RESEARCH ARTICLE

Characterization of resveratrol content in ten wild species of section *Arachis*, genus *Arachis*

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Abstract Peanut is one of the few plants that synthesizes resveratrol, a phenolic compound of the stilbene class, which has been associated with reduced risk of developing chronic diseases, such as cancer, cardiovascular diseases, skin diseases, pulmonary diseases, diabetes and neurological diseases. Resveratrol was detected in different parts of the peanut plant, including roots, leaves, seeds and their derivatives. The wild species of the *Arachis* section are also strong candidates to synthesize resveratrol because they are phylogenetically closely related to cultivated peanut. Our objective was to characterize the resveratrol content in ten wild species of *Arachis* with three different genomes (A, B and K). The plant material was composed of leaves of the ten species treated (test) and not treated (control) with ultraviolet (UV) radiation. The test and control samples were extracted and the identification and quantification of resveratrol was performed using high performance liquid chromatography (HPLC). All species studied synthesized resveratrol and the concentrations ranged from 299.5 µg/g in *A. kempff-mercadoi* to 819.9 µg/g in *A. cardenasii*. DPPH antioxidant activity varied between 18.7 % for *A. duranensis* and 48.2 % in *A. simpsonii*. The results showed that wild *Arachis* species are a potential source of alleles for improvement of cultivated peanut, with the aim of achieving higher resveratrol content in leaves.

Keywords Antioxidant · Fabaceae · Peanut · Stilbene · Wild *Arachis*

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Introduction

Peanut is one of the few plants that synthesize resveratrol (3, 5, 4′–trihidroxistilbene), a phenolic compound of the stilbene class. This bioactive compound has been associated with reduced risk of developing several chronic diseases, such as cancer, cardiovascular diseases, skin diseases, pulmonary diseases, diabetes and neurological diseases (Harikumar and Aggarwal 2008; Fulda 2010; Lopes et al. 2011).

Sobolev and Cole (1999) found resveratrol content between 0.0 and 0.1 µg/g in products derived from roasted peanut, 0.1–0.5 µg/g in peanut butter, and 0.0–7.9 µg/g in boiled peanuts. Resveratrol was also

detected in the hypocotyls in response to fungal infection (Ingham 1976), in cotyledons in response to mechanical injury (Arora and Strange 1991), in stalks in response to ultraviolet C light or biotic stress (Fritzemeier et al. 1983; Yang et al. 2010), in leaves under biotic and abiotic stresses (Chung et al. 2003), in non-stressed seeds (0.0–2.0 μg/g) (Sanders et al. 2000; Tokusoglu et al. 2005; Chukwumah et al. 2012), in UV stressed seeds (2.1–4.7 μg/g) (Potrebko and Resurreccion 2009) and in roots (0.027–0.950 mg/g) (Chen et al. 2002).

Alternative means to increase the resveratrol content in peanut are the identification of accessions with high levels of this substance, which previous studies demonstrate to be unlikely; the use of transgenic plants, which has not been accepted by consumers, and the identification of wild species of *Arachis* with high levels of resveratrol that can be used in peanut breeding programs.

No study on resveratrol in wild species of *Arachis* has been reported. However, wild *Arachis* species of *Arachis* section are strong candidates to synthesize resveratrol, since they are phylogenetically closely to peanut (Cunha et al. 2008; Bechara et al. 2010). Phylogenetic and chemotaxonomic information has proven to be useful in identifying species that produce compounds from well defined chemical classes and pharmacological activities (Brito 1996; Rates 2001), such as resveratrol.

The section *Arachis*, one of nine taxonomic sections of the genus *Arachis*, comprises *A. hypogaea* and 31 wild species that are very closely related to the cultivated peanut, which is an allotetraploid that originated from a spontaneous cross between two diploid species of A and B genomes (Valls and Simpson 2005; Favero et al. 2006; Krapovickas and Gregory 2007; Cunha et al. 2008; Bechara et al. 2010). According to the evidence, *Arachis duranensis* and *A. ipaënsis* are the donors of the genomes A and B respectively (Favero et al. 2006; Grabiele et al. 2012).

The characterization and evaluation studies on wild *Arachis* species have been accompanied by the development of map populations (Moretzsohn et al. 2005; Moretzsohn et al. 2009), synthetic allopolyploids by crossing between A and B wild species (Mallikarjuna et al. 2011; Nielen et al. 2012) and introgression lines resulting from crosses between allopolyploids and *A. hypogaea*.

The objective of this study was to evaluate the resveratrol level by HPLC in leaves of ten wild species

of *Arachis* (*A. batizocoi*, *A. cardenasii*, *A. cruziana*, *A. duranensis*, *A. gregoryii*, *A. ipaënsis*, *A. kempff-mercadoi*, *A. kuhlmannii*, *A. magna*, and *A. simpsonii*) with four different genomes (A, B, AB and K) before and after exposure to UV light. In addition, the leaf extracts were evaluated for their inhibitory activity against the DPPH free radical.

Experimental

General experimental procedures

Stock solutions of *trans*-resveratrol (99 %, Sigma-Aldrich, St. Louis, MO, USA) were prepared at 230–460 μg/mL and phenolphthalein (98 % Sigma-Aldrich, St. Louis, MO, USA) at 507.4–5,854.0 μg/mL in absolute ethanol and stored at −20 °C for up to 3 months in the absence of oxygen and under protection from light. The 2, 2-diphenyl-1-pic-rylhydrazyl was obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (grade HPLC) and phosphoric acid (86 %) were obtained from J. T. Baker (Phillipsburg, NJ, USA), hexane from Merck (Darmstadt, HE, Germany) and absolute ethanol from Vetec (Rio de Janeiro, RJ, Brazil). HPLC analysis was carried out on a ProStar Varian® system (Mulgrave, VIC, Australia) equipped with ternary pump, autosampler and photodiode array detector (PDA PS-240/PS-410/Galaxie PS-335/Software 1.9). The column used was C18 Zorbax XDB Agilent® (250 × 4.6 mm, 5 mm) (Agilent® Technologies, Santa Clara, CA, USA).

Plant material

Seeds of *A. hypogaea* and ten wild species from the active germoplasm bank of *Arachis* (Table 1) were grown in three experimental blocks in the greenhouse at Embrapa Recursos Genéticos e Biotecnologia (Brasília, Brazil) in October 2010. The cultivated peanut, *A. hypogaea*, was used in this experiment as a reference species.

Resveratrol induction

Leaves of each accession were collected between February and March of 2011 in the afternoon using scissors and randomly divided into two sub-samples (control—no treatment with UV; test—treated with

Table 1 *Arachis* species evaluated for resveratrol content, types of genomes of each species and accession number

Species	Genome	Access
A. batizocoi Krapov. et W. C. Greg.	K	K 9484
A. cardenasii Krapov. et W. C. Greg.	A	GKP 10017
A. cruziana Krapov., W. C. Greg. et C. E. Simpson	K	WI 1302-3
A. duranensis Krapov. et W. C. Greg.	A	K 7988
A. gregoryii C. E. Simpson, Krapov. et Valls	A	V 6389
A. ipaënsis Krapov. et W. C. Greg.	B	KG 30076
A. kempff-mercadoi Krapov., W. C. Greg. et C. E.Simpson	A	V 13250
A. kuhlmannii Krapov. et W. C. Greg.	A	V 9214
A. magna Krapov., W. C. Greg. et C. E. Simpson	B	KG 30097
A. simpsonii Krapov. et W. C. Greg.	B	V 13710
A. hypogaea Linnaeus	AB	Cv Caiapó

UV). Resveratrol induction in leaves of wild *Arachis* was done according to methodology previously described for seeds and leaves of *A. hypogaea* (Potrebko and Resurreccion 2009; Chung et al. 2003). Briefly, leaves of each sub-sample were placed in trays (45 cm × 30 cm), which contained a layer of germitest paper under a layer of approximately 1 cm of cotton moistened with 200 ml of water. The tray with test samples was placed in a laminar flow chamber (Trox®—Curitiba, PR, Brazil—Model FLV series: 235-81) with UV lamps (Philips®—São Paulo, SP, Brazil—TUV 30 W/G30 T8 Longlife) for 2 h and 30 min. The tray of the control sample was maintained at room temperature and under white light for the same time as the test sample. After treatment, both samples remained in the dark for 15 h. Later, the leaflets were detached from the petiole and homogenized within each subgroup. One gram of leaflets was weighed in Falcon tubes, protected with aluminum foil. The samples were frozen at −80 °C, for 5–20 days, until extraction of resveratrol.

Resveratrol analysis

The procedure of extracting resveratrol was carried out with light protection according to Potrebko and Resurreccion (2009), with some changes. Maceration of the leaflets was performed in liquid nitrogen by pulverizing with a glass rod directly in the tube. Ten mL of EtOH 80 % was added to the pulverized material, and extraction was performed in a Polytron homogenizer (Kinematica®, Lucerne, LU, Switzerland) for 2 min at 20,000 rpm. After extraction, the stem of the homogenizer was washed with 2 ml of EtOH 80 % to ensure complete recovery of the extracted material. Two ml of the supernatant of the ethanol extract were mixed to 2 mL of hexane in 4 mL vials. The vial was agitated manually and carefully for the two phases to be mixed efficiently. The vial was left to sit for approximately 1 min to separate the two phases. The upper phase, which was intense green due to the presence of chlorophyll, was separated carefully with a Pasteur pipette and discarded. The process of defatting was repeated and the solvent was evaporated on a hot plate (60 °C) with a nitrogen gas jet (35–40 min). Air was removed with a syringe from the vials containing the dry residue, and vials were covered with parafilm and stored for 24 h at −20° C.

Prior to analysis by high performance liquid chromatography (HPLC), the residue was diluted in 0.7 mL of ethanol 15 %, following (Potrebko and Resurreccion 2009) protocol. The vial, protected by aluminum foil, was agitated manually for 30 s, to loosen the extract completely from the wall, then subjected to ultrasound bath inside a plastic container, where it remained for 4 min. The diluted extract was transferred to Eppendorf microtubes of 1.5 mL and centrifuged for 15 min at 25° C at 18,000 rpm. The supernatant of the centrifuged material was transferred to a 2 mL vial, protected from light and injected into HPLC.

As mobile phase, a gradient of acetonitrile and aqueous phosphoric acid 0.02 % was used: acetonitrile 0 min, 13 %; 6–9 min, 15 %; 17 min, 17 %; 28–33 min, 28 %; 40 min, 50 %; 45 min, 60 %; 46–48 min, 80 %, 49–54 min; 13 %; flow 1.0 mL/min. The UV absorption was monitored at 308 nm, 280 nm and also at the length of maximum absorption (PDA). The injection volume was 10 μL. The resveratrol peak was identified by comparison of retention time with the standard, by the profile of the spectrum provided by PDA and co-eluting with the standard. Moreover, this compound was confirmed by comparison of chromatograms of induced and control samples, with a significant increase occurring at the

peak in the retention time, corresponding to the resveratrol, after induction by UV. Resveratrol was quantified by the addition of phenolphthalein to the extracts and standards, as internal standard, according to (Potrebko and Resurreccion 2009).

Antioxidant activity

The antioxidant activity was evaluated by the DPPH (2, 2-diphenyl-1-picryl-hidrazila) method (Miliauskas et al. 2004). The methanol solution of DPPH 6×10^{-5}M was added to aliquots of the ethanol extract of leaves, the same used for resveratrol analysis, remaining in the dark for 60 min. After reaction, the absorbance reading was performed in a Lambda 25 (Perkin Elmer®, Beaconsfield, Bucks, UK) spectrophotometer at 515 nm using ethanol 80 % as blank.

Data analysis

The mean expression of resveratrol was compared between the control and UV treatments by Kruskal–Wallis test. Then, for the UV treatment, the effect of the three experimental blocks was evaluated for the expression of resveratrol. Once it had been detected that the average expression of resveratrol did not differ between the blocks, three blocks were joined and cluster analysis by unweighted pair group method with arithmetic mean (UPGMA) was applied to group species in accordance with their similarity in expression of resveratrol. Due to asymmetry of data, the genomes A, B, AB and K were compared for their mean expression by Kruskal–Wallis test ($\alpha < 0.05$). For data analysis, the program for statistical language R—version 2.13.2 was adopted, from a free download at the site http://www.r-project.org (R 2012).

Results and discussion

Most studies on chemical composition of wild *Arachis* species have focused on oil composition in seeds (Grosso et al. 2000; Wang et al. 2010). This is the first study on resveratrol in wild species of peanut.

We evaluated leaves of A, B, AB and K genome types species (Table 1) and in all of them resveratrol was detected (Table 2). Genome AB, represented by the allotetraploid *A. hypogaea*, had the highest content

Table 2 Resveratrol content (µg/g) in control and test samples of *Arachis* species

Species	Treatment	
	Control	UV
A. batizocoi	Trace[a]	524.5 ± 131.2[b]
A. cardenasii	29.8 ± 5.2[a]	733.3 ± 135.7[b]
A. cruziana	Trace[a]	640.3 ± 197.3[b]
A. duranensis	Trace[a]	581.3 ± 171.2[b]
A. gregoryii	Trace[a]	370.1 ± 103.8[b]
A. ipaënsis	Trace[a]	314.0 ± 76.8[b]
A. kempff-mercadoi	Trace[a]	299.5 ± 89.1[b]
A. kuhlmannii	35.4 ± 26.8[a]	802.3 ± 180.2[b]
A. magna	23.9 ± 20.3[a]	489.2 ± 158.9[b]
A. simpsonii	Trace[a]	318.4 ± 147.0[b]
A. hypogaea	Trace[a]	687.5 ± 229.0[b]

Each result is mean of three blocks, each block evaluated in three repetitions; means followed by same letters in same line do not differ ($\alpha < 0.05$) according to Kruskal–Wallis test

Table 3 Average content of resveratrol (µg/g) per genome type evaluated

Genome	Mean ± SD
A	565.1 ± 291.9[b]
B	394.2 ± 136.7[c]
AB	687.5 ± 229.0[a]
K	595.0 ± 162.9[b]

Means followed by the same letters in the same column do not differ ($\alpha < 0.05$) according to the Kruskal–Wallis test

of resveratrol, A and K genomes had the second highest level of resveratrol content and B genome had the lowest (Table 3). A larger number of species and individuals per species have to be evaluated to be sure if there is any correlation between genome type and resveratrol content. However, some studies have shown higher levels of phenolic compounds in tetraploid individuals than in diploid individuals of some species (Lu et al. 2006; Sanwal et al. 2010; Caruso et al. 2011).

The resveratrol content of all species increased ($\alpha < 0.05$) in relation to the controls, i.e., compared to samples not submitted to UV light treatment (Table 2). Considering only the test induced samples, the concentrations of resveratrol ranged at 15 h after

UV treatment from 299.5 µg/g in *A. kempff-mercadoi* to 819.9 µg/g in *A. cardenasii* (Table 1), with a mean of 516.9 µg/g in wild species.

The mean concentration of resveratrol in leaves of *A. hypoagea* cv Caiapó was 687.5 µg/g, which represented an average of 229-fold increase in resveratrol in relation to control (Fig. 1). Chung et al. (2003) and Tang et al. (2010) also observed an increase in resveratrol levels in leaves of 225 and 196 fold, respectively, after 2 h of UV exposure and 12 h of rest. The highest levels of resveratrol in *A. hypogaea* in this study may be due to the longer exposure time to UV light (2 h and 30 min) and rest time (15 h), combined with the fact that all leaflets had the same exposure to UV since detached leaves were used instead of whole plants as in other studies (Chung et al. 2003; Tang et al. 2010). In stems of *Vitis vinifera* levels were found close to those seen in this study, at 6.0–490.0 µg/g (Melzoch et al. 2001; Balík et al. 2008). Indeed, in roots of *Polygonum cuspidatum*, the

main source of resveratrol, the levels are much higher, reaching 2,960.0–3,770.0 µg/g (Vastano et al. 2000).

Our results showed that leaves are a suitable material to identify species that synthetize resveratrol. Since many studies on *A. hypogaea* have showed that resveratrol is present in leaves and also in many other tissues (seeds, roots, stalks, hypocotyls, cotyledons) we expect to find resveratrol in other tissues of the ten species in whose leaves we found resveratrol.

Peanut leaves are considered residues or by-products of peanut crops for the production of peanut oil or other purposes and, generally, are underutilized. However, the results obtained show that it is possible to add value to by-products (leaves) derived from the cultivation of peanuts, considering the various biological activities of resveratrol and its wide use in pharmaceuticals, cosmetics and food supplements.

Cluster methodology based on data of resveratrol content allowed the clustering of the species in three groups (Fig. 2 and Table 4). The first one comprised
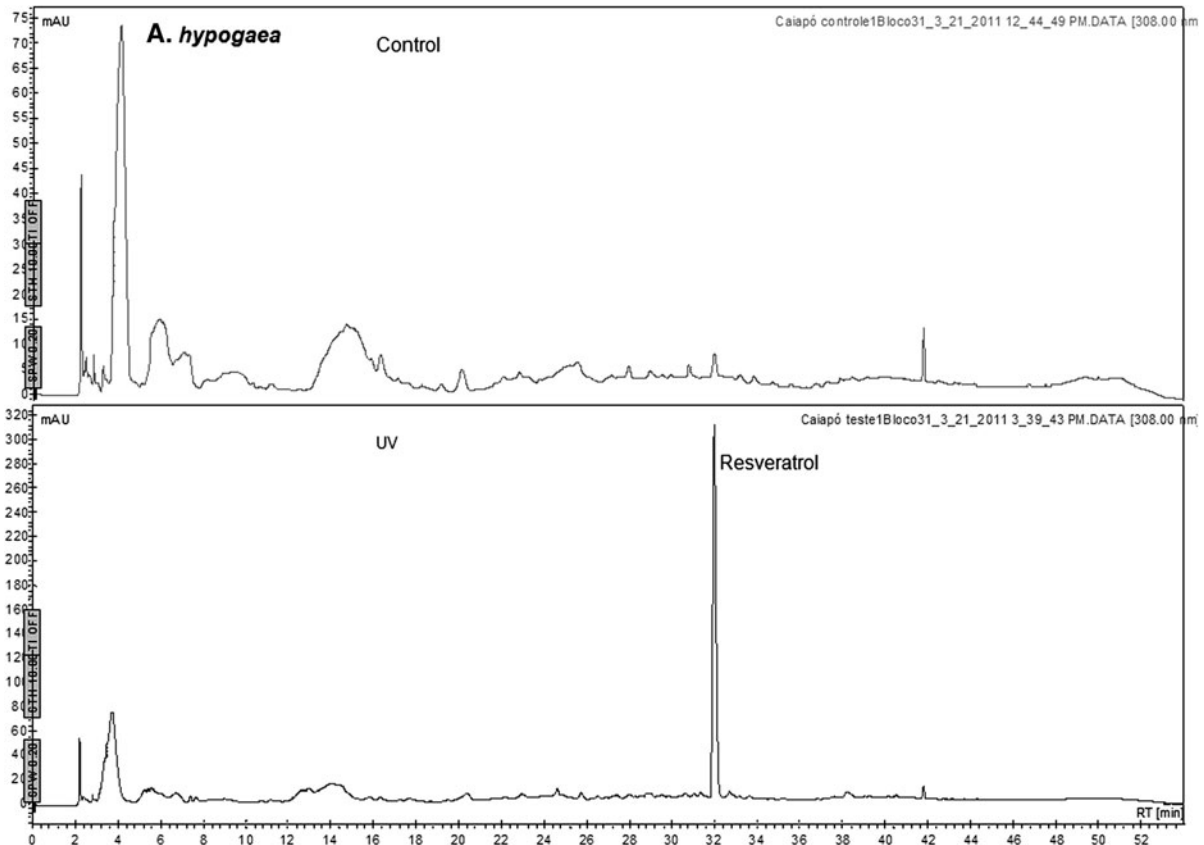
Fig. 1 Chromatograms obtained by HPLC of resveratrol (tR = 31.97 min; purity 99.5 %) in leaf extracts of *A. hypogaea*, control and induced treatments. Chromatographic conditions in the text
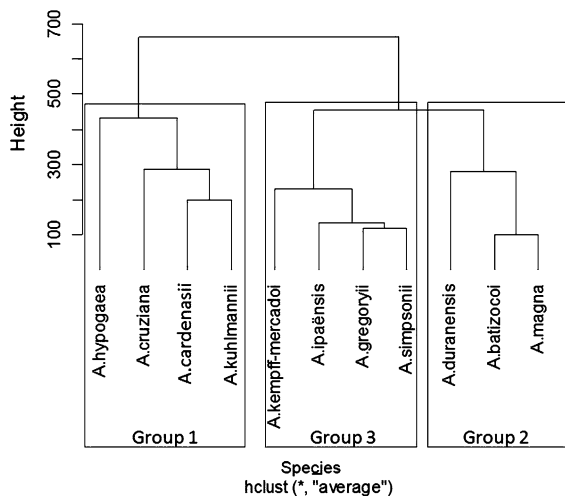
Fig. 2 Dendrogram based on resveratrol content showing the relationship between *Arachis* species. The three clusters observed included species that have different genomes. The first one comprised *A. hypogaea* (AB), *A. cruziana* (K), *A. cardenasii* (A) and *A. kuhlmannii* (A); the second cluster comprised *A. kempff-mercadoi* (A), *A. ipaënsis* (B), *A. gregoryii* (B) and *A. simpsonii* (A); and the third one *A. duranensis*, *A. batizocoi* and *A. magna* (B)

Table 4 Descriptive statistics for the cluster groups showing the similarity of *Arachis* species in relation to resveratrol expression (µg/g)

Group	Mean ± SD	Minimum	Maximum
1	801.9 ± 130.7[a]	596.0	973.0
2	557.2 ± 131.2[b]	314.0	722.0
3	323.9 ± 93.3[c]	198.0	500.0

Means followed by the same letters in the same column do not differ ($\alpha < 0.05$) according to the Kruskal–Wallis test

A. cardenasii (A genome), *A. cruziana* (K genome), *A. kuhlmannii* (A genome) and *A. hypogaea* (AB Genome), species that had the highest resveratrol contents (Fig. 2 and Table 4). *Arachis batizocoi* (K genome), *A. duranensis* (A genome) and *A. magna* (B genome) were placed in a second group, and a third group was formed by *A. gregoryii* (B genome), *A. kempff-mercadoi* (A genome), *A. simpsonii* (A genome) and *A. ipaënsis* (B genome), which presented the lowest levels of resveratrol. The fact the groups comprised species that had different types of genomes, and previous phylogenetic data showed all A, B, D and K genomes species form a monophyletic group, with B, D and K very closely related to each other (Bechara

et al. 2010) suggest that the resveratrol synthase gene is present in all genome types (A, B, D and K) of section *Arachis*. Thus, our data suggest that in section *Arachis* we will find another 21 species that synthesize resveratrol, since this section comprises 32 species (Valls and Simpson 2005; Krapovickas and Gregory 2007) and in this study we have evaluated 10 of them and *A. hypogaea*.

Arachis ipaënsis, the donor of genome B to the cultivated species *A hypogaea* (Bechara et al. 2010; Grabiele et al. 2012), showed a very low mean value for resveratrol compared to other wild species. This species has only one accession available (KG 30076). However, phylogenetic studies showed that *A. ipaënsis* is closely related to *A. magna*, *A. gregoryii*, *A. valida* and *A. williamsii* (Cunha et al. 2008; Bechara et al. 2010); those species have many accessions available, and some of them may have high resveratrol content under stress. In fact, in this study the accession of *A. magna* evaluated was grouped with accessions with intermediary content of resveratrol (Fig. 2). This suggests that there is variability within the B genome group for resveratrol content and that it is possible to find B genome accessions that are able to synthesize higher amounts of resveratrol than *A. ipaënsis*.

The average content of resveratrol found in induced samples of *A. hypogaea* was 668.7 µg/g. None of the wild species of *Arachis* studied presented a significantly higher concentration of resveratrol than *A. hypogaea*. However, previous studies on other plant species indicated the possibility of increasing phenylpropanoid derivatives, such as resveratrol, after polyploidyzation. Phenylpropanoid content was increased significantly in the wild potato *Solanum commersonii* Dun. after polyploidyzation (Caruso et al. 2011). Polyplodization also affected the level of expression of the phenylalanine ammonia-lyase gene (IiPAL), which increased significantly in tetraploid individuals of *Isatis indigotica* (Lu et al. 2006). Thus, the concentrations observed in *A. cardenasii*, *A. cruziana* and *A. kuhlmannii* can be considered high compared to peanut, because they are diploid species. Also, we have to take into account that in this study we compared data at just one time interval after UV induction, and the other species might have had higher resveratrol content before or after 15 h post-UV treatment, since they may have reacted differently to the stimulus because of the differences in leaf morphology observed between the species evaluated.

The differences in leaf morphology between *Arachis* species have been used for species classification (Valls and Simpson 2005; Krapovickas and Gregory 2007).

The extracts of each species were also evaluated for antioxidant activity by DPPH method. The activity varied from 12.6 % for *A. duranensis* to 44.7 % for *A. simpsonii* in control samples and from 18.7 % for *A. duranensis* to 60.7 % in *A. hypogaea*. A significant increase in antioxidant activity was observed after induction by UV radiation in *A. cardenasii, A. cruziana, A. hypogaea, A. kuhlmannii* and *A. batizocoi* species (Table 5). Three of these species (except *A. batizocoi*) were also grouped with those that presented the highest content of resveratrol (Fig. 2), so it may be that the biosynthesis potential of this compound may have influenced the antioxidant potential of the species, evaluated by DPPH. The species *A. batizocoi*, which was grouped together with species showing medium capacity for resveratrol biosynthesis (Fig. 2), and *A. cardenasii,* also showed biosynthesis of other unidentified compounds, which may also have interfered in the antioxidant activity after induction by UV radiation. However, in general no correlation was observed between this antioxidant activity and resveratrol content found in different species evaluated. This lack of correlation can be explained by the low reactivity of resveratrol with the DPPH, as shown by Sanchez-Moreno et al. (1998).

In conclusion, the results showed that all wild species of *Arachis* evaluated in this study have potential for production of resveratrol after induction by UV radiation. The results showed that wild *Arachis* species are a potential source of alleles for improvement of cultivated peanut, if aiming to achieve high contents of resveratrol. Species of genome A and genome B with higher levels of resveratrol could be used to obtain synthetic amphidiploids, which could also be used in production of resveratrol or crossed with *A. hypogaea* to increase the level of production of this compound in cultivated peanut.

Table 5 Mean ± standard deviation of antioxidant activity (%) evaluated in leaf extracts from different *Arachis* species by treatment

Species	Treatment	
	Control	UV
A. batizocoi	29.43 ± 11.80[a]	44.98 ± 11.74[b]
A. cardenasii	28.46 ± 4.62[a]	34.58 ± 10.47[b]
A. cruziana	30.45 ± 9.50[a]	35.61 ± 12.16[b]
A. duranensis	12.64 ± 10.60[a]	18.66 ± 13.39[a]
A. gregoryii	29.11 ± 11.39[a]	31.47 ± 5.37[a]
A. ipaënsis	38.02 ± 13.77[a]	36.34 ± 9.24[a]
A. kempff-mercadoi	29.58 ± 14.85[a]	29.10 ± 15.04[a]
A. kuhlmannii	13.28 ± 5.00[a]	19.61 ± 4.92[b]
A. magna	24.69 ± 11.44[a]	24.79 ± 6.27[a]
A. simpsonii	44.75 ± 29.94[a]	48.16 ± 33.38[a]
A. hypogaea	44.63 ± 22.90[a]	60.69 ± 15.23[b]

Lines with the same letters indicate that treatments do not differ, according to the generalized linear models, assigning Gamma distribution to antioxidant expression ($\alpha < 0.05$)

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