Australian Journal of Crop Science AJCS
AJCS 9(11):1095-1105 (2015) ISSN:1835-2707

Cluster analysis to select peanut drought tolerance lines

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Abstract

The breeding programs focused on semiarid environments significantly invest on selection of early-mature genotypes with the ability to maintain yield at reasonable levels, when drought sets in. The *fastigiata* peanut genotypes have broad physiological adaptation to environments prone to drought, while subsp. *hypogaea* are less tolerant, but are excellent to pod yield. The combination of these intraspecific materials via hybridization often provides robust descendants to breeding works focusing on yield and drought tolerance. In this work we adopted a clustering method to assist in the selection procedures of intraspecific peanut elite lines, based on molecular tools and physiological, biochemical and agronomic traits. Initially, fourteen intraspecific top lines (F_7) were used to identify drought tolerant materials based on SSR markers. Thereafter, the selected lines were submitted to 10 days (d) of water stress and analyzed by physiological (stomata conductance, transpiration and photosynthesis) and biochemical (organic solutes and antioxidative enzymes) traits. These data were used to identify lines tolerant to water stress, based on Canonical variable analysis. Additionally, these same lines were used in validation assays during two year experiments, under irrigated and rainfed conditions. We found that all selected top lines showed osmotic adjustment and antioxidant satisfactory front of disturbances caused by water stress. However, based on the clustering analysis the top line L46 was more suitable for semiarid environment, due to agronomic similarity with drought tolerant-BR 1.

Keywords: *Arachis hypogaea*; water stress; molecular marker; osmotic adjustment; antioxidative enzymes.
Abbreviations: ROS_Reactive Oxygen Species; APX_Ascorbate peroxidase; G x WR_Genotype x Water treatment; GPX_Guaiacol peroxidase; PCR_Polymerase Chain Reaction; UPGMA_Unweighted Pair Group Method with Arithmetic Mean; HI_ Harvest index; DTE_Drought tolerance efficiency.

Introduction

The regular water supply during life cycle is essential to determine the yield in the legumes. In semiarid environments, the irregular rainfall and occurrence *veranicos* (Indian summer) following by high temperature influence the fruit growth, leading to varied damage depending on intensity and duration of drought period. The reproductive phase is more sensible to this situation, although plants use different defense strategies in order to survive and ensuring descendants. The water deficit during flowering and seed development has important effect on the pods and seed yield. Under long-term drought periods (above 14 days), in which the growth is postponed and plants try to overcome the water deficiency by decreasing leaf area or increasing the water use efficiency (Nemeskéri et al., 2010).

The osmotic adjustment is an important mechanism, by which plants synthesize and accumulate compounds acting as osmolytes in cells in response to water deficits (Seki et al., 2007). In order to avoid cell dehydration, plants undergo different biochemical and molecular genetic changes to maintain osmotic adjustment and the structure of cell membranes. In legume crops, several defense strategies have been reported in order minimize the effect of drought during the phenological phase. Water stress induces the accumulation of soluble sugars and increases free proline leading to maintenance of turgor (Babita et al., 2010). Sugars act as osmotic compounds, protecting plants during drought period, contributing to stabilization of cell membrane structures (Streeter et al., 2001). However, accumulation of carbohydrates differs depends on the individual responses of plant species.

An expressive consequence of water stress is the production of ROS that promotes different oxidative actions

in plants (Azevedo Neto et al., 2009; Pereira et al., 2012). According to Nemeskéri et al. (2012), oxidative damage in plant tissues is alleviated by a concerted action of both enzymatic and non-enzymatic antioxidant mechanisms. These antioxidant compounds react with free radicals and neutralize them, overcoming the damage caused by stress.

In semiarid environments, the volume and distribution of rainfall are often unpredictable becoming difficult to draw an agricultural planning without the risks of crop frustration. The breeding programs for drought tolerant crops carried out by research companies worldwide have provided expressive contribution to farmers established in this region, by releasing early mature cultivars, with ability to maintain the yield at satisfactory levels, when rains become scarce.

Peanut (*Arachis hypogaea* L.) is a valuable commercial oilseed, known by broad adaptation to tropical and semiarid climates. The species is commercially divided into three varieties: (a) *fastigiata*, represented by genotypes of Valencia group, whose main traits are upright growth habit, short cycle and 3-4 seeds/pod; (b) *vulgaris*, represented by upright and earliness genotypes of Spanish group, with 1-2 tan seed/pod, and (c) *hypogaea*, represented by runner and late cycle genotypes of Virginia group, with 1-2 large seed/pod (Krapovickas and Gregory, 1994).

Despite to environmental adaptation, peanut yield is jeopardized when plants face water irregularities during flowering and grain filling. In tolerant plants, this effect is reduced by physiological and biochemical adjustments in order to avoid dehydration, such as expansion of the root system, reduced leaf water potential, stomatal closure and osmotic adjustment (Furlan et al., 2012; Junjittakarn et al., 2014; Kottapalli et al., 2009; Thangella and Rao, 2013). Additionally, to minimize the damage caused by ROS, the cells trigger a neutralizing process led by antioxidative enzymes that modulate their activities depending on level of plant tolerance (Akcay et al., 2010; Pereira et al., 2012; Sankar et al., 2007). Sensitive genotypes also face this process; however, the cell machinery is less efficient.

The breeding program for drought tolerance in peanut, headed by Brazilian Company of Agricultural Research (EMBRAPA), has focused on selection to earliness and pod yield under field conditions, located at semiarid environments. Several robust cultivars are commercially available, such as BR 1, an earliness-short cycle of Valencia type, with broad adaptation to drought and salinity (Gomes et al., 2007; Graciano et al., 2011).

Although earliness is an important trait for peanut adaptation to a wide range of cropping systems in semiarid environment, early maturity is also disadvantageous because plant has shorter growing period to develop, manufacture and store nutrient materials (Sleper and Poehlman, 2006). A strategy to overcome this challenge is through hybridization, by using subsp. *fastigiata* × *hypogaea* genotypes in order to broaden the genetic basis of progenies and favoring the selection procedures to drought-adapted and productive genotypes, in field conditions. As drought tolerance is driven by multigenic factors, the improvement is often slow. Then, other approaches must be adopted in order to assist in identifying plants that meet the goals of the breeding program.

In this work, we used physiological and biochemical approaches to identify peanut lines tolerant to drought. Clustering analysis was adopted to assist selection procedures. Additionally, we validated the results in field trials during two years of experiments in semiarid environment.

Result and Discussion

Genetic similarity of peanut top lines

The primer sets for the genetic analysis of peanut lines were contributive to identify divergent groups in studied genotypes. An average of 23 bands/primer were obtained, with polymorphism rate of 88% (Supplementary Table 1). Fig 1. shows the pattern of bands obtained with the more contributive primers. The combinations #4, #8 and #30, exhibited polymorphism rate of 100%. It is noteworthy that although peanut is an autogamous species with cleistogamic flowers (Coffelt et al., 1989), the polymorphism obtained with primers set was expressive, considering the genetic basis of the intraspecific population studied. This result attests the contribution of gene banks to assist the breeding of several crops.

The genetic similarity analysis obtained by matrix of amplicons revealed formation of three groups (Fig 2): Group 1, composed of *cv*. IAC Caiapó and L81, both late cycle and more adapted to tropical weather (Santos et al., 2012b); Group 2, clustered *cv*. BR 1, LBR White, Runner, L59, L46 and L67, all flowering at 24-26 d and pod maturing at 87 and 110 d; and Group 3, composed of L51, L75, L60, L66 and L73, all with intermediate cycle of 115 d (Table 1). The remainder genotypes were more divergent and remained isolated. Among them, the early mature-drought tolerant *cv*. 55437 (Boote et al., 1982; Duarte et al., 2013; Pereira et al., 2012) and the high yield-late runner LViPE (Santos et al., 2012a) were distinctively detected. Based on agronomic and commercial attributes of *cv*. BR 1, the lines LBR Branco, L59 and L46 were selected for further assays in greenhouse and field trials.

Physiological and biochemical responses in peanut lines submitted to water stress

Six genotypes (3 lines, the parents and cv. 55437) were grown in greenhouse and submitted to 10 d of water suppression. Physiological and biochemical traits were measured in leaf and root tissues. Genotypes (G) and water treatments (WT) were statistically different for most traits in both tissues, however, in leaves, interaction effect (G x WT) was seen only to biochemical variables indicating that behavior of genotypes was not dependent on water treatments to physiological traits adopted in this work. In roots, G x WT effect was seen in all traits, excepting to enzyme CAT (Table 2).

The averages of traits generated by G × WT interactions are found in Fig 3 and 4. In general, all lines showed adequate adjustment to disturbances caused by water stress, increasing the concentrations of organic solutes (Fig 3) to minimize the cytotoxic damages caused by ROS and favoring the action of antioxidative enzymes (Fig 4). This profile was also seen in earliness *cvs.* 55437 and BR 1, agreeing with Thangella and Rao (2014). They reported that in defense processes the antioxidative enzymes act along with other non-enzymatic components to provide protection to cell structure. Sankar et al. (2007) exposed peanut plants to 10 d of water suppression and found that tolerant genotype showed simultaneous increase in SOD (40%), APX (14%) and CAT (40%) and also in ascorbic acid (34%), α-tocopherol (46%) and reduced glutathione (15%). The combined action of enzymatic and non enzymatic complex favored tolerant plant in defense metabolism.

Table 1. Genealogy and some descriptors of the studied population.

Access	Genealogy / Origin	GH	SC	NSP	SS	BF (dae)	Cicle (dae)	O (%)
55437	Cultivar/África	E	B	1-2	S	21	75-80	43-45
IAC Caiapó	Cultivar/SP, Brasil	R	B	1-2	L	32	120-125	48-50
BR 1	Cultivar/PB, Brasil	E	R	3-4	M	24	87-90	45-46
LViPE-06	Land race, PE, Brasil	R	B	1-2	EL	33	120-130	51-53
LBR Branco	F_6 line[1]/PB, Brasil	R	Wh	3-4	L	26	100-114	47-49
L Runner	F_6 line/PB, Brasil	SR	R	1-2	L	26	100-110	48-50
L37	F_6 line/PB, Brasil	SE	B	1-2	S	29	100-115	45-47
L 46	F_6 line/PB, Brasil	E	B	2-3	M	24	90-95	50-51
L 51	F_6 line/PB, Brasil	E	R	2-3	M	27	114-116	46-48
L 59	F_6 line/PB, Brasil	R	R	2-3	L	25	100-114	50-52
L 60	F_6 line/PB, Brasil	R	B	2-3	M	31	114-116	48-50
L 66	F_6 line/PB, Brasil	R	Wh	2-3	M	33	114-116	46-48
L 67	F_6 line/PB, Brasil	E	B	1-2	M	26	100-110	45-47
L 68	F_6 line/PB, Brasil	SE	B	1-2	M	35	115-120	48-50
L73	F_6 line/PB, Brasil	E	Wh	2-3	M	30	114-116	45-47
L 75	F_6 line/PB, Brasil	E	B	2-3	M	30	114-116	46-48
L 77	F_6 line/PB, Brasil	SE	B	1-2	M	30	115-120	46-48
L 81	F_6 line/PB, Brasil	SR	B	1-2	L	27	118-120	48-50

GH- growth habit: E- erect, SE- semi erect, R- runner, SR- semi runner; SC- seed color: R- red, T- tan, W- white; NSP- number of seeds/pod; SS- seed size: S- small, M- medium, L- large, EL- extra-large; F- flowering; O (%) - oil content; Dae- days after emergence.

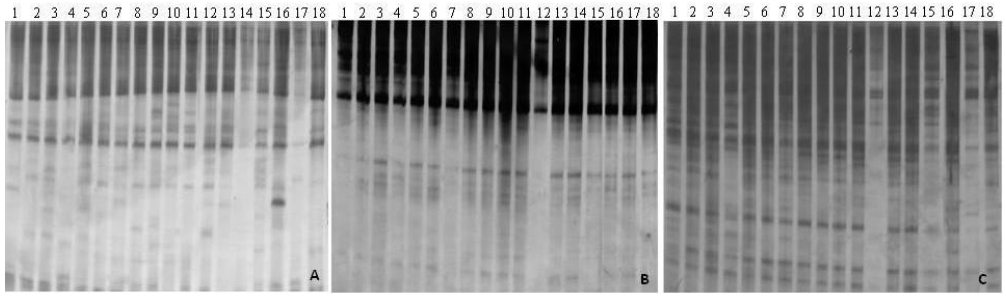

Fig 1. Electrophoretic profiles obtained by SSR-PCR from DNA leaves of peanut 18 genotypes. SDS-PAGE gel stained with Silver nitrate (0.2%). Set of primers: A- # 4, B- # 8, C- # 30. Samples: 1- 55437, 2- IAC Caiapó, 3- BR 1, 4- LViPE-06, 5- LBR Branco, 6- L Runner, 7- L37, 8- L 46, 9- L 51, 10- L 59, 11- L 60, 12- L 66, 13- L 67, 14- L 68, 15- L73, 16- L 75, 17- L 77, 18- L 81.

In legume, the activity of antioxidative enzymes are often increased in tolerant plants submitted to abiotic stress, mainly peroxidases and catalase (Akcay et al., 2010; Pereira et al., 2012; Sankar et al., 2007). In sensitive plants, the responses are reduced or even nulls depending on damage suffered by cell membrane due to stress (Cavalcanti et al., 2007; Prakash and Kumar, 2014). In this work, we found that the ability of late-runner LViPE-06 to activate the antioxidant system in leaves was limited possibly due to faster loss of turgor exhibited in plants during water stress (data not shown). Therefore, plants showed slow growth. A daily monitoring of chlorophyll content was carried out in plants during water stress period. The *cvs*. BR 1 and 55437 maintained almost the same pattern of chlorophyll, differing from 8 d water suppression (Fig 5), while in other genotypes changes were found from 5 d. Phenotypically, these plants showed loss of turgor. According to Arunyanark et al. (2008), stability in chlorophyll content is reported as an indicator of drought tolerance in peanut and changes during water stress is related to the plant-defense response. Drought tolerant genotypes often have higher levels of chlorophyll in any condition or, a less significant reduction when the plants are water-stressed (Anjum et al., 2011; Talebi et al., 2013). Such statements justify the patterns found here with *cvs*. BR 1 and 55437, which have been reported as drought tolerant (Boote et al., 1982; Duarte et al., 2013; Gomes et al., 2007; Nogueira et al., 1998). According to O'Neill et al. (2006), genotypes with high content of photosynthetic pigments under water

Table 2. Mean square of variance analysis of physiological and biochemical traits in leaves and roots of peanut.

Leave

SV	DF	A	gs	E	Carb	PT	Prol	APX	GPX	CAT
G	5	63.41ns	.004ns	2.16ns	409.24**	36293178*	1548**	.003**	.01**	.004**
WR	1	542.37**	.22**	102.20**	12893.68**	6353956ns	62379**	.002**	.05**	.0003ns
G X WR	5	11.34ns	.003ns	0.70ns	326.14**	3212691ns	1549**	.0001*	.01**	.0013**
Error	30	21.49	0.001	0.94	84.83	1684320	155,11	0,006	.001	.0002
Total	47									
M		25.02	.10	2.43	45.83	6705.68	39.42	8.15	0.25	0.05
CV (%)		18.52	41.22	39.86	20.09	19.35	31.63	9.49	13.61	7.6

*; ** Significant at 0.05 and 0.01; NS- not significant by F test; SV- Source of variation; DF- Degree of freedom; G genotype; WR water treatment; CV (%) - Coefficient of variation; A- photosynthesis rate; gs- Stomata conductance; E- transpiration rate; Carb- carbohydrate content; PT- protein content; Prol- proline content; APX- activity of ascorbate peroxidase; GPX- guaiacol peroxidase activity; CAT- catalase activity.

Root

SV	DF	CARB	PROT	PROL	APX	GPX	CAT
G	5	680.98**	21129726ns	160.14**	.003**	.05**	.0001**
WT	1	26611.97**	53174936**	6826.82**	.097**	.71**	.0009**
G X WT	5	948.64**	5208688**	155.33**	.001**	.02**	.0001ns
Error	30	88.90	1020583.07	27.12	.0001	.001	0.0001
Total	47						
Mean		41.44		12.52	0.12	0.40	3.44
CV (%)		22.75		41.58	8.66	9.54	10.59

*; ** Significant at 0.05 and 0.01; NS- not significant by F test; SV- Source of variation; DF- Degree of freedom; G genotype; WR water treatment; CV (%) - Coefficient of variation; A- photosynthesis rate; gs- Stomata conductance; E- transpiration rate; Carb- carbohydrate content; PT- protein content; Prol- proline content; APX- activity of ascorbate peroxidase; GPX- guaiacol peroxidase activity; CAT- catalase activity.

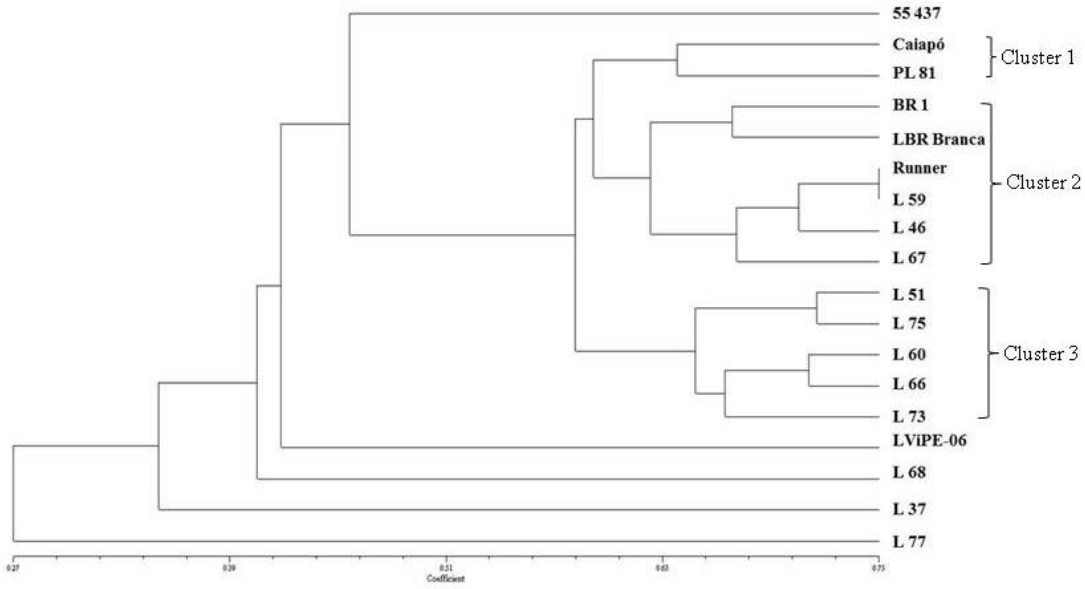

Fig 2. Dendrogram obtained by hierarchical clustering method UPGMA, from matrix generated with 18 peanut genotypes. Cophenetic correlation coefficient: 0.829. Similarity index above 60% ($p \leq 0.01$, F test).

stress are more able to tolerate dry condition due to interrelationship with chlorophyll, photosynthetic potential and productivity. Duarte et al. (2013) exposed early-mature and late peanut genotypes to 27 d of water suppression, including BR 1, 55437 and LViPE-06, and found Drought Tolerance Index of 42%, 54% and 36%, respectively. The reduction in harvest index in earliness cultivars was close to 12%, while in the late genotype was 27%.

Clustering of drought tolerant lines by canonical variables

Data obtained from physiological and biochemical traits of six genotypes in both water treatments were analyzed by multivariate method using canonical variables, which allows discriminating individuals maintaining homogeneity within the group and heterogeneity between groups. Only traits that showed statistically significant G × WT interactions were

Table 3. Variance (eigenvalues), percent (PV) and accumulated variances of canonical variables obtained from matrix formed by physiological and biochemical traits in peanut genotypes submitted to water stress.

Canonical variables	S.j	PV (%)	Acumulated %
CV1	2149.97	95.60	95.60
CV2	1797.59	2.22	97.83
CV3	1666.33	1.12	98.95
CV4	625.37	0.70	99.73
CV5	431.39	0.21	100.0
CV6	312.52	0.07	100.0
CV7	193.49	0.04	100.0
CV8	225.82	0.02	100.0
CV9	168.79	0.02	100.0
CV10	50.19	0.02	100.0

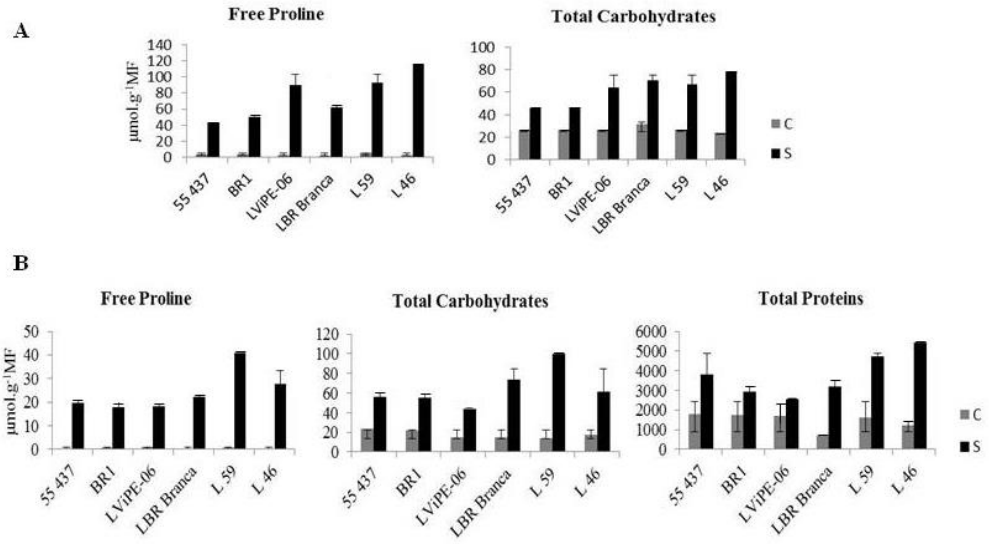

Fig 3. Organic solute in leaves (A) and roots (B) of peanut genotypes submitted to water stress. C- control and S- stressed treatments. Bar indicates standard deviation.

Table 4. Combined analysis of production components and drought tolerance efficiency of peanut genotypes grown under irrigated and rainfed conditions. Barbalha, CE (2013/14).

Genotype	PY (tha^{-1})		DR	SY (tha^{-1})		DR	HI (%)		DR	DTE (%)
	I	R		I	R		I	R		
BR 1	2.71c	1.75b	36	1.98c	1.22c	38	44b	36a	18	65a
L46	2.89c	1.91a	34	2.01c	1.34b	33	45b	38a	16	66a
L59	3.21b	1.89a	41	2.34b	1.32b	44	45b	37a	18	59b
LBR Branco	3.36b	2.01a	40	2.35b	1.35b	42	47a	37a	21	60b
LViPE-06	4.68a	2.10a	55	3.28a	1.40a	57	51a	33b	35	45c
55437	2.16d	1.65b	24	1.58d	1.22c	23	41c	36a	12	76a
Mean	3.16	1.88		2,26	1.31		45.5	36.17		62
CV (%)	21.4	24.6		12.1	10.33		9.56	8.98		9.32

I-irrigated, R- rainfed, PY- pod yield, SY- seed yield, HI-harvest index, DTE- drought tolerance efficiency. DR- relative difference based on irrigated crop. Means followed by the same letter are not statistically different (Tukey, $p \leq 0.05$).

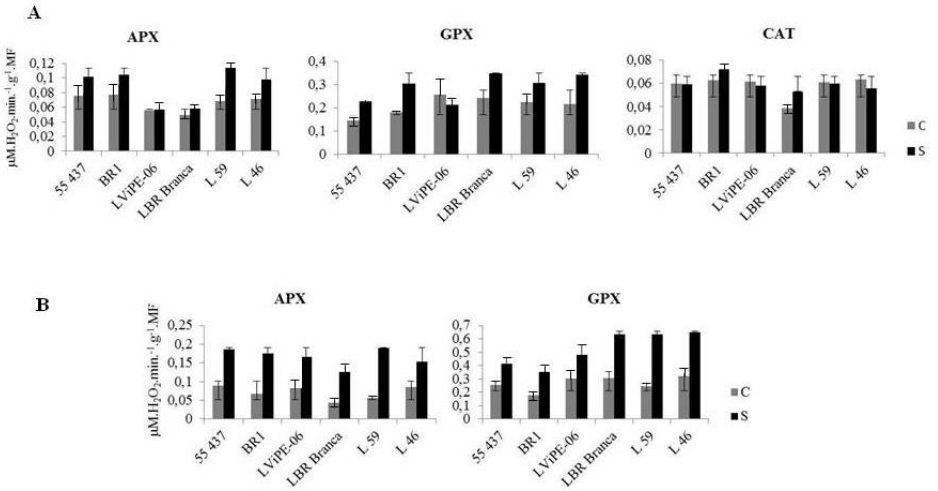

Fig 4. Activity of antioxidant enzymes in leaves (A) and roots (B) of peanut genotypes submitted to water stress. C- control and S- stressed treatments. Bar indicates standard deviation.

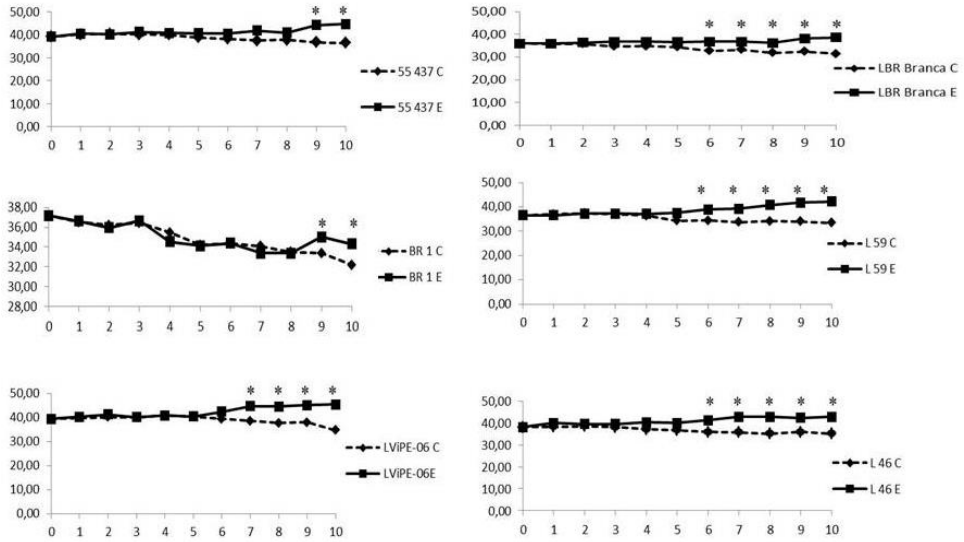

Fig 5. Monitoring of chlorophyll (µmol.m-2) in peanut genotypes during 10 d of water stress. C- control and S- stressed treatments. (*) Indicates statistical difference between treatments ($p \leq 0.05$, Tukey test).

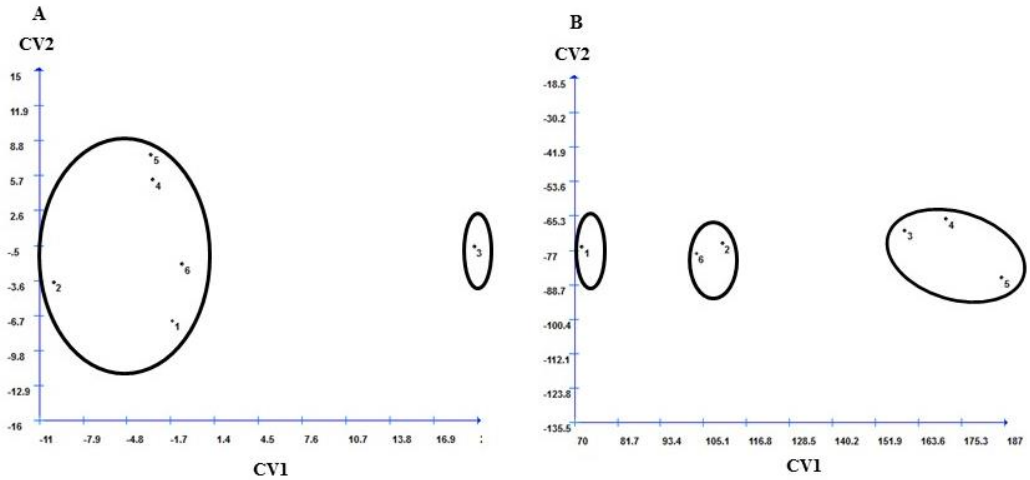

Fig 6. Graphical dispersion, in relation to two axes representing the first two Canonical Variables (CV1 and CV2), obtained from physiological and biochemical traits in control (A) and water stressed (B) treatments of six peanut genotypes. 1- 55437, 2- BR 1, 3- LViPE-06, 4- LBR Branco, 5- L59, 6- L46.

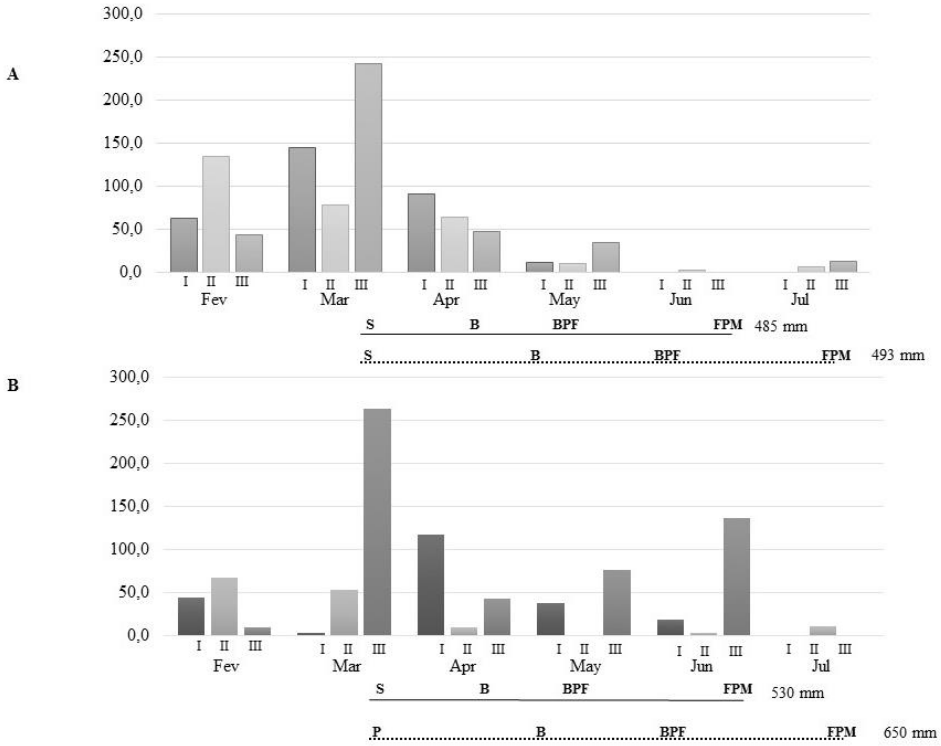

Fig 7. Graphical representation of the rainfall (mm) in Barbalha (CE) during peanut cycle in 2014(A) and 2013(B). I, II and III: total rainfall to each 10 days. Main events in peanut phenology: S- sowing, B- blooming, BFP- beginning of pod formation, FPM- full pod maturation. Solid and dotted lines- upright and runner genotypes, respectively.

used, indicating that the genotypes responded differently to water treatments. Table 3. presents the estimation of individual and accumulated eigenvalues of canonical variables obtained from matrix, generated by physiological and biochemical traits. Each canonical variable is the linear combination of the independently measured variables and is orthogonal to the others. In this analysis, the first two canonical variables were significant ($p \leq 0.001$) and accounted for 97.83% of the total variation among genotypes. According to Cruz et al. (2012), this value allows to represent all variance in a two-dimensional plot. The variables that most contributed to the genetic diversity based on D^2 technique were: Free proline, GPX and Carbohydrate, in roots; proline in leaves; APX in roots and leaves; GPX and Carbohydrate in the leaves; protein in the roots, and catalase in leaves.

The dispersion graphic obtained from physiological and biochemical traits in control and water stressed treatments of peanut genotypes is shown in Fig 6. Canonical variables clustered the genotypes of control treatment into two groups: one containing only LViPE-06, and other with the remaining genotypes (Fig 6A). This separation agrees perfectly with agronomical profile of genotypes selected in this work, indicating the robustness of canonical analysis to discriminating botanical groups in autogamous species.

In graphic of stressed treatment (Fig 6B), three groups were formed whose overall result also corroborate with the genetic nature of the materials, when submitted to drought: Group 1, represented by only *cv*. 55437, confirming results in Fig 2, due its high earliness and tolerance to drought (Clavel et al., 2004; Kanyika et al., 2015; Pereira et al., 2012), Group 2, clustering *cv*. BR 1 and L46, which are the more promising material (lines) to advance in drought tolerant-peanut breeding, and Group 3, represented by intermediates L59 and LBR Branco, and late LViPE-06.

In both treatments, canonical analysis was useful to identify the genetic variation and the traits that better describe the variation among genotypes tolerant to drought. Cluster analysis was contributive in differentiating the lines with perspective to meet the goals of breeding program focused on yield and adaptation to semiarid environment.

Yield components and drought tolerance efficiency

In order to validate the results generated by clustering analysis (Fig 6B), the genotypes used in physio-biochemical assays were also tested during two years in semiarid environment, grown under irrigated and rainfall conditions. Pod and seed yield, HI and DTE were estimated in both situations. The combined analysis of variance carried out during two years showed statistically difference between genotypes (G), treatments (rainfed and irrigated treatments) and G × T interactions, indicating that genotypes responded differently to water availability in field conditions.

The averages of traits obtained during two years in irrigated and rainfed crops are found in Table 4. The availability of water established in irrigated crop (680 mm), contributed to high pod yield of genotypes, standing out LViPE-06 (4.68 tha^{-1}), LBR Branco (3.36 tha^{-1}) and L59 (3.21 tha^{-1}). The same trend was followed for seed yield, estimated in 3.28 $tha^{-1,}$ 2.35 tha^{-1} and 2.34 $tha^{-1,}$ respectively. In rainfed treatment, the total volume of rainfall was satisfactory for both upright and runner genotypes; however, the phenology was impaired due to distribution and irregularity of rains (Fig 7). All genotypes showed reduction in pod and seed yield, more expressively for LViPE-06 (mean: 56%, Table 4). As it is a

late cycle genotype (120-130 d), it faced long drought period in vital phases of crop phenology, such as flowering and early pod formation (Puangbut et al., 2010; Santos et al., 1997), in both experimental assays. LBR Branco and L59 (110-114 d) were also faced water stress during this period; however, they were able to adjust to low volume of rainfall and complete reasonably the pod maturation. The pod and seed yield losses were recorded ~42%.

The upright genotypes faced minor difficulties during dry period, mainly because the beginning of pod formation was already established. No genotypes revealed an adjustment pattern as 55437 showed only 24% of reduction in pod and seed yield, confirming high drought tolerance and adaptation to semiarid environments (Boote et al., 1982; Duarte et al., 2013; Nogueira et al., 1998; Reddy et al., 2003). Among the lines, the L46 showed production close to BR 1 in irrigated treatment, and 34% of reduction in rainfed season. This good performance reflected in reduced losses of HI measured in the rainy season, which was close to *cv*. 55437.

Regarding to DTE, the *cv.* 55437 showed high efficiency (76%), followed by L46 (66%) and BR 1 (65%) that confirmed the genetic similarity in clustering analysis (Fig 6B). Therefore, L46 proves to be a highly promising material to advance in peanut breeding focused on semiarid region.

The lines LBR Branco (60%) and L59 (59%) showed intermediate behavior between parents and, as both line are runner types, they could be further promising alternative to environment with rainfall up to 400 mm. In Brazilian semiarid region, few commercial runner cultivars are available due to narrow adaptation and high sensitivity to drought. According to Santos et al. (2012a), the yield of runner types are quite dependent on well stablished water availability and recommendation of genotypes adapted to semiarid region could provide better competitiveness to farmers that also demand for this botanical types.

Materials and Methods

Genetic resources

In order to identify lines tolerant to drought, a previous analysis of genetic divergence was carried out based on molecular assays, using eighteen peanut genotypes. Fourteen (LBR Branco, L Runner, L 37, L 46, L 51, L 59, L 60, L 66, L 67, L 68, L 73, L 75, L 77 and L81) are high yield- elite lines (F_7), provided by Peanut Breeding Program, from EMBRAPA. The others genotypes are: BR 1 (*fastigiata*) and LViPE-06 (*hypogaea*), are drought tolerant and high yield genotypes, respectively, and parents of elite lines (Luz et al., 2014; Pereira et al., 2012), IAC Caiapó (*hypogaea*) is a high yield Virginia-type, developed by Agronomic Institute of Campinas, Brazil (IAC), and recommended to tropical environments (Godoy et al., 1999), and 55 437 (*vulgaris*) is a drought tolerant Spanish type, from Institut sénégalais de recherches agricoles – Centre de coopération internationale en recherche agronomique pour le développement (Isra-Cirad) (Clavel et al., 2004; Boote et al., 1982). The genealogy, origin and other agronomical traits are described in Table 1.

Fifteen seeds from each genotype were sown in pots in a greenhouse. Young leaves from 15d- plants were collected for each accession and immediately placed in liquid nitrogen for molecular assays.

Extraction of DNA and SSR-PCR assays

DNA from leaves was extracted using DNeasy Plant Mini Kit (Qiagen), following manufacturer' instructions. The PCR assays were performed in a Mastercycler Gradient (Eppendorf, Germany). The reaction mixture (25 µL) contained 10X PCR buffer (Fermentas), 25 mM $MgCl_2$, 100 mM dNTP, 20 ng of each primer, 20 ng of genomic DNA and 1 unit of Taq DNA polymerase (Fermentas). The SSR primers were provided by biotechnologist team from Embrapa Algodão (Supplementary Table 1), which were specific for drought tolerance and deposited in gene bank (Fragoso, 2010). The samples were subjected to a initial cycle of denaturation at 94°C /5 min, followed by 30 repeats of the following cycle: denaturation at 94°C/45 sec, annealing at 55-57°C/30 sec, and extension at 72°C/30 sec min. A final extension cycle was added to reaction at 94°C/5 min. The amplified products were analyzed by electrophoresis on 7% SDS-polyacrylamide gel in 1X TBE buffer at 60V for 3 h. The gel was stained with Silver nitrate (0.2%), following Creste et al. (2001) procedures.

Statistical analysis

SSR markers obtained from 18 genotypes were scored for their presence '1' or absence '0' of bands for each primer. The binary data were used to estimate levels of polymorphism by dividing the polymorphic bands by the total number of scored bands. Pair-wise similarity matrix was generated by Jaccard's coefficient (Sneath and Sokal, 1973), using NTSYS-pc (Rohlf, 2002). A dendrogram was constructed by UPGMA method to identify the phenetic representation of genotypes. The accuracy of clustering was evaluated by cophenetic correlation coefficient (CCC) and the significance of the groups was tested with 1,000 simulations. Lines grouped in same cluster of BR 1 were chosen for further physiological and biochemical assays.

Water stress assays in greenhouse

Seeds of lines selected in previous assay were grown in greenhouse in order to select lines tolerant to water stress using physiological and biochemical traits. The parents and *cv*. 55437 were also added to assays. Sowing was carried out in pots (10 kg) containing sandy loam soil previously fertilized with NPK, 20:60:30, ammonium sulfate, single superphosphate and potassium chloride. Fourteen days after emergence, seedlings were thinned to two per pot. The watering was daily until seedlings aged 20 days, when water treatments were established: C- control (100% field capacity) and E- plants subjected to 10 d of withholding water. Field capacity was determined by gravimetric method after 72 h of draining. Pots of both treatments were weighted daily. In order to prevent the losses by evaporation, surface of pots was covered with polyethylene discs. In control treatment, the water lost by transpiration was replaced. A completely randomized design with bi-factorial scheme was adopted (6×2), with 6 replications. Temperature (28-34 °C) and relative humidity (57-68%) were collected daily during assay period.

Physiological and biochemical traits

After 10 d of water stress, roots and leaves of treatments were collected for physiological and biochemical assays. Stomata conductance, transpiration and photosynthesis were estimated using LI-6400XT Portable Photosynthesis System (LICOR). The stomata monitoring was carried out at 10 h - 12 h in fully expanded leaves. Total chlorophyll was estimated daily using ClorofiLOG CFL 1030 (Falker, Porto Alegre, RS, Brazil).

For biochemical analysis, a crude extract (25%) of leaves and roots was prepared in phosphate monobasic buffer (100 mM) and EDTA (0.1 mM), pH 7.0. Organic solutes and antioxidative enzymes were estimated by spectrophotometry (Biomate 3, USA): total protein (Bradford, 1976), at 595 nm; free proline (Bates et al., 1973), at 520 nm; total carbohydrates (Dubois et al., 1956), at 490 nm; catalase (Beers Junior and Sizer, 1952), at 240 nm; ascorbate peroxidase (Nakano and Asada, 1981), at 290 nm; guaiacol peroxidase (Urbanek et al., 1991), at 470 nm. Data were subjected to analysis of variance using GENES software, version 5.1.2013 (Cruz, 2013). Tukey's test ($p \leq 0.05$) was adopted to mean comparisons.

Cluster analysis by canonical discriminant analysis (CDA)

Data obtained from physiological and biochemical traits in control and stressed treatments were analyzed by CDA-multivariate methodology. Canonical variables are linear combinations of the original quantitative measurements that contain the highest possible multiple correlation with each group. In CDA, the differentiation of groups is based on the correlation among the independent variables (traits) and their relationships with the dependent variable (genotypes) (Vaylay and van Santen, 2002). The Mahalanobis distance (D^2) was used in a nonhierarchical clustering procedure to quantify the relative importance of the traits for genetic diversity and to identify the main descriptors associated with tolerance. The scores of the eigenvalues and eigenvectors were plotted in two-dimensional projection. Analyses were performed using GENES software, version 5.1.2013 (Cruz, 2013).

Yield estimation and efficiency of drought tolerance in field condition

In order to validate the physiological and biochemical results, genotypes used in greenhouse assay were grown in a 2-year experiment (2013/2014), carried out in a representative region of semiarid weather, located at Experimental Field of EMBRAPA (Brazilian Company of Agricultural Research), in Barbalha, CE (07°18'18"S; 39°18'07"W, 414 m). Genotypes were grown under rainfed (March/July) and irrigated (Aug/Dez) conditions aiming to estimate yield traits and the efficiency of tolerance to drought, following methodology reported in Arunachalam and Kannan (2013) and Ndunguru et al. (1995). Soil (Vertisoil type), was previously limed and fertilized (NPK, 20:60:30, ammonium sulfate, single superphosphate and potassium chloride). Genotypes were sown in plots (3 rows of 5 m length), spaced in 70×20 cm, in a randomized complete block design with five replications. Four seeds were sown per hill. After emergence they were thinned to only two seedlings. The experiment was surrounded by guard rows to avoid damage and boarder effects. The crop was grown by adopting recommended package of practices, described by Santos et al. (2006). The total rainfall recorded during peanut growing in rainfed season (4 months) was 650 mm in 2013 and 493 mm in 2014. To irrigated treatment, the estimative of water requirements to peanut crop in Barbalha, CE, was 680 mm, according recommended by Barreto and Luz (2006).

Harvest was started from 85 d until 120 d, based on full maturation of pods estimated randomly in the plots. The following traits were recorded: pod and seed yield, harvest index (HI) and drought tolerance efficiency (DTE). The HI was estimated by pod yield/total biomass production ratio, based on dry weight of the plants (Painawadee et al., 2009). The DTE was estimated by ratio of pod yield obtained in rainy/irrigated seasons, as suggested in Fischer and Wood (1981). According to authors, genotypes with high value of DTE are considered as drought tolerant. Data were subjected to analysis of variance using GENES software, version 5.1.2013. Means were compared by Tukey test ($p \leq 0.05$).

Conclusions

Water stress induced changes in gas exchange, osmotic adjustment and antioxidant system in peanut top lines. Based on clustering analysis using physiological, biochemical and agronomical traits, the top line L46 was more suitable to semiarid environment, due to agronomic behavior and efficiency to drought tolerance.

Acknowledgements

To REPENSA/Embrapa for financial support and to CNPq and CAPES for grants.

Conflict of Interest

The authors declare no conflict of interest between the partners with the dissemination of results.

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