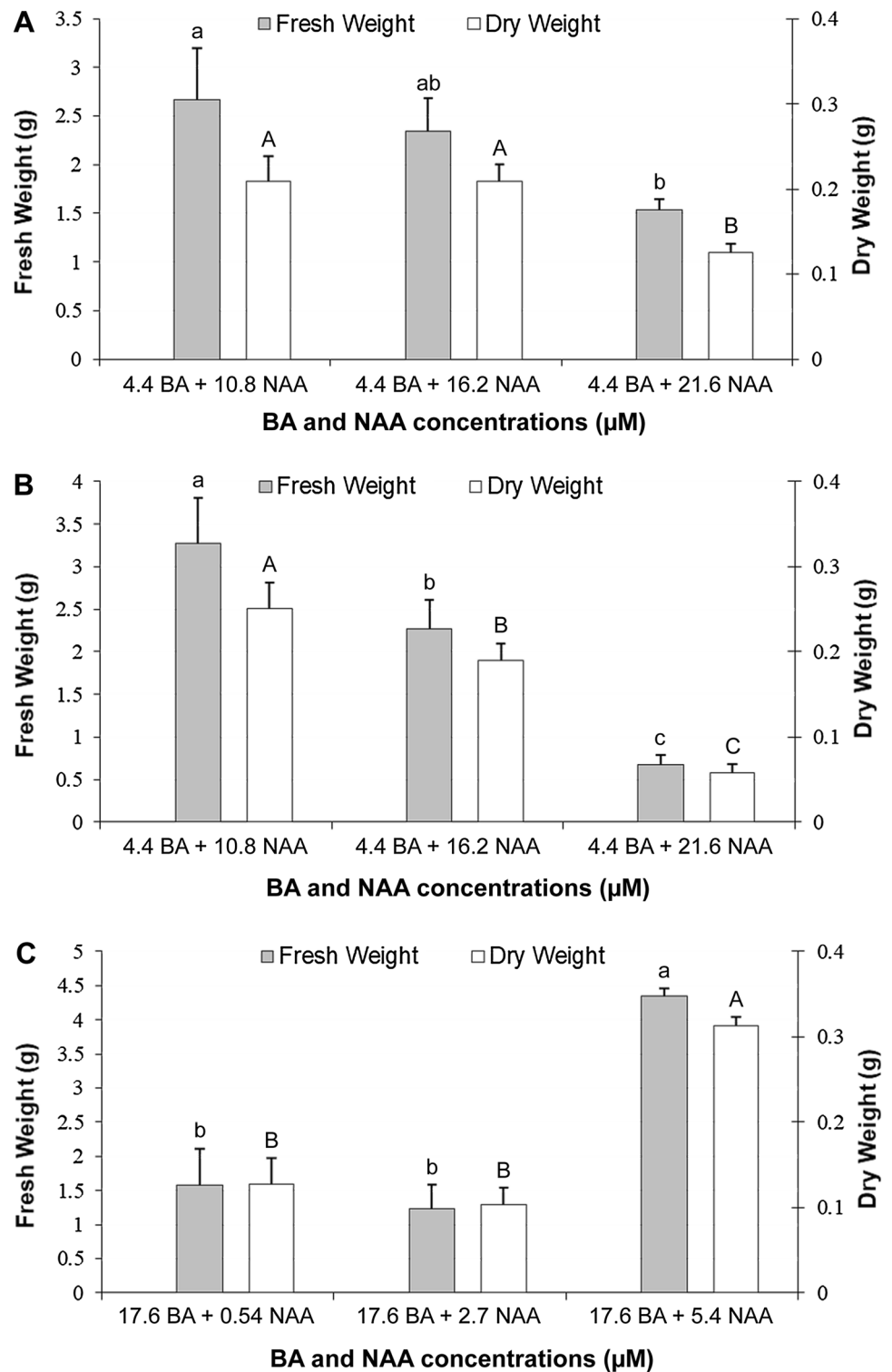
Table 1 Callus formation from seed explants of *Arachis pintoii* in response to PIC, 2,4-D or TDZ in the presence or absence of light

Plant growth regulators (μM)	Callus induction (%)					
	Cotyledons		Embryonic leaflets		Embryonic axes	
	Light	Dark	Light	Dark	Light	Dark
2,4-D						
4.52	–	15	–	–	–	–
13.56	25	40	–	–	15	20
22.6	25	20	–	–	10	10
31.64	5	25	–	–	20	10
45.2	–	10	–	–	–	10
PIC						
4.14	45	70	–	5	35	30
12.42	35	90	10	10	15	20
20.7	45	50	–	20	10	50
28.98	50	60	–	55	10	70
41.4	40	40	–	70	5	60
TDZ						
4.54	–	–	10	20	30	10
13.62	–	–	–	–	10	20
22.7	10	70	–	10	50	60
31.78	50	60	–	20	70	50
45.4	50	80	–	30	40	80

Table 2 Callus formation from seed explants of *Arachis pintoii* in response to combinations of BA and NAA in the presence or absence of light

Plant growth regulators (μM)		Callus induction (%)					
		Cotyledons		Embryonic leaflets		Embryonic axes	
BA	NAA	Light	Dark	Light	Dark	Light	Dark
0.44	10.8	50	–	10	66.6	20	50
0.44	16.2	50	40	100	100	60	70
0.44	21.6	50	50	100	33.3	50	80
2.2	10.8	57.1	60	66.6	66.6	50	70
2.2	16.2	20	50	33.3	33.3	40	60
2.2	21.6	40	30	33.3	100	90	90
4.4	10.8	30	30	50	33.3	70	70
4.4	16.2	50	50	100	66.6	70	60
4.4	21.6	50	60	66.6	33.3	50	80
8.8	0.54	37.5	80	33.3	33.3	80	42.8
13.2	0.54	50	28.6	33.3	50	71.4	70
17.6	0.54	62.5	100	33.3	100	42.8	50
8.8	2.7	50	60	66.6	33.3	71.4	71.4
13.2	2.7	40	80	100	66.6	70	70
17.6	2.7	80	100	66.6	100	50	60
8.8	5.4	60	60	66.6	100	33.3	100
13.2	5.4	60	100	33.3	100	70	57
17.6	5.4	80	100	66.6	100	71.4	57

Fig. 2 Biomass accumulation (g) of *A. pintoii* calluses obtained in response to MS medium supplemented with combinations of BA and NAA, after 60 days in the dark. **a** Cotyledons, **b** embryonic axes and **c** immature leaflets. Different letters within graphs indicate statistically significant differences in the fresh weight (lower case) or dry weight (upper case) values (Tukey test, $p \leq 0.05$)



presented higher accumulation (0.313 ± 0.03 g DW) in response to $5.4 \mu\text{M}$ NAA plus $17.6 \mu\text{M}$ BA (Fig. 2). These calluses were chosen for phytochemical analysis, considering the possibility of their use for establishing cell suspension cultures.

Phenols and *trans*-resveratrol contents, and antioxidant potential of seeds and calluses

Extracts from friable calluses showed 10× higher yields (379.76 – 436.13 mg/g of DW) than those obtained from seeds (39.1 – 49.35 mg/g of DW). In contrast, total phenolic

Table 3 Total phenolic and *trans*-resveratrol contents, and antioxidant activity of extracts from seeds and calluses of *A. pintoii*

Sample	Yield (mg/g DW)	TPC (mg GAE/g FW)	<i>Trans</i> -resveratrol ($\mu\text{g/g FW}$)	Antioxidant activity EC_{50} (mg/mL)
Seeds with testa	39.10	1.27 ± 0.15^b	ND	11.75 ± 1.45^a
Seeds without testa	39.29	1.37 ± 0.20^b	ND	30.11 ± 1.59^b
Cotyledons	49.35	1.68 ± 0.20^a	ND	28.26 ± 1.80^b
Cotyledon callus	379.76	0.53 ± 0.02^c	8.37 ± 0.23^a	13.90 ± 1.36^a
Embryo axes callus	406.65	0.57 ± 0.04^c	0.62 ± 0.02^b	14.32 ± 0.62^a
Embryo leaflet callus	436.13	0.39 ± 0.04^d	0.69 ± 0.00^b	37.78 ± 2.37^c

Data expressed as mean \pm SE. Different letters indicate data statistically different within columns (Tukey test, $p \leq 0.05$)

ND: not detected

contents (TPC) was higher in seeds extracts, and the value obtained for calluses derived from cotyledons was 3 \times lower than that obtained from the original explant (Table 3). Extracts from calluses derived from embryonic axes and cotyledons displayed similar TPC (0.57 ± 0.04 and 0.53 ± 0.02 mg GAE/g FW, respectively), whereas those obtained from embryonic leaflets showed a significant lower value (0.39 ± 0.04 mg GAE/g FW) (Table 3).

Trans-resveratrol was only detected in extracts from calluses (Fig. 3), and those derived from cotyledons showed more than 10 \times the content exhibited by calluses derived from the other seed explants (Table 3). In the qualitative analysis (total run time = 90 min), chromatograms of callus extracts were complex and rich in compounds of medium polarity (tR = 10–20 min), among which signals characteristic of

stilbenoids ($\lambda_{\text{max}} = 306$ nm) were observed at 20 min. Chromatograms of callus extracts were similar to those obtained from seeds. These extracts displayed polar, medium polarity and apolar compounds and the majority of stilbens was found in tR around 7–10 min (Fig. 4).

The highest antioxidant activity was obtained in the extracts of seeds with testa. This activity was almost 3 \times higher than the values showed by extracts from seeds without testa or cotyledons. Also, the activity of extracts from calluses derived from cotyledons and embryonic axes was higher than that displayed by calluses originated from embryonic leaflets. No statistical difference was observed among the EC_{50} values of the extracts from the different calluses (Table 3).

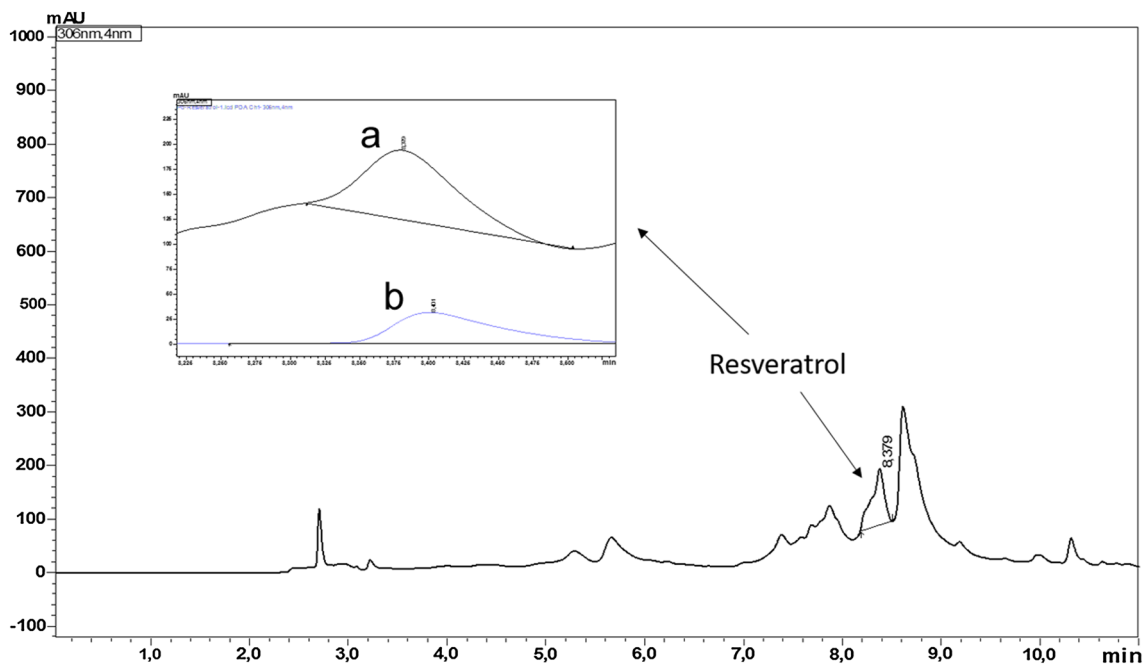


Fig. 3 Chromatogram of extract from cotyledon calluses (total run time = 11 min) with expansion of the *trans*-resveratrol peak (tR = 8.4 min): *a* sample, *b* standard

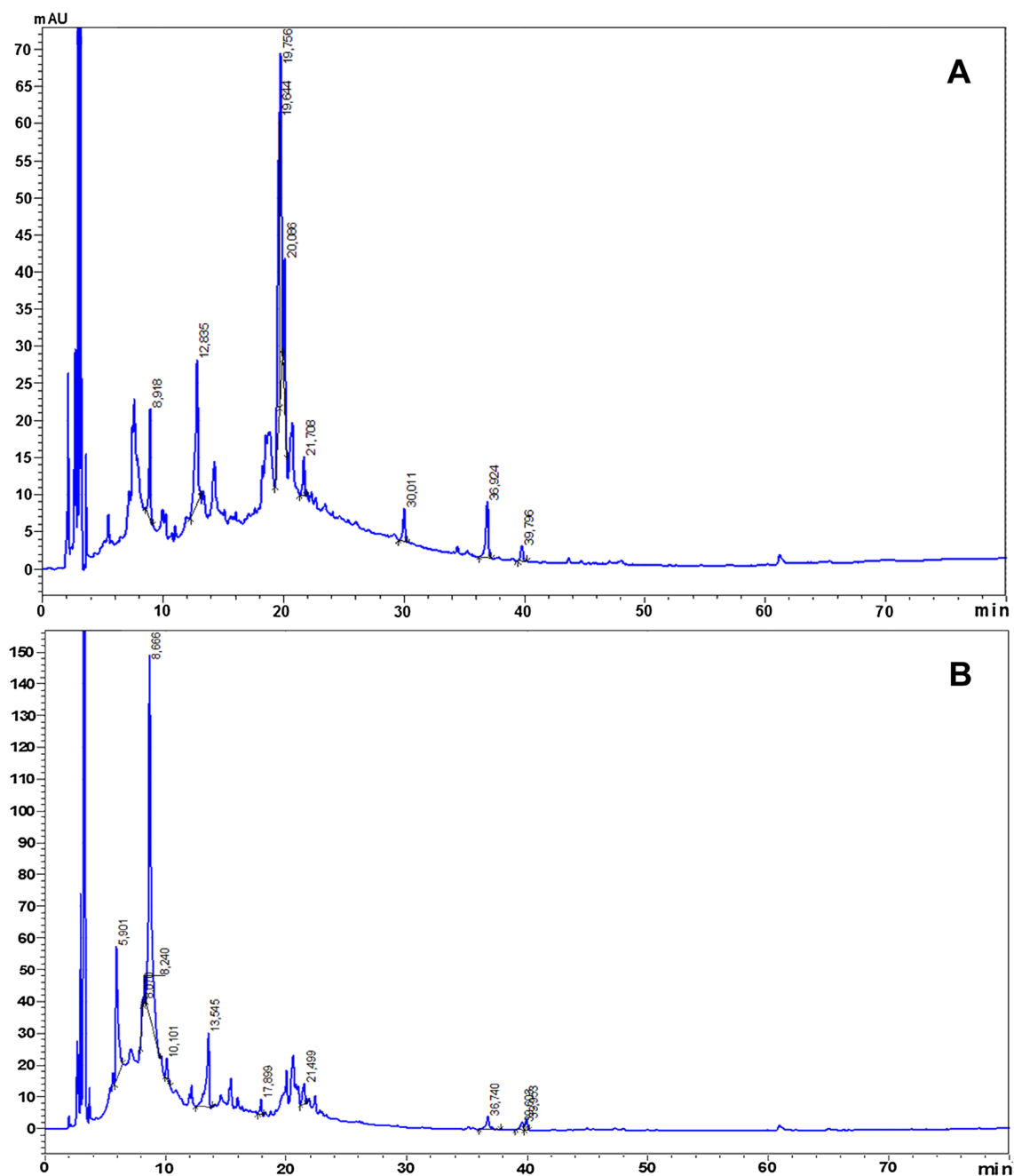


Fig. 4 Chromatograms (total run time=90 min, $\lambda_{\text{máx}} = 306$ nm) of extracts from **a** calluses derived from cotyledons and **b** seeds with testa

Discussion

Peanuts have been recognized as rich sources of stilbenes, including *trans*-resveratrol, which is associated to numerous human health benefits (Aune et al. 2017; Mallikarjuna et al. 2016). Phytochemical studies mainly performed in *Arachis hypogaea* have demonstrated the production of *trans*-resveratrol and other stilbenes by different organs of plants grown in natural conditions (Holland et al. 2011; Zorzete et al. 2011; Marka et al. 2013; Aljuhaimi and Ozcan 2018),

Agrobacterium-transformed roots (Medina-Bolívar et al. 2007; Kim et al. 2008; Condori et al. 2010; Halder and Jha 2016), and calluses (Ku et al. 2005; Yang et al. 2010a, b). However, similar investigations are scarce for other cultivated species of the genus.

In the present work, we studied callus culture systems derived from *A. pintoi* seed explants as influenced by explant type, PGR and light condition, in order to evaluate the production of stilbenoids and other compounds of pharmacological interest. Seeds proved to be an excellent source of

explants, with the advantages of great availability and rapid regenerative response. Calluses with different morphogenic potentials, colors and consistencies were obtained according to the culture conditions.

Auxins and cytokinins are especially important for biomass accumulation and secondary metabolites production in calluses, and can be used alone or in combination (Coimbra et al. 2017). Among the auxins evaluated here, 2,4-D was entirely ineffective in inducing morphogenic responses from embryonic leaflets, and produced low callogenesis rates on the other explant types tested. Although 2,4-D has been used for callus induction and shoot regeneration in other *Arachis* species (Srinivasan et al. 2010), it seemed to be less efficient than combinations of NAA and BA in leaf explants excised from plants of *A. pintoii* grown in natural conditions (Rey et al. 2000).

In the present work, seed explants cultivated in the presence of PIC gave rise to mucilaginous calluses with globular regions that did not display further development, in contrast to the induction of somatic embryogenesis from leaf explants reported previously (Rey et al. 2000; Rey and Mroginski 2006). On the other hand, organogenic calluses obtained in response to TDZ became oxidized, impairing the development of shoots. Similar results were reported for leaves of *A. hypogaea*, *A. correntina* (Mroginski et al. 2004), and *A. villosa* (Fontana et al. 2009). In addition, cotyledonary nodes of *A. hypogaea* showed the development of morphologically abnormal shoots in response to high concentrations of TDZ (Hsieh et al. 2017).

BA and other cytokinins were found to influence the content of phenolic compounds, as well as the antioxidant and antimicrobial activities of *in vitro*-grown materials (Baskaran et al. 2014; Castro et al. 2016; Karalija et al. 2017). Regarding media supplemented with combinations of BA and NAA tested here, the use of a higher BA concentration (4.4 μM) in combination with 10.8–21.6 μM NAA for cotyledons and embryonic axes explants cultured in the absence of light resulted in the formation of heterogeneous calluses with a large friable region. On the other hand, embryonic leaflets showed different requirements of PGRs when compared to the other types of explant. In contrast to the friable masses obtained from cotyledons and embryonic axes in response to low ratios BA/NAA, these explants produced entirely friable calluses in response to high ratios BA/NAA. The balance of these PGRs was studied by other authors, who found results similar to those obtained with cotyledons and embryonic axes, when using mature leaflet explants of *A. pintoii* (Burtnik and Mroginski 1985; Rey et al. 2000). This difference can be associated to the specificities of the tissues, particularly regarding the internal amount of PGRs in the explants.

Light is other important factor for callus induction, cell growth, and secondary metabolites production. Hence, it is

important to carefully investigate the influence of PGRs and light conditions, since these two parameters can affect callus growth rate, color and consistency (Coimbra et al. 2017; Mohlakola et al. 2017). In our work, although PGRs played a major role in callogenesis, light significantly influenced the phenotypic aspect of the calli, which were more friable and pale when grown in the dark. These differences were already reported for other species such as *Pyrostegia venusta* (Bignoniaceae), whose calluses were compact and green in the presence of light, and yellow in the dark (Coimbra et al. 2017). Olgunsoy et al. (2017) also observed that friable calluses obtained from *Rosa damascena* Mill. petals in the presence of light became harder in texture and produced green and pink pigmentation. These authors also compared the production of volatile compounds in the absence and presence of light, demonstrating that dark treatments were superior in terms of tocopherol production and that monoterpenoid α/β -pinene synthesis was significantly higher in the presence of light.

Considering the potential of friable calluses for the establishment of industrial systems, friable masses originated from cotyledons, embryonic axes and leaflets in response to the combinations of BA/NAA cited above were selected for phytochemical analysis. Whereas the presence of *trans*-resveratrol was not detected in seeds, calluses derived from seed explants presented amounts ranging between 0.62 ± 0.02 to 8.37 ± 0.23 $\mu\text{g/g}$ FW. Despite this difference, seed and cotyledon extracts displayed higher values of TPC when compared with calluses extracts. The low content or even the absence of *trans*-resveratrol in non-treated seeds of *A. hypogaea* was reported by some authors, who detected other polar compounds, such as bound and free phenolic acids (Wang et al. 2005; Sobolev 2013). Additionally, previous works using *in vitro* systems (Ku et al. 2005; Yang et al. 2010a, b; Xu et al. 2015; Pilaisangsuree et al. 2018) found *trans*-resveratrol production only with elicitation. This strategy has been widely used for secondary metabolites production *in vitro* using various elicitors, such as methyl jasmonate (MeJA), salicylic acid (SA), H_2O_2 , sodium acetate (NaOAc), cyclodextrin (CD), and ultraviolet irradiation (UV) of B and C types (Tyunin and Kiselev 2016; Pilaisangsuree et al. 2018).

The values found for the *trans*-resveratrol content vary widely among the different *in vitro* systems established for *A. hypogaea*. Calluses originated from stem segments produced between 0.25 and 11.97 $\mu\text{g/g}$ FW of *trans*-resveratrol in response to ultraviolet (UV) irradiation (Ku et al. 2005). Yang et al. (2010a) obtained values up to 58.84 ± 21.16 $\mu\text{g/g}$ FW in hypocotyl-derived calluses elicited by sterilized fungal spores, while Yang et al. (2010b) reported contents between 1.77 and 2.72 and 5.86 to 10.43 $\mu\text{g/g}$ FW, using bacteria and chitin as elicitors, respectively. An important aspect of our work is that calluses obtained from *A. pintoii*

cotyledons were able to produce *trans*-resveratrol up to $8.37 \pm 0.23 \mu\text{g/g}$ FW without any treatment.

The antioxidant activity observed in seed testa extracts of *A. hypogaea* has been associated to the presence of phenolic compounds, such as chlorogenic acid, caffeic acid, coumaric acid, ferulic acid, flavonoids and stilbenes (Yu et al. 2005; Attree et al. 2015), quinic acid (Francisco and Resurrección 2009), catechin, procyanidins and proanthocyanidins (Ma et al. 2014). However, in the present work, TPC could not be correlated to the EC₅₀ values observed in DPPH assays, since extracts from seeds with testa showed the lowest TPC, in spite of presenting the highest antioxidant activity. Similarly, Sang et al. (2014) observed a negative correlation between EC₅₀ and TPC values of leaves from different legumes, including *A. pintoi*. These results could be probably related to the presence of other phenolics without antioxidant capacity. In addition, qualitative analyses evidenced different groups of stilbenoids and other polar compounds that could be associated to the antioxidant activity.

In conclusion, the use of seed explants of *A. pintoi* provided different callus lines that can be useful for in vitro production of secondary metabolites. Large friable masses potentially useful for establishing cell culture systems were obtained in the absence of light, in response to different BA and NAA ratios. Our results also revealed the ability of calluses to produce *trans*-resveratrol and other stilbenes, which were not found in seeds. Considering that elicitation is an efficient strategy to increase secondary metabolites production in vitro, as discussed above, further studies will be undertaken to improve the synthesis of these compounds in callus systems developed from *A. pintoi* seeds with the use of biotic and abiotic elicitors.

Acknowledgements The authors acknowledge the Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) for financial support. Isabela B. de Sousa-Machado is a recipient of a scholarship from the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). E. Mansur is a recipient of a research fellowship from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

References

- Adjolohoun S, Bindelle J, Adandedjan C, Toleba SS, Nonfon WR, Sinsin B (2013) Reproductive phenology stages and their contributions to seed production of two *Arachis pintoi* ecotypes (CIAT 17434 and CIAT 18744) in Sudanian savanna region of Benin, West Africa. *Agric Sci Res J* 3(6):152–157
- Aljuhaimi F, Özcan MM (2018) Influence of oven and microwave roasting on bioproperties, phenolic compounds, fatty acid composition, and mineral contents of nongerminated peanut and germinated peanut kernel and oils. *J Food Process Preserv* 42(2):e13462
- Attree R, Du B, Xu B (2015) Distribution of phenolic compounds in seed coat and cotyledon, and their contribution to antioxidant capacities of red and black seed coat peanuts (*Arachis hypogaea* L.). *Ind Crops Prod* 67:448–456. <https://doi.org/10.1016/j.indcrop.2015.01.080>
- Aune D, Giovannucci E, Boffetta P, Fadnes LT, Keum NN, Norat T, Greenwood DC, Riboli E, Vatten LJ, Tonstad S (2017) Fruit and vegetable intake and the risk of cardiovascular disease, total cancer and all-cause mortality—a systematic review and dose-response meta-analysis of prospective studies. *Int J Epidemiol* 46(3):1029–1056. <https://doi.org/10.1093/ije/dyw319>
- Baskaran P, Moyo M, Van Staden J (2014) *In vitro* plant regeneration, phenolic compound production and pharmacological activities of *Coleonema pulchellum*. *S Afr J Bot* 90:74–79
- Brand-Williams W, Cuvelier ME, Berset C (1995) Use of a free radical method to evaluate antioxidant activity. *LWT Food Sci Technol* 28:25–30. [https://doi.org/10.1016/S0023-6438\(95\)80008-5](https://doi.org/10.1016/S0023-6438(95)80008-5)
- Bresolin APS, Castro CM, Herter FG, Oliveira AC de, Carvalho FIF de, Pereira FB, Vieira CL, Bertoli RF (2008) Tolerância ao frio do amendoim forrageiro. *Ciência Rural* 38:1154–1157. <https://doi.org/10.1590/S0103-84782008000400041>
- Burtnik O, Mroginski L (1985) Regeneración de plantas de *Arachis pintoi* (Leguminosae) por cultivo in vitro de tejidos foliares. *Oleagineux* 40:609–611
- Carvalho MA, Quesenberry KH (2009) Morphological characterization of the USA *Arachis pintoi* Krap. and Greg. collection. *Plant Syst Evol* 277:1–11. <https://doi.org/10.1007/s00606-008-0089-9>
- Casimiro GS, Mansur E, Pacheco G, Garcia R, Leal ICR, Simas NK (2017) Allelopathic activity of extracts from different Brazilian Peanut (*Arachis hypogaea* L.) cultivars on lettuce (*Lactuca sativa*) and weed plants. *Sci World J* 2017:1–7. <https://doi.org/10.1155/2017/2796983>
- Castro AHF, Braga KDQ, Sousa FMD, Coimbra MC, Chagas RCR (2016) Callus induction and bioactive phenolic compounds production from *Byrsonima verbascifolia* (L.) DC. (Malpighiaceae). *Rev Cienc Agron* 47(1):143–151
- Coimbra MC, César R, Chagas R (2017) Influence of plant growth regulators and light on callus induction and bioactive phenolic compounds production in *Pyrostegia venusta* (Bignoniaceae). *Indian J Exp Biol* 55:584–590
- Condori J, Sivakumar G, Hubstenberger J, Dolan MC, Sobolev VS, Medina-Bolivar F (2010) Induced biosynthesis of resveratrol and the prenylated stilbenoids arachidin-1 and arachidin-3 in hairy root cultures of peanut: Effects of culture medium and growth stage. *Plant Physiol Biochem* 48:310–318. <https://doi.org/10.1016/j.plaphy.2010.01.008>
- Fontana ML, Mroginski LA, Rey HY (2009) Organogenesis and plant regeneration of *Arachis villosa* Benth. (Leguminosae) through leaf culture. *Biocell* 33:179–186
- Francisco ML de, Resurrección AVA (2009) Development of a reversed-phase high performance liquid chromatography (RP-HPLC) procedure for the simultaneous determination of phenolic compounds in peanut skin extracts. *Food Chem* 117:356–363. <https://doi.org/10.1016/j.foodchem.2009.03.110>
- Garcia L, Garcia R, Pacheco G, Sutili F, Souza R de, Mansur E, Leal I (2016) Optimized extraction of Resveratrol from *Arachis repens* Handr. by ultrasound and microwave: a correlation study with the antioxidant properties and phenol contents. *Sci World J* 2016:1–10. <https://doi.org/10.1155/2016/5890897>
- Halder M, Jha S (2016) Enhanced *trans*-resveratrol production in genetically transformed root cultures of Peanut (*Arachis hypogaea* L.). *Plant Cell Tiss Organ Cult* 124:555–572
- Holland KW, Balota M, Eigel WN III, Mallikarjunan P, Tanko JM, Zhou K, O’Keefe SF (2011) ORAChromatography and total phenolics content of peanut root extracts. *J Food Sci* 76:380–384. <https://doi.org/10.1111/j.1750-3841.2011.02069.x>
- Hsieh YF, Jain M, Wang J, Gallo M (2017) Direct organogenesis from cotyledonary node explants suitable for

- Agrobacterium*-mediated transformation in peanut (*Arachis hypogaea* L.). Plant Cell Tiss Organ Cult 128:161–175
- Iqbal MM, Nazir F, Iqbal J, Tehrim S, Zafar Y (2011) *In vitro* micropropagation of peanut (*Arachis hypogaea*) through direct somatic embryogenesis and callus culture. Int J Agric Biol 13:811–814
- Karalija E, Čavar Zeljković S, Tarkowski P, Muratović E, Parić A (2017) The effect of cytokinins on growth, phenolics, antioxidant and antimicrobial potential in liquid agitated shoot cultures of *Knautia sarajevensis*. Plant Cell Tiss Organ Cult 131:347–357
- Kim JS, Lee SY, Park SU (2008) Resveratrol production in hairy root culture of peanut, *Arachis hypogaea* L. transformed with different *Agrobacterium rhizogenes* strains. African J Biotechnol 7:3788–3790. <https://doi.org/10.5897/AJB08.499>
- Krapovickas A, Gregory WC (1994) Taxonomia del genero *Arachis*. (Leguminosae) Bonplandia. Taxon 8:1–179
- Ku KL, Chang PS, Cheng YAC, Lien CYI (2005) Production of stilbenoids from the callus of *Arachis hypogaea*: A novel source of the anticancer compound piceatannol. J Agric Food Chem 53:3877–3881. <https://doi.org/10.1021/jf050242o>
- Ladeira MM, Rodriguez NM, Borges I, Gonçalves LC, de Oliveira Simões Saliba E, Corrêa Brito S, Pinto de Sá LA (2002) Avaliação do feno de *Arachis pintoi* utilizando o ensaio de digestibilidade in vivo. Rev Bras Zootec 31:2350–2356. <https://doi.org/10.1590/S1516-35982002000900025>
- Lopes RM, Agostini-Costa T, da S, Gimenes, Silveira MA D (2011) Chemical composition and biological activities of *Arachis* species. J Agric Food Chem 59:4321–4330. <https://doi.org/10.1021/jf104663z>
- Lopes RM, Silveira D, Gimenes MA, Vasconcelos PAS, de Alves RBN, Silva JP, da Agostini-Costa TS (2013) Characterization of resveratrol content in ten wild species of section *Arachis*, genus *Arachis*. Genet Resour Crop Evol 60:2219–2226. <https://doi.org/10.1007/s10722-013-9987-y>
- Ma Y, Kosinska-Cagnazzo A, Kerr WL, Amarowicz R, Swanson RB, Pegg RB (2014) Separation and characterization of phenolic compounds from dry-blanched peanut skins by liquid chromatography-electrospray ionization mass spectrometry. J Chromatogr A 1356:64–81. <https://doi.org/10.1016/j.chroma.2014.06.027>
- Mallikarjuna G, Rao TSRB., Kirti PB (2016) Genetic Engineering for Peanut Improvement: Current Status and Prospects. Plant Cell Tiss Organ Cult 125:399–416
- Marka R, Talari S, Penchala S, Rudroju S, Nann RS (2013) Preliminary phytochemical analysis of leaf, stem, root and seed extracts of *Arachis hypogaea* L. Int J Pharm Sci Rev Res 20(1):134–139
- Matand K, Wu N, Wu H, Tucker E, Love K (2013) More improved peanut (*Arachis hypogaea* L.) protocol for direct shoot organogenesis in mature dry-cotyledonary and root tissues. J Biotech Res 5:24–34
- Medina-Bolivar F, Condori J, Rimando AM, Hubstenberger J, Shelton K, O'Keefe SF, Bennett S, Dolan MC (2007) Production and secretion of resveratrol in hairy root cultures of peanut. Phytochemistry 68:1992–2003. <https://doi.org/10.1016/j.phytochem.2007.04.039>
- Mohlakola EM, Cheng C, Lin Y, Guo R, Min KT, Chen Y, Lai Z (2017) Effects of 2,4-dichlorophenoxy acetic acid and light on growth of gerbera (*Gerbera jamesonii* cv. Daxueju) callus. J Agr Sci Tech 18(3):385–393
- Mroginski E, Rey HY, Gonzalez AM, Mroginski LA (2004) Thidiazuron promotes in vitro plant regeneration of *Arachis correntina* (Leguminosae) via organogenesis. J Plant Growth Regul 23:129–134. <https://doi.org/10.1007/s00344-004-0038-y>
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497. <https://doi.org/10.1111/j.1399-3054.1962.tb08052.x>
- Olgunsoy P, Ulusoy S, Akçay U (2017) Metabolite production and antibacterial activities of callus cultures from *Rosa damascena* Mill. petals. J Res Pharm 21(3):590–597. <https://doi.org/10.12991/marupj.319331>
- Pacheco G, Gagliardi RF, Carneiro LA, Callado CH, Valls JFM, Mansur E (2007) The role of BAP in somatic embryogenesis induction from seed explants of *Arachis* species from Sections *Erectoides* and *Procumbentes*. Plant Cell Tissue Organ Cult 88:121–126. <https://doi.org/10.1007/s11240-006-9169-0>
- Pacheco G, Gagliardi RF, Valls JFM, Mansur E (2009) Micropropagation and in vitro conservation of wild *Arachis* species. Plant Cell Tiss Organ Cult 99:239–249. <https://doi.org/10.1007/s11240-009-9599-6>
- Pilaisangsuree V, Somboon T, Tonglairoum P, Keawracha P, Wongsat T, Kongbangkerd A, Limmongkon A (2018) Enhancement of stilbene compounds and anti-inflammatory activity of methyl jasmonate and cyclodextrin elicited peanut hairy root culture. Plant Cell Tiss Organ Cult 132:165–179
- Pittman RN, Banks DJ, Kirby JS, Mitchell ED, Richardson PE (1983) *In vitro* culture of immature peanut (*Arachis* spp.) leaves: Morphogenesis and plantlet regeneration. Peanut Sci 10:21–25. <https://doi.org/10.3146/i0095-3679-10-1-7>
- Poeaim A, Poeaim S, Pongtongkam P (2015) Callus induction and cell suspension cultures of Rhizome Peanut (*Arachis glabrata*) cultivars: arbrook. J Agric Technol 11(8):2481–2488
- Rey HY, Mroginski LA (2003) Regeneration of plants from apical meristem tips and nodal segments of *Arachis pintoi*. Peanut Sci 30:75–79
- Rey HY, Mroginski LA (2006) Somatic embryogenesis and plant regeneration in diploid and triploid *Arachis pintoi*. Biol Plant 50:152–155. <https://doi.org/10.1007/s10535-005-0093-7>
- Rey HY, Scocchi AM, Gonzalez AM, Mroginski LA (2000) Plant regeneration in *Arachis pintoi* (Leguminosae) through leaf culture. Plant Cell Rep 19:856–862. <https://doi.org/10.1007/s00290000198>
- Sang SY, Jamharee F, Prasad KN, Azlan A, Maliki N (2014) Influence of drying treatments on antioxidant capacity of forage legume leaves. J Food Sci Technol 51:988–993
- Santana SH, Valls JFM (2015) *Arachis veigae* (Fabaceae), the most dispersed wild species of the genus, and yet taxonomically overlooked. Bonplandia 24:139–150
- Silva SC, Martins MIG, Santos RC, Peñaloza APS, Filho PAM, Benko-Iseppon AM, Valls JFM, Carvalho R (2010) Karyological features and banding patterns in *Arachis* species belonging to the *Heterantheae* section. Plant Syst Evol 285:201–207. <https://doi.org/10.1007/s00606-010-0278-1>
- Sobolev VS (2013) Production of phytoalexins in peanut (*Arachis hypogaea*) seed elicited by selected microorganisms. J Agric Food Chem 61:1850–1858. <https://doi.org/10.1021/jf3054752>
- Srinivasan T, Kumar KRR, Kirti PB (2010) Establishment of efficient and rapid regeneration system for some diploid wild species of *Arachis*. Plant Cell Tiss Organ Cult 101:303–309. <https://doi.org/10.1007/s11240-010-9689-5>
- Tyunin AP, Kiselev KV (2016) Alternations in VaSTS gene cytosine methylation and *t*-resveratrol production in response to UV-C irradiation in *Vitis amurensis* Rupr. Cells. Plant Cell Tiss Organ Cult 124:33–45. <https://doi.org/10.1007/s11240-015-0872-6>
- Wang KH, Lai YH, Chang JC, Ko TF, Shyu SL, Chiou RYY (2005) Germination of peanut kernels to enhance resveratrol biosynthesis and prepare sprouts as a functional vegetable. J Agric Food Chem 53(2):242–246. <https://doi.org/10.1021/jf048804b>
- Xu A, Zhan J-C, Huang W-D (2015) Effects of ultraviolet C, methyl jasmonate and salicylic acid, alone or in combination, on stilbene biosynthesis in cell suspension cultures of *Vitis vinifera* L. cv. Cabernet Sauvignon. Plant Cell Tiss Organ Cult 122:197–211
- Yang MH, Kuo CH, Hsieh WC, Ku KL (2010a) Investigation of microbial elicitation of *trans*-resveratrol and *trans*-piceatannol in peanut

- callus led to the application of chitin as a potential elicitor. *J Agric Food Chem* 58:9537–9541. <https://doi.org/10.1021/jf1022725>
- Yang MH, Lin YJ, Kuo CH, Ku KL (2010b) Medicinal mushroom *Ganoderma lucidum* as a potent elicitor in production of *t*-resveratrol and *t*-piceatannol in peanut calluses. *J Agric Food Chem* 58:9518–9522. <https://doi.org/10.1021/jf102107p>
- Yu J, Ahmedna M, Goktepe I (2005) Effects of processing methods and extraction solvents on concentration and antioxidant activity of peanut skin phenolics. *Food Chem* 90:199–206. <https://doi.org/10.1016/j.foodchem.2004.03.048>
- Zorzete P, Reis TA, Felício JD, Baquião AC, Makimoto P, Corrêa B (2011) Fungi, mycotoxins and phytoalexin in peanut varieties, during plant growth in the field. *Food chem* 129(3):957–964