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The effect of tetraploidization of wild *Arachis* on leaf morphology and other drought-related traits

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ABSTRACT

Cultivated peanut is an allotetraploid (genome type AABB) with a very narrow genetic base, therefore wild species are an attractive source of new variability and traits. Because most wild species are diploid, the first step of introgression usually involves hybridization of wild species and polyploidization to produce a synthetic allotetraploid (AABB) that is sexually compatible with peanut. This study investigates drought-related traits such as leaf morphology, transpiration profile, chlorophyll meter readings (SCMR), specific leaf area (SLA) and transpiration rate per leaf area for two wild diploids (*Arachis duranensis* and *Arachis ipaënsis*) that could be of interest for improvement of the peanut crop. Furthermore, the inheritance of the traits from the diploid to the tetraploid state was investigated. Results showed that whilst some diploid traits such as SCMR, are maintained through hybridization and polyploidization, most characters, such as the leaf area, stomata size, trichome density and transpiration profile, are substantially modified. The study concludes that direct evaluations of drought-related traits in wild diploids may be useful for evaluation of wild species to be used in introgression. However, evaluations on wild-derived synthetic tetraploids are likely to be more informative.

1. Introduction

Cultivated peanut (*Arachis hypogaea*) is an allotetraploid (AABB) which most probably originated via hybridization of two diploid wild species followed by a spontaneous duplication of chromosomes or fusion of unreduced gametes (Halward et al., 1991). Due to difference in ploidy from the diploid wild species, peanut became reproductively isolated from its wild relatives. This led to lack of diversity for some traits of agricultural interest and very low DNA polymorphism. Consequently the wild diploid species of *Arachis* harbor many "lost alleles" and have potential for broadening the genetic base of the peanut crop.

Historically, for peanut, the transfer of genes from wild species by sexual crossing was hindered by the ploidy differences. This can be overcome by artificial hybridizing A and B genome wilds followed by induced chromosome duplication to restore fertility through the tetraploid or hexaploid route (Stalker and Wynne, 1979; Simpson et al., 1993). Furthermore, improved knowledge of *Arachis* species relationships has been gained in recent years by more detailed cytogenetic and molecular phylogenetic studies (Kochert et al., 1996; Burow et al., 2001; Robledo and Seijo, 2010). These have provided a much better understanding of the relationships of the wild and cultivated species. Linkage drag and difficulties in confirming hybrid identities and tracking introgressed segments have also hindered progress. However, over the last few years very significant advances have been made in the development of the genetic tools needed to overcome these problems.

Many wild *Arachis* species have been found to be resistant to biotic stresses (Leal-Bertioli et al., 2010; Nelson et al., 1989). At least in principle, the introgression of disease resistances from wild relatives into crop plants is relatively simple, because generally, one or a few genes confer disease resistance (Hajjar and Hodgkin, 2007).

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So far, genetically characterized wild chromosome segments that confer improved nematode, leaf-spot and rust resistance have been introgressed into peanut (Simpson and Starr, 2001). With active research in this area we can expect more successes in the coming years (Khedikar et al., 2010).

Peanut is widely cultivated in the tropics, where drought is one of the most limiting factors for production. This together with concerns about climate change and yield stability has led to increased interest in improved drought tolerance. The wild species of peanut are found in extremely diverse environments, ranging from swamps to grasslands, to rocky ground in semi-arid conditions (Krapovickas and Gregory, 2007; Bertioli et al., 2011). Therefore, it is possible that wild species do harbor genes that could confer improved performance under certain water limited conditions. However, the inheritance of characters associated with drought adaptation is likely to be genetically complex, and therefore unlikely to be tractable with alternating cycles of backcrossing and phenotyping/genotyping. Perhaps because of this, so far, little effort has been made towards the use of wild species to improve this aspect of the peanut crop.

One promising route for the introgression of improved drought-related traits using synthetic allotetraploids could be the construction of chromosome substitution lines (Foncéka et al., 2009). Such lines are powerful tools for revealing cryptic beneficial alleles from the wild species but are very labor intensive to construct, and only possible to make with a limited number of wild accessions/species. Considering the resources needed to obtain new synthetics and backcrossed lines, a pertinent question is what wild diploid parentals to choose and whether their direct evaluations can be used to identify desirable drought adaptation traits that have potential for improving cultivated peanut. Potentially, wild diploid species identified by such evaluations (Nautiyal et al., 2008; Upadhyaya et al., 2011) could be used for the production of synthetic allotetraploids and introgression.

In this study, we initiated an investigation on drought-related characters in wild *Arachis* accessions. Focus was placed on two accessions of *Arachis ipaënsis* and *Arachis duranensis*, originated from regions with relatively low rainfall and their derived synthetic, using cultivated peanut as a parameter for comparison. The overall aim of the study was to investigate some drought-related characters and how far direct evaluations of wild diploid species may be useful to identify desirable traits that could be introgressed into cultivated peanut to improve its drought tolerance.

2. Materials and methods

2.1. Plant material

Arachis seeds were obtained from the Active Germplasm Bank of Embrapa Genetic Resources and Biotechnology (Cenargen, Brasília, Brazil). Seeds were bulked up in greenhouse conditions. An initial experiment of progressive water deficit was carried out with 10 wild diploid accessions, two cultivated tetraploid genotypes and a synthetic allotetraploid (*A. duranensis* V14167 × *A. ipaënsis* KG 30076)$^{4\times}$ (Fávero et al., 2006) referred to here as synthetic, all annual genotypes (Supplementary File 1). Subsequently, the following genotypes were chosen for more detailed study: *A. duranensis* V14167; *A. ipaënsis* KG30076, the synthetic and *A. hypogaea* subsp. *hypogaea* var. *hypogaea* 'IAC-Runner' (referred here as 'Runner'). Seeds were germinated in germitex paper, with 2% Ethrel (2-chloroethylphosphonic acid) to break dormancy and 0.05% Thiram® to prevent fungal contamination. Plantlets were transferred to pots of 15 cm diameter and 1200 g of dry soil capacity. Wild accessions show greater seed dormancy and initiate growth at a lower rate; therefore they were planted two weeks before the cultivated genotype and one week before the synthetic. All plants were kept in greenhouse conditions. Plants were periodically treated for mites and fungal diseases prior to water stress imposition. Temperature and relative humidity were recorded hourly using Electronic Datalogger Sato SK-L200TH II (Sato, Japan).

2.2. Leaf morphology

Features observed on the abaxial and adaxial surfaces included stomata type according to Metcalfe and Chalk (1950), stomata length and width, stomata and epidermal cells density (number of stomata or epidermal cells mm^{-2}), number of trichomes, leaflet and thickness of water storage cells layer. Leaves from ninety-day old plants were used. Portions of two leaflets of first expanded leaf from five plants of each genotype were dissociated (Berlyn and Miksche, 1976). Six epidermal dissociations (three for adaxial and three for abaxial surface observations) were mounted for each plant with glycerinated gelatin and observed in Zeiss Axiophot phase contrast microscope (Carl Zeiss, Germany). Images were recorded in AxioCam Zeiss system (Carl Zeiss, Germany).

Features observed on the abaxial and adaxial surfaces included stomata length and width, stomata and epidermal cells density (number of stomata or epidermal cells mm^{-2}), number of trichomes, leaflet and spongy parenchyma thickness. To determine stomata density, the numbers of epidermal cells and stomata were determined in three observations of each surface, of each of the leaflets of five plants in an area of 0.33 mm^2. Stomatic index was calculated as: $IE = [NE/(CE + NE)] \times 100$, where NE corresponds to the number of estomata and CE to the number of epidermal cells (Cutter, 1986). Other samples were collected and processed for JB4® resin embedding. Transversal astra blue and safranin stained semithin sections (2–4 μm thick) were analyzed to determine leaf cell types and estimate leaflet and hypodermis thickness. Leaf length and width were determined using a digital caliper and leaf area was estimated considering that leaflets are ellipse-like. Statistical analyses for genotype comparisons were performed using multiple observations with the non-parametric test of Tukey with significance levels of 5%. For biplot of multivariate data based on Principal Components Analysis (PCA), the method of Principal Components with GH factoring column metric preserving was used (Gabriel, 1971). Analyses were performed using the statistical software *R* (R Development Core Team, 2010).

2.3. Drought-related traits

2.3.1. Transpiration profile (dry-down)

Evaluations of transpiration profile in the initial experiment of progressive water deficit experiment—dry down (Sinclair and Ludlow, 1986) in *Arachis* genotypes (Supplementary File 1) were conducted in June 2007 in temperature-controlled greenhouse. Further evaluations of four selected genotypes were conducted in February/March 2010. Dry-down experiments were initiated at 8, 9, and 10 weeks after sowing the cultivated, synthetic, and wild diploid genotypes, respectively, after plants had initiated reproductive stage. At field capacity (FC), pots contained approximately 1200 g of dried soil and 350 g of water. Until water stress imposition plants were grown under well-watered conditions. Plants were divided into different sets of five repetitions of each genotype: one set was harvested to assess biomass at the time of stress imposition, one set was used as control with well-watered treatment (WW), and the last set was used for dry-down (DS).

During the experiment, all plant pots were weighed every day at around 9 AM. WW plants were kept at approximately 70% FC by compensating water losses due to transpiration. For DS plants, a loss of no more than 10 g of water per day was allowed, so that stress imposition was gradual. Daily transpiration rate (TR) was

calculated by dividing transpiration of individual plants by the mean transpiration of the WW plants of the same genotype. Normalized transpiration rate (NTR) was calculated by dividing TR values by the average TR for the first three days for each individual plant. The purpose of this second normalization was to reduce the effect of plant variation in size within a particular genotype. For a given DS plant, the dry-down was terminated when its NTR fell below 0.1, considered the point where there is no more water available for transpiration. The difference between the initial pot weight and the weight at the end of the experiment gave the total transpirable soil water (TTSW).

Changes in NTR during the dry-down experiment were expressed as a function of the fraction of soil transpirable water (FTSW). FTSW was calculated daily for each individual plant as the ratio between the water remaining in the soil and TTSW, so that:

$$\mathrm{FTSW} = \frac{\text{daily weight} - \text{final weight}}{\mathrm{TTSW}} \quad (1)$$

Plots of NTR against FTSW were generated for each genotype including individual replicated data on each day for all plants. The relationship between the normalized transpiration rate (NTR) and the FTSW were fitted by nonlinear regression (Muchow and Sinclair, 1991), as follows:

$$\mathrm{NTR} = \frac{1}{[1 + \alpha * \exp(\beta * \mathrm{FTSW})]} \quad (2)$$

The estimates of the parameters of Eq. (2) were fitted by the Gauss–Newton algorithm using the *n*/*s* function of *R* statistical package. Additionally, a plateau regression procedure was used to estimate a specific FTSW threshold value where NTR begins to decline (Ray and Sinclair, 1997). The plateau regression was performed on each replicate plant and a break-point and its standard error were estimated for each genotype.

The rate of water loss over unit of leaf area was measured in WW plants over the last three days of the dry-down experiment, and expressed as $g\,cm^{-2}$. Leaf area was then assessed soon after harvesting the plants on the last day of the experiment.

2.3.2. SCMR, SLA and $\delta^{13}C$

Soil plant analysis development (SPAD) chlorophyll meter readings (SCMR) were made with a SPAD meter (Konica Minolta Sensing, Inc., Japan) on three month old plants. Four SCMR were recorded in each leaflet of the second fully expanded leaf of the main stem or lateral branches and averaged. Only healthy plants were evaluated, and care was taken to avoid leaf veins. After recording SCMR, leaves were soaked into water for at least 2 h to arrive in full turgor. Leaf area was calculated as previously described (Item 2.2). Leaves were oven dried at 80 °C for 72 h and were then weighed. Specific leaf area (SLA) was estimated as the ratio of leaf area and leaf dry weight and expressed in $cm^2\,g^{-1}$. Both SCMR and SLA measurements were taken from leaves of WW plants. A minimum of 34 leaves from at least 20 plants per genotype were measured.

For $\delta^{13}C$ analyses, leaf samples were oven-dried at 60 °C for at least 48 h and then ground in a Wiley mill to a fine powder. The samples analyzed were the bulk of five leaves of at least three plants. A 1.5–2 mg sub-sample of ground leaf material were placed and sealed in a tin capsule and loaded into a ThermoQuest-Finnigan Delta Plus isotope ratio mass spectrometer (Finnigan-MAT; CA, USA) in line with an Elemental Analyzer (Carlo Erba model 1110; Milan, Italy). Stable isotope ratios of Carbon were measured relative to established international standards. The reference material (Atropine from LECO Corporation) was used as an internal working calibration standard and was analyzed every ten samples to account for drift during run and to perform nonlinearity correction due to variation in sample weight. The carbon isotope discrimination is expressed in "delta" notation (‰), where the isotopic composition of a material relative to that of a standard on a per mill deviation basis is given by $\delta‰ = (R\ \text{sample}/\text{R standard} - 1) \oplus 1000$, where *R* is the molecular ratio of heavy to light isotope forms ($^{13}C/^{12}C$).

3. Results

Differences in leaf morphological and other drought related traits were observed between wild diploid, synthetic and the cultivated tetraploid genotypes studied here. Changes in plant structure upon induced hybridization followed by tetraploidization of the same wild diploids were also observed, such as increase in individual leaf area and thickness (Supplementary File 2), plant size (not shown) and dry biomass. Total dry biomass of three months old 'Runner' plants (8.29 g ± 1.75) was twice that of the synthetic (4.14 g ± 1.19), which, by its turn, was almost twice that of the wild diploid parentals: *A. duranensis* (2.5 g ± 0.54) and *A. ipaënsis* (2.03 g ± 0.32).

3.1. Leaf morphology

The majority of the stomata (92–98%) of all genotypes were of paracytic type (stoma surrounded by two subsidiary cells parallel to the long axis of the pore) present on both leaf surfaces. Other stomata types were mostly anisocytic (stoma surrounded by three cells of which one is distinctly smaller than the other two) and anomocytic (surrounded by four subsidiary cells) (data not shown). Stomatal dimensions (length and width) were comparable on both leaf surfaces of all genotypes. In addition, trichome density was greater in the abaxial than the adaxial leaf surface of all genotypes (Supplementary File 2). Adaxial trichome density was not statistically different in the synthetic, 'Runner' and *A. duranensis*. Density in *A. ipaënsis* was significantly lower than the other genotypes. Abaxial trichome density was similar in the synthetic and *A. ipaënsis*. The density in the synthetic was significantly lower than those in 'Runner' and *A. duranensis* (Supplementary File 2). Stomatal measurements, length and width, on both adaxial and abaxial leaf surfaces of the synthetic were similar to, and not statistically different from 'Runner' in all cases, whereas the stomata of wild diploid were somewhat smaller that the tetraploids (Supplementary File 2).

Significantly lower adaxial densities of stomata and epidermal cells were observed in the synthetic than either of the wild diploids. The densities of the synthetic were similar to 'Runner'. Adaxial stomatic index values were also similar for the tetraploids, and lower than for the diploids, but the difference was less marked, indicating that cell size was generally larger in the tetraploids (Supplementary File 2). The abaxial stomatal density was lowest in the synthetic but not statistically different from *A. duranensis*. Densities in *A. ipaënsis* and 'Runner' were significantly higher than for the synthetic. The density of epidermal cells in the abaxial surface was statistically different for all genotypes, being highest for *A. ipaënsis*, followed by *A. duranensis*, the synthetic and finally 'Runner'. As a result, *A. duranensis* and the synthetic showed significantly lower stomatic index than the other genotypes (Supplementary File 2).

Individual leaf area of the synthetic was similar to 'Runner', which both were significantly larger than the wild diploids (Supplementary File 2). Similar general leaf structure was observed in all genotypes, with the thickest leaves being those of the synthetic, which was statistically similar to 'Runner'. Leaves were delimited by a single layered epidermis in both adaxial and abaxial surfaces, and covered by a thin cuticle. The chlorenchyma showed a large palisade parenchyma above the upper epidermis, composed by uniform, cylindrical cells that were intercalated with vascular

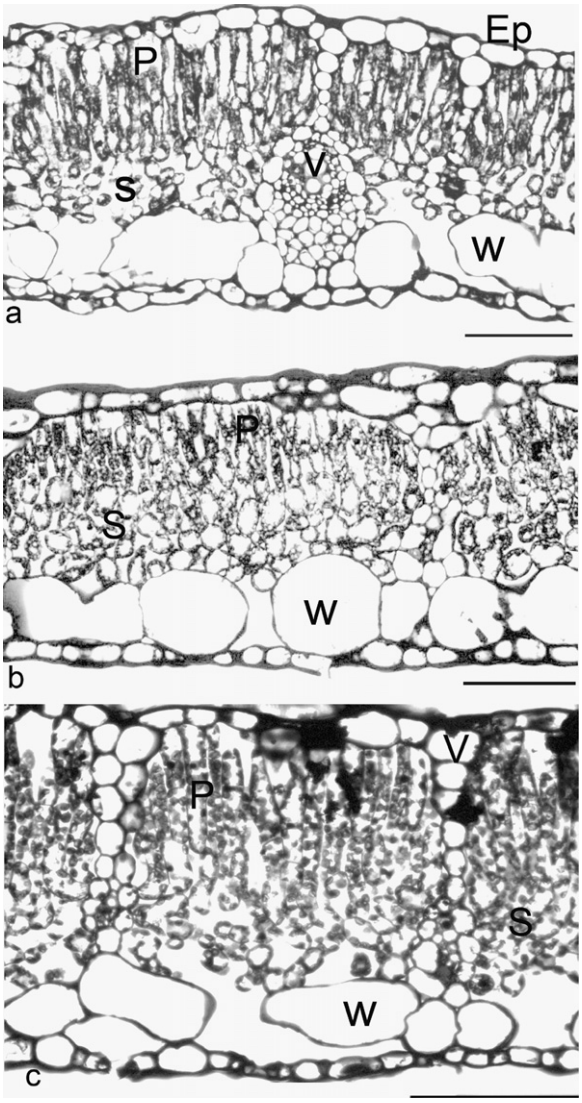

Fig. 1. Transversal semi-thin sections of *Arachis* leaf portions stained with astra blue and safranin. (a) synthetic allotetraploid (KG30076 × V14167)$^{4\times}$, (b) *A. hypogaea* subsp. *hypogaea* var. *hypogaea* 'IAC-Runner 886', (c) *A. ipaënsis* KG30076. General leaf profile is similar in all genotypes. They show a single layered epidermis (Ep) covered with a thin cuticle in both adaxial and abaxial surfaces; large palisade parenchyma (P) with numerous cylindrical cells intercalated by vascular bundles (V). A less compact palisade parenchyma is observed in *A. ipaënsis* (c) when compared to the other tetraploids. The spongy parenchyma (S) occupies around 20% of the total leaf thickness and is formed by irregular shaped cells, with variable volume and they are distributed within irregular intercellular cavities and vascular bundles. Bars correspond to 100 μm.

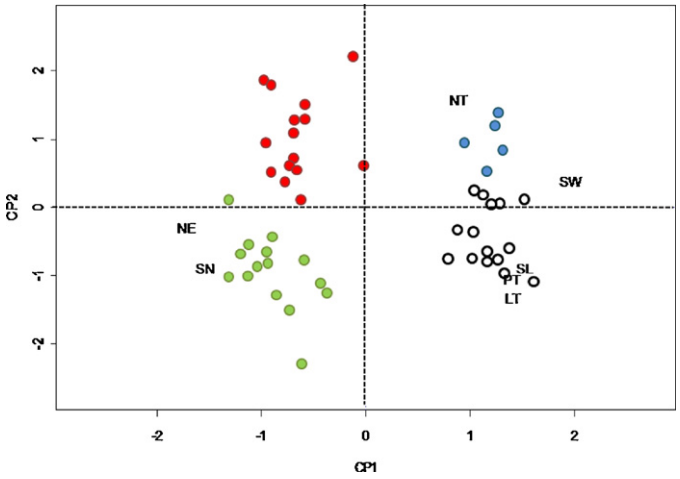

Fig. 2. Principal component analysis of leaf morphological characters. Adaxial number of trichomes (NT), number of epidermal cells (NE), stomata length (SL), width (SW) and number (SN), leaf thickness (LT) and parenchyma thickness (PT). The first two principal components explained 78% of the total variation. Red dots for *A. duranensis* V14167, green dots for *A. ipaënsis* KG30076, white dots for the synthetic allotetraploid (*A. duranensis* V14167 × *A. ipaënsis* KG30076)$^{4\times}$ and blue for *A. hypogaea* subsp. *hypogaea* var. *hypogaea* 'IAC-Runner 886'. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

bundles. Above that, a smaller spongy parenchyma composed by roundish large cells was observed, being more or less abundant depending on the genotype. A single layer of achloroplast cells, separated by large intercellular cavities was observed lying directly above the lower epidermis (Fig. 1). Cells of this layer are considered to be water storage cells (Reddy and Rao, 1968). The average width of this layer corresponded to approximately 24% of the leaflet thickness in the synthetic and, around 20% for the other genotypes (Supplementary File 2).

In sum out of the 15 characters evaluated in the synthetic in comparison with the wild diploids and 'Runner', three were similar to the parental *A. duranensis* (trichome density on the adaxial surface, stomata density and stomatic index on the abaxial surface); only trichome density in the abaxial surface was similar to *A. ipaensis*. Eight characters were different to both wild diploids, but similar to the cultivated tetraploid (individual leaf area and thickness, stomata length and width in both surfaces, stomata and epidermal cells density in the adaxial surface); two characters were similar to both 'Runner' and one of the wild parentals (thickness of water storage cell layer and adaxial stomatic index); and finally, only density of epidermis cells on the abaxial surface differed from all genotypes above (Supplementary File 2).

The principal component analysis (PCA) for characteristics of the adaxial surface corroborated this: the first principal component (vertical line) was able to separate synthetic and 'Runner' (group 1) from both wild diploids (group 2). For this component, group 1 showed high values for number of trichomes, stomata width and length, leaf and spongy parenchyma thickness, and low values for the number of epidermal cells and stomata on the adaxial surface. The group 2 showed the opposite behavior. Moreover, the second principal component (horizontal line) is able to separate *A. duranensis* and 'Runner' (group 3) from *A. ipaënsis* and synthetic (group 4): The group 3 has high values for number of trichomes and low values of leaf and thickness of water storage cells layer, width and stomata length, all in the adaxial surface, and group 4 had inverse values (Fig. 2). Similar analysis for abaxial surface did not produce distinct groups as for adaxial surface (data not shown).

3.2. Drought-related traits

3.2.1. Transpiration profile

During the initial dry down experiment, temperature ranged between 15.6 and 28.9 °C, relative humidity, 96–28%, and midday VPD between 0.47 and 1.4 KPa with average of 0.81 KPa. During the subsequent experiments, temperature ranged between 16.10 and 34.2 °C, relative humidity, 95.3–33.9% and midday VPD between 0.78 and 1.90 KPa and averaged 1.42 KPa (Supplementary File 3). In both experiments, VPDs were considered moderate.

The transpiration curve of the *Arachis* genotypes here studied under gradual exposure to water deficit followed the same pattern as for cultivated and other cultivated tropical legume plant species (Sinclair and Ludlow, 1986; Devi et al., 2010; Zaman-Allah et al., 2011) (Figs. 3 and 4).

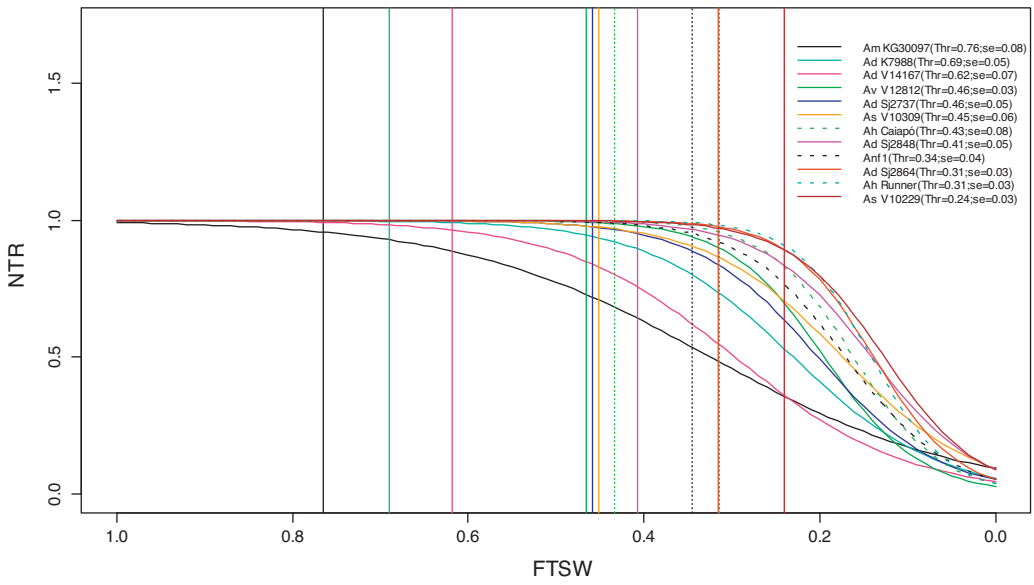

Fig. 3. Normalized transpiration rate vs. fraction of transpirable soil water of 12 *Arachis* genotypes. Data is the mean of at least five plants. Thr = FTSW threshold for NTR decline, Ad = *A. duranensis*, Am = *A. magna*, As = *A. stenosperma*, Av = *A. villosa*, Anf1 = synthetic amphidiploid (*A. duranensis* V14167 × *A. ipaënsis* KG 30076)$^{4\times}$, Ah = *A. hypogaea*.

The initial dry down experiment revealed a large variation in the FTSW threshold for NTR decline in different genotypes, even from the same species and varied from 0.24 (*A. stenosperma* V10229) and 0.76 (*A. magna* KG30097) (Fig. 3). In general, the wild diploids tend to have higher FTSW threshold than the tetraploids. The synthetic revealed a FTSW threshold more similar to the cultivated peanut than to one of its wild parentals, *A. duranensis* (Fig. 3). For this reason, this genotype and its parentals were the focus of the second dry down experiment. In the second experiment, the synthetic transpiration response to progressive exposure to water deficit confirmed to be different from both of the wild diploid parentals. The FTSW threshold value where NTR begins to decline was significantly lower for the synthetic (0.30 ± 0.05) than for the wild parental genotypes (0.62 and 0.71) (Fig. 4).

The rate of water used for transpiration per total leaf area (WU/LA) per day was higher for the tetraploids than for the wild diploids: *A. duranensis* transpired 0.55 g/cm^{-2} d^{-1}, *A. ipaënsis*, 0.74 g/cm^{-2} d^{-1}, synthetic, 0.9 g cm^{-2} d^{-1} and 'Runner', 0.94 g cm^{-2} d^{-1}, and (Fig. 5).

3.2.2. SCMR, SLA and $\delta^{13}C$

Values of SCMR and δ^{13}C for the synthetic were generally closer to the wild diploid parentals than to 'Runner'. For SLA and SCMR, there was little variation among genotypes. The synthetic had the same SLA value as the wild *A. duranensis* and higher than *A. ipaënsis*. SCMR values observed for the synthetic and the wild diploids were similar, differing from 'Runner', which had higher value (Supplementary File 2).

Mean foliar δ^{13}C (measured only in WW plants) varied from −25.47‰ (synthetic) to −28.31‰ ('Runner') (range of 2.84‰) (Supplementary File 2). Synthetic foliar δ^{13}C value was very similar to the wild diploids and distinct from that of 'Runner'. Although, from a statistical point of view, the reduced sample size did not

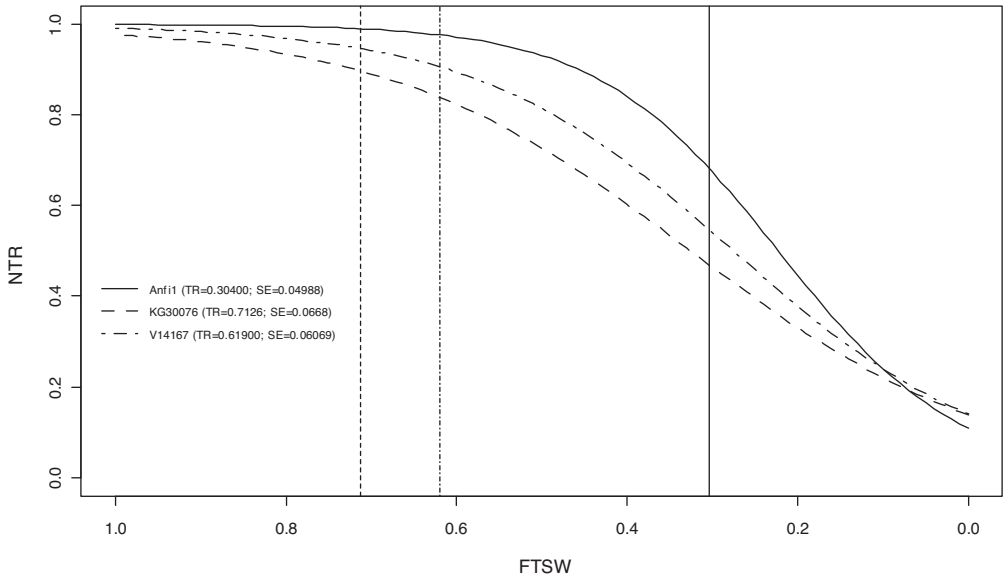

Fig. 4. Normalized transpiration rate (NTR) vs. fraction of transpirable soil water (FTSW) of *A. duranensis* (V14167), *A. ipaënsis* (KG30076) and the synthetic allotetraploid (*A. ipaënsis* KG30076 × *A. duranensis* V14167)$^{4\times}$, (Anf1). Data is the mean of five plants.

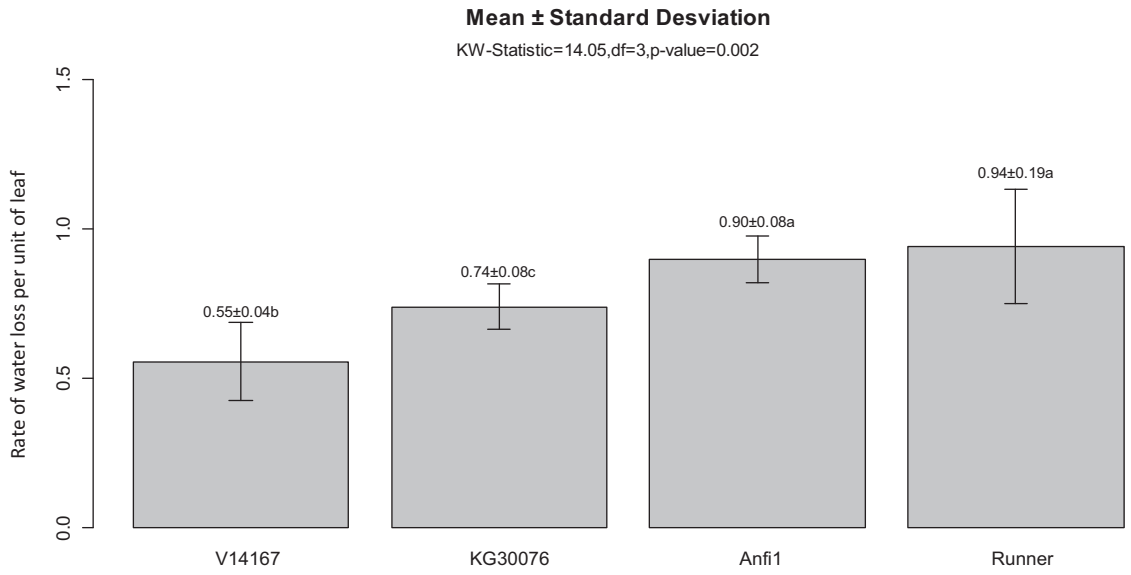

Fig. 5. Daily rate of water loss (g) per unit of leaf area (cm^2) (WU/LA) over a period of three days. Data are the mean of at least four plants. *A. duranensis* (V14167), *A. ipaënsis* (KG30076), the synthetic allotetraploid (*A. ipaënsis* KG30076 × *A. duranensis* V14167)$^{4\times}$ (Anf1) and *A. hypogaea* subsp. *hypogaea* var. *hypogaea* 'IAC-Runner 886' (Runner).

allow correlation, a reduction of $\delta^{13}C$ when WU/LA increases was visually evident (Fig. 6).

4. Discussion

The introduction of new genetic diversity through crosses with wilds is an attractive route to overcoming genetic limitations and introducing improved characteristics to crops. However there are numerous obstacles that may be found whilst navigating this route. Wild plants are not agronomically adapted. Multiple cycles of backcrossing are usually required to eliminate undesirable wild genes and even then, wild genes with pleiotropic effects and linkage drag can reduce the agronomic performance of progeny derived from cultivated × wild crosses. At the beginning of this journey it would be prudent to make as informed a choice as possible regarding which wild relatives to use. This rationale has served well for the successful introgression of wild disease resistances into crop plants (Hajjar and Hodgkin, 2007). In this case, direct evaluations of wild plants are often very informative, because single, or a few genes often confer disease resistance (e.g. Foncéka et al., 2009; Leal-Bertioli et al., 2009). Drought adaptation would be expected to be more genetically complex. Nevertheless, direct evaluation of wild species' natural habitat, phenotype and performance under water limited growth conditions are frequently used as a guide in the choice of wild species for introgression of improved drought adaptation (e.g.Canci and Toker, 2009; Chetelat et al., 2009).

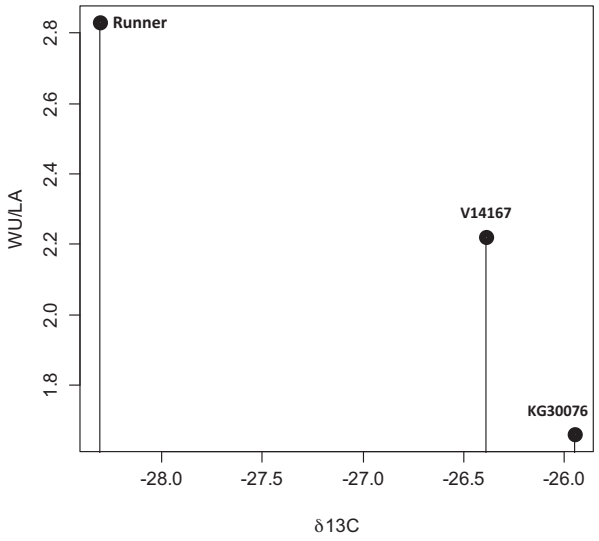

Fig. 6. Graphic representation of daily rate of water loss per unit of leaf area (WU/LA) ($g\,cm^{-2}\,d^{-1}$)and leaf stable carbon isotope composition ($\delta^{13}C$) of *A. hypogaea* subsp. *hypogaea* var. *hypogaea* 'IAC-Runner 886' (Runner), *A. duranensis* (V14167) and *A. ipaënsis* (KG30076), over a three day period.

In the case of peanut, the direct evaluation of wild relatives for desirable drought adaptation traits has an extra complicating factor. Whilst most wild relatives are diploid, cultivated peanut is tetraploid. Therefore, introgression routes in peanut rely on hybridization and the induction of polyploidy, which are known to cause changes in genome structure, gene expression, cell size and growth rates, size and shape of organs, and reproductive systems (Adams and Wendel, 2005; Otto and Whitton, 2000; Masterson, 1994). However, such changes are by no means universal or predictable and polyploid plants may have characteristics that are similar to either of their parentals; intermediate between them; or have characteristics that are absent in, or exceed those of the contributing parentals (Liu et al., 2007; Xu et al., 2007).

Because of the inherent problems of comparing wild *Arachis* and cultivated plants which differ in ploidy, phenology, architecture and cycle (Krapovickas and Gregory, 2007), emphasis was placed on leaf morphology and other drought tolerance related traits. Careful analysis is needed to evaluate the usefulness of the features in an agronomic context, as well as the possibility of their introgression. For this, investigation was performed on a synthetic allotetraploid and its two diploid parentals (*A. duranensis* and *A. ipaënsis*).

Cultivated peanut, which most probably originated from the same two wild species (Kochert et al., 1996) was used as a point of comparison.

The soil moisture level (FTSW) at which transpiration starts to decrease under gradual water deficit (FTSW threshold) is one of the key variables in the way that plants respond to limited water availability (Jones, 1992). Genotypes with NTR threshold at relatively high soil water content can be said to be "conservative" with water use (Gollan et al., 1986). In the initial dry down experiment, the large variation in the FTSW threshold for NTR and the tendency for the wild diploids to have higher soil moisture threshold for the transpiration decline than the tetraploid cultivated suggested

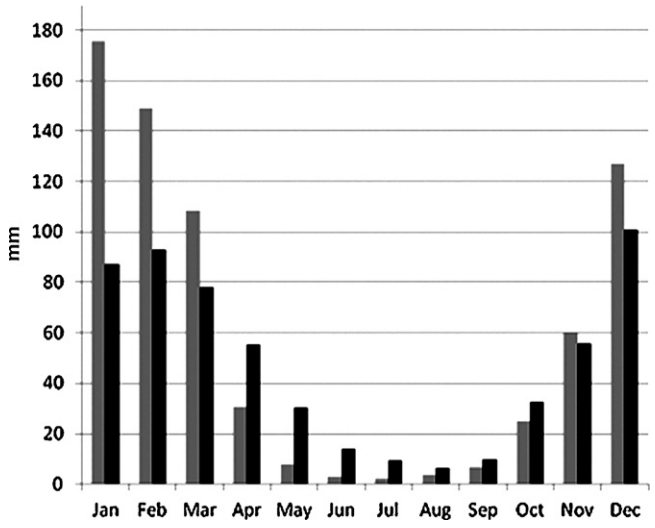

Fig. 7. Average rainfall (mm) in the sites of origin of *A. duranensis* (V14167, grey) and *A. ipaënsis* (KG30076, black).

a more conservative response to water deficit in the wild diploid species, in particular, *A. magna* KG30097. In contrast, the tetraploids maintained stomata opened until the soil was dryer. This could not have been predicted to be present in the synthetic by assays on the wild diploids and this response seems to be mainly related to ploidy change. Subsequently, we paid special attention to the accessions of *A. ipaënsis* KG30076 and *A. duranensis* V14167, which had higher threshold for the decline of transpiration than its synthetic allotetraploid. Here, the synthetic allotetraploid had a profile of transpiration response that was clearly closer to that of 'Runner'. These two wild diploids originate from regions with relatively low rainfall (699 and 575 mm/year, respectively (Fig. 7); average World rainfall is about 1050 mm/year (www.worldclimate.com). Therefore, it was expected that water saving features such as restricting transpiration at relatively high level of soil moisture would be present in these accessions. This could be useful to improve the cultigens, where water saving traits have recently been shown to be important (Ratnakumar and Vadez, 2012), but where the range of variation for the transpiration response to water deficit is relatively limited in the cultivated germplasm (Devi et al., 2010). It is also important to note that more moderate use of soil water could improve crop performance where water is limited, but also would be related to less photosynthetic activity and growth and a yield penalty where water availability is higher (Vadez et al., 2008).

The FTSW threshold data was also consistent with the data on WU/LA, which is a proxy for stomata conductance. Lower stomata conductance relates to terminal drought tolerance where plants depend on stored soil moisture at the end of the crop cycle (Kholová et al., 2010). Plants with this characteristic save water and make it available for later stages of the crop cycle, especially at the grain filling stage (Passioura, 1977; Ratnakumar et al., 2009). The wild diploids after flowering had a consistently lower WU/LA, when compared to 'Runner'. However, here also, tetraploidization appeared to offset the characteristics of the wild diploids and the synthetic was more similar to the cultivated genotype ('Runner') than to the wild parentals. For these characteristics the use of direct measurements on diploids for choosing wild parentals for crosses would not be appropriate.

Leaf morphological characteristics here evaluated, such as stomatal density and dimensions, leafleat area and layers thickness and trichomes density can all be associated with drought adaptation. These features can indeed affect gas exchange rates, photosynthesis levels, water loss and air movement, therefore, interfering in gas and water exchange at the leaf surface (Veiga et al., 1994; Mauseth, 2009). Overall, these characteristics of the synthetic tended to be closer to the 'Runner' than to either wild parentals, which suggests that many of the traits are more influenced by tetraploidization than by the diploid parental heritage. Interestingly, the synthetic shows a thicker leaf structure and higher ratio of water storage cells layer/leaf thickness than either the wild parentals. The layer of water storage cells observed for all genotypes is similar to that described for *A. hypogaea* (Pallas, 1980) and constitute an air-filled lower surface (Reddy et al., 2003) The water storage property of these cells is associated with lower conductivity, larger vapor diffusion path (Reddy et al., 2003) and radiation reflectance properties (Babu and Rao, 1983) as previously shown for peanut leaflet lower layer. Therefore, a relative higher stomatal resistance is expected there than in the upper layer. This is useful whenever drought stress is underway and as one of the consequences, is the exposition of the lower surfaces of the leaflets to the environment due to parahelionastic movements (Chung et al., 1997).

Most of the characteristics shared between the tetraploids were inversely related to the FTSW threshold (data not shown). For other characteristics such as SCMR and $\delta^{13}C$, the synthetic retains the characteristics of its wild parentals. These characters may be favorable candidates for introgression.

We show that tetraploidization changes many, but not all aspects of general architecture and response to drought. The synthetic that is derived from the wild diploids is more similar to the cultivated peanut 'Runner' than its parentals for most characteristics analyzed, which were lost in the first steps towards introgression, artificial hybridization and manipulation of ploidy. Some other characters of the synthetic allotetraploid exceed those of the wild parentals and 'Runner', such as the ratio of spongy parenchyma to leaf thickness. These characters could not have been predicted by evaluations on the wild diploids. However, notwithstanding complex genetics, they are still candidates for transferring to cultivated peanut by introgression.

What then would be the best way to identify wild species or accessions to be used in introgression to improve the performance of cultivated peanut under drought stress? This is not a question with easy answers: although it would seem justifiable to pay attention to potential parental plants that have evolved under periodic water-limited conditions, it is clear that evaluations of traits of interest on wild-derived synthetics are essential. Further experiments with new synthetics derived from other wild parentals will provide more information on the potential of introgression of characteristics of interest.

5. Conclusions

This is the first comparison of accessions of wild *Arachis* species and their derived synthetic allotetraploid on characteristics related to water deficit. The present analysis revealed variation in morphological and physiological features relevant to drought adaptation in wild, wild-derived synthetic allotetraploid and cultivated *Arachis*. Many characteristics are potentially more favorable in the wild diploids than the cultivated tetraploid and could potentially be useful in improving the performance of cultivated peanut under drought stress. However, most drought related characteristics studied here are substantially modified by polyploidization. Therefore, evaluations of traits of interest on wild-derived synthetics are likely to be more informative than direct evaluations on wild diploids.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.envexpbot.2012.04.005.

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