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Metabolic features involved in drought stress tolerance mechanisms in peanut nodules and their contribution to biological nitrogen fixation

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ABSTRACT

Legumes belong to the most important crops worldwide. They increase soil fertility due their ability to establish symbiotic associations with soil microorganisms, known as rhizobia, capable of fixing nitrogen from the atmosphere. However, they are frequently exposed to abiotic stress conditions in particular drought. Such adverse conditions impair the biological nitrogen fixation (BNF) and depend largely on the legume. Therefore, two peanut cultivars with contrasting tolerance to drought, namely the more tolerant EC-98 and the sensitive Granoleico, were investigated to elucidate the relative contribution of BNF to the tolerance to drought. The tolerant cultivar EC-98 sustained growth and BNF similar to the control condition despite the reduced water potential and photosynthesis, suggesting the functioning of distinct metabolic pathways that contributed to enhance the tolerance. The biochemical and metabolomics approaches revealed that nodules from the tolerant cultivar accumulated trehalose, proline and gamma-aminobutyric acid (GABA), metabolites with known function in protecting against drought stress. The amide metabolism was severely affected in nodules from the sensitive cultivar Granoleico as revealed by the low content of asparagine and glutamine in the drought stressed plants. The sensitive cultivar upon rehydration was unable to re-establish a metabolism similar to well-watered plants. This was evidenced by the low level of metabolites and, transcripts and specific activities of enzymes from the carbon (sucrose synthase) and nitrogen (glutamine synthetase) metabolism which decreased below the values of control plants. Therefore, the increased content of metabolites with protective functions under drought stress likely is crucial for the full restoration upon rehydration. Smaller changes of drought stress-related metabolites in nodule are another trait that contributes to the effective control of BNF in the tolerant peanut cultivar (EC-98).

1. Introduction

Legumes are important sources of oil, fiber, micronutrients, minerals, and vegetable proteins suitable for livestock feed and human consumption [1,2]. Besides, they can fix nitrogen as a result of their ability to form symbiotic associations with rhizobia. However, they are frequently exposed to drought stress conditions affecting plant yield and productivity around the world [3]. Considering the predicted increase of the global population and the expansion of semi-arid regions [4], the study of processes underlying drought stress acclimation of crops is of great interest. Nitrogen fixation is impaired in dehydrated tissue [5–10]. This inhibition may contribute to yield losses under drought and, therefore, is an important topic for research. The proposed mechanisms responsible of inhibition of biological nitrogen fixation (BNF) in legumes are inadequate oxygen supply, carbon shortage and nitrogen (N) feedback. More recently, the role of oxidative stress and sulfur metabolism has been discussed [11]. For all these mechanisms, which can occur in a simultaneous manner, it is important to consider that species and cultivars behave different and, till now, it is impossible to describe a unique mechanism underlying nodule metabolic responses to drought. Regarding oxygen levels, the closure of the O_2 diffusion barrier with a subsequent decrease in O_2 availability for bacteroid respiration and the consequent lack of energy to support the highly demanding BNF process were reported for nodules of soybean and bean [12–15]. Besides, the content of leghemoglobin (Lb), the O_2-binding protein, was diminished at the transcript level in soybean [16] and at

Abbreviations: BNF, biological nitrogen fixation; DW, dry weight; GS, glutamine synthetase; NDFA, nitrogen derived from the atmosphere; SS, sucrose synthase; ROS, reactive oxygen species

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the protein level in *Vicia faba*, pigeon pea, and common bean [13,17,18]. Both mechanisms can explain the O_2-dependent restrictions in the bacteroid, responsible of the ATP limitation for respiration and in the last instance reduced BNF. However, exceptions for those observations were reported [7,9,19,20] suggesting that O_2 limitation plays no central regulatory role in the regulation of BNF [11]. An alternative mechanism proposed is the availability of carbon fixed by photosynthesis which is essential to sustain the bacteroid respiration and nitrogenase activity. However in some cases the photosynthetic activity is maintained longer than BNF [21]. Some authors proposed that the sucrose supply can be supported due to starch hydrolysis [16,19,22]. Inside the nodule, the enzyme sucrose synthase (SS) is responsible for sucrose cleavage for subsequent synthesis of organic acids, mainly malate, that provide carbon skeletons for the fixing activity. The decline in SS activity was proposed to cause a limited BNF in drought-stressed soybean, pea and common bean [4,19,20,21,22–24]. However, the decline in SS activity does not explain the low BNF in forage legumes from the genus *Medicago* [9,11]. Accumulation of nitrogen-containing compounds in nodules was linked to inhibition of export. Thus, decreased water efflux from nodules has been proposed as an alternative explanation of impaired BNF by an N-feedback regulation. Efforts were made to decipher molecules responsible for this inhibition such as ureides [25,26], glutamine [27], asparagine [26,28], and aspartate [29]. However, recent reports point to a more complex explanation that involves the complete pool of aminoacids rather than a single molecule [20,30,31]. It is important to notice that most of the references available are from ureide-exporting legumes, thus there is a need to study amide-exporting legume species. Recently, alterations in sulfur metabolism related to declines in BNF were proposed by Larrainzar et al. [8,10] and Irar et al. [32] who showed a decline in the enzymes responsible for the biosynthesis of methionine and S-adenosylmethionine (SAM) in *M. truncatula* and pea. SAM is required for the synthesis of ethylene and aspartate-related aminoacids and former research showed that the levels of this phytohormone were reduced upon drought stress conditions. Although S availability does not seem to be a limiting factor for BNF in drought-stressed *M. truncatula* plants, the reduced ethylene production could have some implications in N fixing signaling which demands future research [33]. Finally, a role of reactive oxygen species (ROS) was proposed to lower BNF due to oxidative damage of biomolecules including proteins with antioxidant activity [34,35] and the autooxidation of leghemoglobin and nitrogenase [9,36]. On the other hand, the oxidative stress had a direct effect on the functionality of SS at both the transcriptional and post-transcriptional level by the processes of reversible sulfenylation [37,38].

Considering the profound differences among legumes, generalizations of findings and conclusions often are inappropriate. Therefore, it is necessary to elucidate the behaviour of a distinctive legume such as peanut which is a tropical legume that forms determinate nodules and exports N-products mainly in the form of amides [39]. Peanut (*Arachis hypogaea* L.) establishes symbiosis with bacteria from the genus *Bradyrhizobium* sp. constituting an important source of N supply for the soil. Peanut crops are economically important in Argentina due the incoming benefits from exportation, since almost the 80% of the production is destined to the European Union, Russia and China [40]. However, it is noteworthy that the crop areas suffer intermittent periods of water deficit affecting the yield and the consequent economic activities [41]. To counteract the decreased food production and quality due to environmental constraints it is necessary to develop new crop varieties adapted to environmental changes [4]. Efforts were made to develop peanut cultivars with improved performance under environmental conditions such as drought; therefore, peanut cultivars differing in drought stress tolerance are now available for producers. Peanut genotypes exposed to drought stress revealed phenotypic variation in terms of BNF [42,43] which is a major trait affecting legume productivity under environmental constraints [44]. Therefore, in order to elucidate the metabolic features involved in drought stress tolerance mechanisms in peanut nodules, physiological, biochemical and metabolomics approaches were performed in two peanut cultivars with contrasting tolerance to drought.

2. Materials and methods

2.1. Plant material and treatments

Seeds of the two peanut cultivars Granoleico and EC-98, previously characterized by Faustinelli et al. [45] as sensitive and tolerant genoytpes, respectively, were obtained from Criadero El Carmen (General Cabrera, Córdoba, Argentina). To establish the symbiosis, the strain able to infect peanut plants *Bradyrhizobium* sp. SEMIA6144, was provided by MIRCEN (Porto Alegre, Brazil). The assay was performed as described before [46]. Briefly, sterilised and pre-germinated seeds were transferred to pots filled with sterile sand:perlite (2:1) and seven days after sowing they were inoculated with 4 ml of yeast extract–mannitol culture containing 10^8 CFU of *Bradyrhizobium* sp. ml^{-1}. Plants were grown in a controlled growth chamber and irrigated in order to keep the field capacity. Thirty days after sowing plants were separated at random into three experimental groups: (a) the control, where plants were kept under normal irrigation conditions (soil water content at field capacity: 13%); (b) drought stress, where the irrigation was suspended for 14 d and (c) drought stressed and rehydrated, where plants subjected to 14 d drought stress were re-irrigated for 72 h. At harvest, the fully expanded second nodal leaves were used to measure the water potential using a pressure bomb (Model 10, Bio-Control, Buenos Aires, Argentina) [47]. Nodules were harvested into liquid nitrogen and stored at −80 °C until use.

2.2. Physiological status indicators

At the end of the water-deficit period (14 d without irrigation) and the rehydration treatment (14 d without irrigation followed by 72 h of re-irrigation), the effective quantum yield of PSII (ΦPSII) was determined in fully expanded second nodal leaves using the Mini-PAM Fluorometer (Walz, Germany). Light intensity was set to 230 µmol quanta $m^{-2}\,s^{-1}$. Measurements were done between 10 and 12 am.

2.3. Growth and biological nitrogen fixation variables

Shoots and roots were dried at 70 °C until constant weight to determine the dry weight (DW). Nodules were counted and the dry weight was recorded. The nitrogen content per plant and the proportion of nitrogen derived from the atmosphere (NDFA) were calculated as estimate of BNF [48,49]. The oven-dried leaves were pulverized in a Wiley mill using a 0.5 mm mesh (Arthur H Thomas, California, USA). Between 2.1 and 2.2 mg of each sample was weighed into 8 mm by 5 mm tin capsules (Elemental Microanalysis Ltd., Devon, UK) on a Sartorius microbalance (Sartorius, Göttingen, Germany). The isotopic ratio of $\delta^{15}N$ was calculated as $\delta = (R_{sample}/R_{standard}\text{-}1)*1000$, where R is the molar ratio of the heavier to the lighter isotope of the sample and standards as defined by Farquhar et al. [50]. The $\delta^{15}N$ values for the nitrogen gases released were determined on a Finnigan Matt 252 mass spectrometer (Finnigan MAT GmbH, Bremen, Germany), which was connected to a CHN analyser by a Finnigan MAT Conflo control unit. Three standards were used to correct the samples for machine drift; two in-house standards (Merck Gel and Nasturtium) and one IAEA (International Atomic Energy Agency) standard-$(NH_4)_2SO_4$. NDFA (%) was calculated according to Shearer and Kohl [51]: %NDFA = 100(($\delta^{15}N$ reference plant − $\delta^{15}N$ legume)/($\delta^{15}N$ reference plant − B value)). Where the reference plant was maize (*Zea mays* L.) grown under the same glasshouse conditions. The B-value is the $\delta^{15}N$ natural abundance of the N derived from biological N-fixation of the above-ground tissue of *Lupinus luteus*, grown in N-free solution [52,53].

2.4. Metabolite profiling

2.4.1. Metabolite extraction

10 mg of lyophilized nodules were homogenized using a ribolyzer (3 cycles: 3 × 45 s, 6.5 m s^{-1}) with 0.5 g of zirconia beads (1 mm diameter, Roth) and 1 ml 80% methanol containing 10 µM ribitol as internal standard. The homogenate was centrifuged at 12.000 x*g* for 20 min at room temperature and 750 µl of supernatant was dried in a stream of nitrogen gas. Samples were derivatized at 37 °C by dissolving them in 75 µl methoxylamin-hydrochloride (20 mg ml^{-1} in pyridine, Sigma–Aldrich) for 90 min and afterwards addition of 75 µl *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (Macherey–Nagel) with alkane retention time standards dissolved in pyridine (C12, C15, C18, C19, C22, C28, C32 at 0.55 mg ml^{-1} final concentration each and C36 at 1.1 mg ml^{-1} final concentration) (Sigma Aldrich) for 30 min.

2.4.2. Gas chromatography–mass spectrometry

GC–MS was done for high abundant compounds by a TRACE GC Ultra gas chromatograph coupled to an ITQ 900 GC–Ion Trap MS (both Thermo ScientificTM). The instrument was equipped with a Rtx®-5MS column (30 m, iD 0.25, df 0.25 µm; Restek). One µl sample was injected (splitless) for GC–MS analysis. The oven program was: 3 min at 80 °C, ramp with 5 °C/min up to 325 °C, 2 min at 325 °C. Transfer line temperature was set to 250 °C and ion source on 220 °C. Mass spectra were recorded from m/z = 50 to 750. Replicate samples were derivatized and measured separately with intervals of at least three days. A blank to check carry over of metabolites was run every five to six samples. To evaluate constant chromatographic performance and sensitivity, a defined complex biological sample derived from germinating *Medicago truncatula* seeds was measured once a week. Samples were measured at least in technical triplicates on one biological replicate.

2.4.3. Metabolite identification and analysis

Raw data were converted to cdf-files by Xcalibur software (Thermo) and uploaded to the MeltDB software [54]. Peak detection in chromatograms was done with a signal to noise ratio of 5 followed by a multiple profiling to identify common MS patterns and to tag unknown compounds. Metabolites were identified according to retention index [55] and additional mass spectra fitting to separately measured reference substances. Quantification was performed on peak areas of characteristic compound masses, normalized to ribitol (m/z 217) and material dry weight. Ribitol was proven to be absent in the biological samples by triplicate measurements of selected samples extracted without ribitol. Principal component analysis (PCA) was performed in the software R v. 2.25.2 using all the recognized metabolites from nodules of all the treatments in the two cultivars. The differentially accumulated metabolites were clustered using the euclidean distance and average linkage in Cluster 3.0 [56]. Heat maps were generated using Java Treeview [57].

2.5. RNA isolation, cDNA synthesis and transcript profiling

RNA isolation and the subsequent cDNA synthesis were performed according to Wormuth et al. [58]. Semiquantitative reverse transcription–PCR analysis was carried out as previously described by Finkemeier et al. [59]. The used primer combinations were designed by comparison of annotated genes of legumes and the peanut database and are listed in Supplementary Table S1 in the online version at DOI: http://dx.doi.org/10.1016/j.plantsci.2017.06.009. The identity of all PCR products was confirmed by sequence analysis at the Center of Biotechnology of Bielefeld University (Bielefeld, Germany). Quantitative real-time PCR (q-PCR) analysis was carried out on the iCycler Thermal Cycler (Bio-Rad, USA) with the iQ SYBR Green Supermix (Bio-Rad) in a final volume of 20 µl according to the manufacturer's instructions. The iCycler was programmed to 94 °C for 3 min, 45 × (94 °C for 15 s, 57 °C or 58 °C for 30 s, 72 °C for 45 s) and 72 °C for 10 min followed by a melting curve program (55–95 °C with increments of 0.5 °C). The efficiency of each reaction was calculated using LinRegPCR software [60]. Signal values were subsequently derived from the threshold cycles (the average background was subtracted) using the equation of Pfaffl [61].

2.6. Specific activities of carbon and nitrogen metabolism enzymes

The glutamine synthetase (GS) activity of the supernatant was measured using the ATP-dependent biosynthetic assay described by Bielawski [62]. Nodule tissue was ground in a mortar with 50 mM Tris-HCl (pH 8), 5 mM EDTA, 1 mM magnesium acetate, 1 mM DTT, 10% glycerol, and 5% ethylene glycol containing 1% polyvinylpolypyrrolidone (10 ml g^{-1} fresh weight) and the homogenate was clarified by centrifugation for 20 min at 12.000 *xg*, 4 °C. A 1.0-ml reaction contained 100 mM Tris-HCl buffer (pH 7.7), 1 mM EDTA, 5 mM $MgCl_2$, 8 mM ATP, 80 mM glutamic acid, 6 mM hydroxylamine, and 0.01-0.1 ml enzyme. The reaction was terminated after 30 min of incubation at 30 °C by the addition of 0.25 ml of 0.5 M TCA. The precipitated protein was centrifuged at 6.000 *xg* for 5 min and 0.5 ml 0.57 M $FeCl_3$ in 1 M HCl was added to 1 ml supernatant. The absorbance was read at 540 nm in the presence of the control sample (without ATP). One unit of GS was defined as formation of 1 nmol y-glutamyl hydroxamate (GH) min^{-1}.

To quantify the sucrose synthase (SS) specific activity the procedure by Morell and Copeland [63] was followed. Nodules were homogenized with pestle in a mortar with 50 mM MOPS, 10 mM 2-mercaptoethanol, 4 mM $MgCl_2$, pH 7, 4 °C (10 ml g^{-1} fresh weight). The homogenate was centrifuged for 20 min at 12.000 *xg*, 4 °C. Sucrose synthase specific activity was determined in microplates in a final volume of 50 µl, at 30 °C and 340 nm for 10 min in the assay media containing 50 mM HEPPS pH 7.0, 5 mM $MgCl_2$, 100 mM sucrose, 1 mM ATP, 2 mM $NADP^+$, and 2 units each of hexokinase, phosphoglucose isomerasae and glucose-6-phosphate dehydrogenase. The reaction was started by addition of uridine diphosphate (UDP) to a final concentration of 2 mM. Controls lacking UDP were performed simultaneously. The protein concentration was determined by the dye-binding method of Bradford [64] using BSA as a standard. One unit of SS was defined as 1 nmol NADPH formed min^{-1}.

2.7. Statistical analysis

Experiments were conducted in a completely randomised design and repeated three times. The data were analysed using ANOVA and Duncan's test at $P < 0.05$. Assuming that genotype and drought/rehydration treatments are two *a priori* independent factors that possibly interact, ANOVA was applied using a 2 × 4 group design. If the p-value associated with the interaction was significant at $P < 0.05$, the means of the factor level A were compared for those treatments that received the same level of factor B and *vice versa* (Supplementary Table S3 in the online version at DOI: http://dx.doi.org/10.1016/j.plantsci.2017.06.009). Prior to the test of significance, the normality and homogeneity of variance were verified using the modified Shapiro–Wilk and Levene tests, respectively. If homogeneity of variance was not given, data were transformed using an appropriate function.

3. Results

3.1. Physiological status of peanut cultivars with contrasting drought tolerance

Peanut plants exposed to drought stress had lower water potential than well-irrigated plants. Water potentials of re-irrigated plants recovered to that of well-watered control plants. Both cultivars behaved in the same way upon the treatments (Fig. 1). The effective quantum yield of PSII was severely reduced by 45% in the tolerant cultivar (EC-

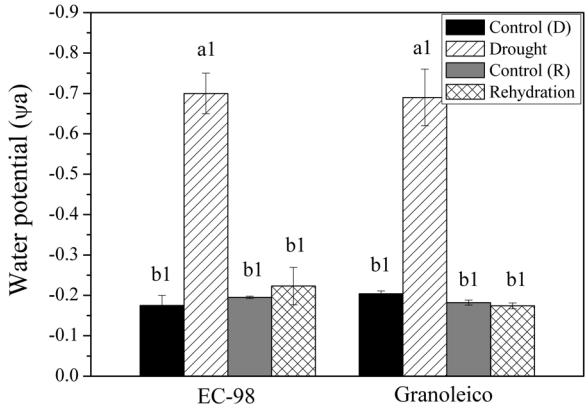

Fig. 1. Water potentials in leaves of peanut cultivars (EC-98 and Granoleico) differing in drought stress tolerance after exposure to drought stress for 14 d and rehydration for 3 d. Values are means ± SE (n = 9). Different letters in columns indicate significant differences between treatments for each cultivar at $P < 0.05$ according to Duncan's test. No differences between cultivars for each treatment at $P < 0.05$ were found according to Duncan's test.

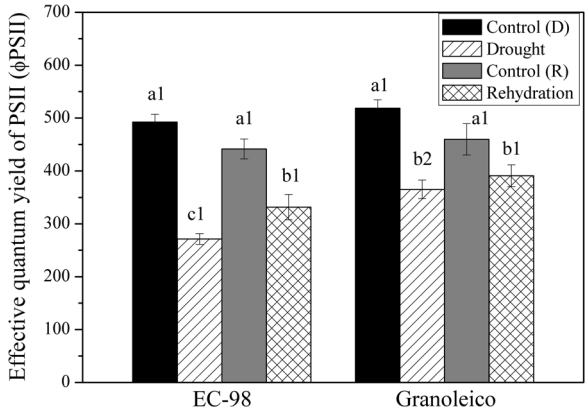

Fig. 2. Effective quantum yield of PSII (ΦPSII) in leaves of peanut cultivars (EC-98 and Granoleico) differing in drought stress tolerance during a drought stress and rehydration experiment.
Values are means ± SE (n = 9). Different letters in columns indicate significant differences between treatments for each cultivar at $P < 0.05$ according to Duncan's test. Asterisks in columns indicate differences between cultivars for each treatment at $P < 0.05$ according to Duncan's test.

98) exposed to drought stress in comparison with the controls but after 72 h of rehydration plants showed a significant increase, although smaller than control (well-irrigated) plants. In the sensitive cultivar Granoleico exposed to drought stress, the ΦPSII was 30% lower than in control plants but interestingly still higher than in the drought-stressed plants from the tolerant cultivar. Rehydrated plants were not able to restore the ΦPSII in similar level than controls exhibiting a similar value than drought-stressed ones (Fig. 2). This likely indicates that the tolerant EC-98 activated energy dissipation mechanisms more efficiently than Granoleico under drought, but also relieves this inhibition upon rehydration.

3.2. Growth and biological nitrogen fixation in peanut cultivars exposed to drought stress and rehydration

The root dry weight of peanut plants from the tolerant cultivar (EC-98) exposed to drought stress was significantly increased (25%) in comparison with well-irrigated and rehydrated plants. In the sensitive cultivar (Granoleico) a similar behaviour was observed. Plants from the cultivar Granoleico had a higher root biomass compared to plants from the cultivar EC-98 irrespective of the treatment (Table 1). The shoot dry weight of plants exposed to drought stress and subsequent rehydration did not show differences among the treatments in the tolerant cultivar EC-98. The shoot dry weight of the sensitive cultivar Granoleico was reduced by 25%, and plants rehydrated for 72 h maintained a lower shoot dry weight compared to well-irrigated plants. The root/shoot ratio was increased in drought-stressed plants from Granoleico but it was statistically unaltered in EC-98 (Table 1).

The nodulation process decreased by around 30% in treated peanut plants as revealed by the nodule number in drought-stressed and rehydrated plants compared to well-irrigated plants in both cultivars. However, the nodule dry weight did not show variations in EC-98 while it decreased by around 35% in drought-stressed as well as rehydrated plants from the sensitive cultivar Granoleico. In a similar manner, the nitrogen content of peanut plants from the tolerant cultivar was unaffected upon exposure to the different treatments but in the sensitive cultivar was decreased by 32% and 27% after the exposure to drought stress and rehydration for 72 h, respectively, compared to well-watered plants. The nitrogen derived from the atmosphere (NDFA) was unaltered in treated (drought-stressed and rehydrated) plants from the tolerant cultivar. However, in the sensitive cultivar, the NDFA was inhibited by 15% in response to drought stress and rehydration compared to control plants. Therefore, the NDFA values of treated plants were higher in the tolerant cultivar than in the sensitive one (Table 2).

3.3. Metabolite profiles of peanut nodules exposed to drought stress and subsequent rehydration

Nodules developed by the interaction between peanut and *Bradyrhizobium* sp. SEMIA 6144 were analysed by GC–MS for high abundant compounds which resulted in the identification of 58 metabolites (Supplementary Table S2 in the online version at DOI: http://dx.doi.org/10.1016/j.plantsci.2017.06.009). It is important to consider that the metabolites analysed constitute a mixture with contributions of both plant and microorganisms. The statistical analysis showed that in the tolerant EC-98 eleven metabolites (18% of the total analysed metabolites) exhibited significant differences in response to drought stress (10 metabolites) and/or rehydration (3 metabolites). However, 22 metabolites corresponding to 38% of the analysed metabolites showed statistically differences in the sensitive Granoleico upon exposure to drought stress (16 metabolites) and/or rehydration (7 metabolites) (Table 3). Almost all metabolites that showed statistical differences in the sensitive cultivar exposed to drought stress were reduced compared to well-irrigated plants. Contrastingly, all metabolites that exhibited statistical differences at 72 h post-rehydration were increased above the control (well-irrigated) plants. It is noteworthy that the tolerant cultivar exhibited a different tendency where, approximately half of the metabolites that showed statistical differences upon exposure to drought stress, were above the levels from well-irrigated plants. After rehydration (72 h), the responding-metabolites were below control levels. In order to reduce the multivariate data complexity, the principal component analysis (PCA) was performed. This method allows for identifying patterns highlighting similarities and differences between samples [65]. In this study, PCA was applied to the information gathered from the metabolomics analysis of control, drought-stressed and rehydrated nodules from two peanut cultivars with varied tolerance to drought, in order to validate the differences among the metabolite profiles of the differently treated tissues and to identify the metabolites that may explain the different physiology. The PCA analysis revealed that the first two principal components explained 40.1% of the total variation between cultivars and treatments. The identified metabolites contributing to the explanation of the variance of the first principal component, namely glycerate 2P, homoserine, S-methylcysteine, alanine, cysteine, asparagine, glycolate, valine are metabolites that were also decreased statistically upon exposure to drought stress according to the statistical analysis in the sensitive Granoleico. Among the metabolites contributing to the second principal

Table 1
Growth parameters of peanut cultivars with contrasting drought stress tolerance and after the exposition to drought stress and rehydration.

Cultivar	Treatment	Root dry weight (g)	Shoot dry weight (g)	Root/Shoot ratio
EC-98	**Control (D)**	0.24 ± 0.02 b	1.23 ± 0.10 a	0.195 ± 0.061 a
	Drought stress	0.32 ± 0.02 a	1.20 ± 0.07 a*	0.266 ± 0.038 a
	Control (R)	0.24 ± 0.01 b	1.12 ± 0.10 a	0.214 ± 0.021 a
	Rehydration	0.28 ± 0.02 a	0.90 ± 0.09 a	0.311 ± 0.035 a
Granoleico	**Control (D)**	0.31 ± 0.02 b*	1.13 ± 0.10 ab	0.277 ± 0.028 b
	Drought stress	0.41 ± 0.02 a*	0.85 ± 0.07 c	0.458 ± 0.022 a*
	Control (R)	0.29 ± 0.03 b	1.40 ± 0.12 a	0.210 ± 0.057 b
	Rehydration	0.35 ± 0.01 ab*	0.99 ± 0.05 bc	0.339 ± 0.035 ab

Values are means ± SE (n = 9). Different letters in columns indicate significant differences between treatments for each cultivar at $P < 0.05$ according to Duncan's test. Asterisks in columns indicate differences between cultivars for each treatment at $P < 0.05$ according to Duncan's test.

component, trehalose and saccharose exhibited an increased amount in response to drought stress in the tolerant EC-98 (Fig. 3).

Hierarchical clustering allowed the identification of groups of metabolites belonging to distinct categories: the first group included ß-alanine, citrate and 4-aminobutyrate (GABA) which were accumulated in the tolerant cultivar both under drought and rehydration; the second group included metabolites that were subtly decreased during drought and increased during rehydration in the sensitive cultivar; the third group was formed by the low-responding (< 2 log fold change) or non-responding metabolites; the fourth group (serine, pantothenic acid, citrulline/ornithine/arginine, tryptophan, phenylalanine, glucuronic acid) was composed of metabolites showing an increase during rehydration in the sensitive cultivar; the fifth group contained metabolites with similar trend during drought in both cultivars, namely a decrease in alanine, homoserine, S-methylcysteine, α-ketoglutarate, pyruvate, glycerate, lactate, adenosine and glycolate contents, and an increase in some of them upon rehydration in the sensitive cultivar. The last group included the sugars xylose, sucrose and trehalose which accumulated during drought in both cultivars and decreased upon rehydration in the tolerant cultivar (Fig. 4). The marked increase in the levels of proline resulted in the formation of a separate group that remained distantly separated from all the other studied metabolites.

3.4. Expression and activity of enzymes involved in N and C metabolism in response to drought stress and subsequent rehydration in peanut nodules

A literature search for legumes resulted in the identification of several isoforms corresponding to both glutamine synthetase and sucrose synthase. The available information in the open databases was used to design the peanut nodule primers. After confirming the identity of the obtained fragments, two isoforms of GS (*GS1b* and *GS2a*) and three isoforms of SS (*SS3*, *SS4a* and *SS4b*) were analysed. The qPCR analysis revealed that both GS isoforms respond similarly in the same genotype under all treatments. However a pronounced difference was seen between both cultivars in response to drought stress. *GS1b* and *GS2a* transcript levels decreased in the tolerant EC-98 and increased in the sensitive Granoleico. After 72 h of rehydration, the tolerant cultivar had values similar to control (well-irrigated) plants but in the sensitive cultivar the transcript levels were significantly below the control levels (Fig. 5A).

The different sucrose synthase isoforms were differentially affected by treatments. While *SS3* and *SS4b* decreased after the exposure to drought stress in the tolerant cultivar they increased significantly above the control level in well-irrigated plants in the sensitive cultivar. The *SS4a* isoform did not revealed changes during drought stress. After rehydration (72 h) the peanut nodules showed a significant increment for all three analysed *SS* isoforms in the tolerant cultivar; however, with one exception (*SS4a*), the SS transcript levels were similar to well-irrigated plants in the sensitive cultivar (Fig. 5B).

The specific activity of the ammonium assimilating glutamine synthetase was significantly increased in the tolerant cultivar exposed to drought stress. This response was reversed upon rehydration. In the sensitive cultivar Granoleico the specific activity of GS was decreased in drought-stressed and rehydrated plants in compare to well-watered plants. The most remarkable difference was the opposite trend of the GS levels in drought-stressed plants, being significantly increased in the tolerant cultivar and decreased in the sensitive one, when compared to control (well-irrigated) plants (Table 4).

The sucrose synthase showed a specific activity lower to control plants in response to drought stress in the tolerant cultivar EC-98; however it was increased reaching the levels of well-irrigated plants after rehydration. In the cultivar Granoleico, there were no differences in SS specific activity between plants exposed to drought stress and those maintained under optimal irrigation, but it was significantly decreased in rehydrated plants. Thus the behaviour of the SS activity in the two cultivars was opposite, being significantly lower in the tolerant cultivar in response to drought stress in comparison to the sensitive one. In a contrasting manner, EC-98 plants that underwent drought stress and later recovered showed higher SS activity than Granoleico (Table 4).

Table 2
Nodulation efficiency and biological nitrogen fixation activity in peanut cultivars differing in drought stress tolerance during a drought stress and rehydration cycle.

Cultivar	Treatment	Nodule number	Nodule DW (mg)	N content (mg plant^{-1})	NDFA (%)
EC-98	**Control (D)**	63.38 ± 4.13 a	33.85 ± 3.62 a	29.52 ± 2.14 a	83.31 ± 2.25 a
	Drought stress	43.43 ± 1.32 b	37.46 ± 2.24 a*	28.46 ± 2.42 a	83.49 ± 1.82 a*
	Control (R)	65.89 ± 4.39 a	35.80 ± 3.64 a	27.67 ± 0.58 a	88.08 ± 1.24 a
	Rehydration	46.50 ± 3.04 b	35.70 ± 3.16 a*	24.66 ± 0.49 a	85.45 ± 1.71 a*
Granoleico	**Control (D)**	70.00 ± 3.87 a	26.73 ± 3.69 a	35.14 ± 2.26 ab	82.00 ± 2.41 a
	Drought stress	47.64 ± 2.66 b	16.16 ± 1.64 b	24.24 ± 2.02 c	69.69 ± 3.10 b
	Control (R)	75.96 ± 6.93 a	26.17 ± 2.92 a	41.66 ± 3.02 a*	82.84 ± 3.33 a
	Rehydration	52.76 ± 2.49 b	16.10 ± 2.17 b	30.93 ± 1.51 b*	71.82 ± 2.49 b

Values are means ± SE (n = 9). Different letters in columns indicate significant differences between treatments for each cultivar at $P < 0.05$ according to Duncan's test. Asterisks in columns indicate differences between cultivars for the same treatment at $P < 0.05$ according to Duncan's test. D: control for drought; R: control for rehydration; NDFA: nitrogen derived from the atmosphere.

Table 3
Metabolites showing significant changes in peanut cultivars differing in drought stress tolerance during a drought stress and rehydration experiment.

	EC-98				Granoleico			
	FC_D/CD	t-Student (p)	FC_R/CR	t-Student (p)	FC_D/CD	t-Student (p)	FC_R/CR	t-Student (p)
Aminoacids								
Alanine	0.6	*0.11*	1.0	*0.88*	**0.4***	***0***	1.5	*0.18*
Asparagine	0.7	*0.39*	1.2	*0.39*	**0.7***	***0***	1.0	*0.99*
Citrulline_Ornithine_Arginine	0.6	*0.07*	1.4	*0.24*	1.0	*0.98*	**1.7***	***0.05***
Cysteine	0.9	*0.52*	1.1	*0.48*	**0.8***	***0***	1.3	*0.24*
Glutamate	1.0	*0.81*	1.0	*0.88*	**0.8***	***0.02***	1.0	*0.54*
Glutamine	0.9	*0.87*	1.1	*0.58*	**0.7***	***0***	1.1	*0.61*
Homoserine	0.6	*0.24*	1.1	*0.81*	**0.4***	***0.01***	1.3	*0.41*
Isoleucine	0.6	*0.15*	**0.6***	***0.02***	0.7	*0.33*	1.5	*0.21*
L-Aspartate	0.7	*0.2*	0.9	*0.3*	0.8	*0.16*	**1.4***	***0.04***
Lysine	0.8	*0.09*	0.9	*0.64*	1.0	*0.7*	**1.3***	***0.05***
Proline	**16.0***	***0***	1.0	*0.66*	**28.9***	***0***	1.3	*0.34*
S-Methylcysteine	0.6	*0.38*	1.0	*1*	**0.6***	***0.02***	1.3	*0.33*
Tryptophan	**0.7***	***0.02***	1.1	*0.8*	1.0	*0.92*	2.0	*0.07*
Valine	**0.7***	***0.04***	1.0	*0.88*	0.7	*0.08*	1.4	*0.09*
Organic acids								
a-Ketoglutarate	**0.6***	***0.05***	1.2	*0.18*	0.7	*0.11*	1.4	*0.2*
Citrate	0.9	*0.66*	1.9	*0.07*	1.0	*0.9*	**1.4***	***0.03***
Glycerate	**0.6***	***0.01***	1.0	*0.84*	0.8	*0.14*	1.1	*0.71*
Glycolate	0.7	*0.11*	0.9	*0.51*	**0.6***	***0.02***	0.9	*0.64*
Lactate	**0.5***	***0.05***	1.1	*0.85*	**0.7***	***0.04***	0.9	*0.67*
Pyruvate	**0.6***	***0.03***	0.8	*0.26*	**0.4***	***0***	0.9	*0.39*
Carbohydrates								
Glucuronic acid	0.9	*0.66*	1.1	*0.46*	1.3	*0.23*	**1.9***	***0.01***
Sucrose	**1.7***	***0***	**0.6***	***0***	1.5	*0.17*	1.0	*0.75*
Trehalose	**2.3***	***0.02***	**0.6***	***0***	1.2	*0.48*	0.9	*0.76*
Xylose	1.6	*0.13*	0.8	*0.24*	**1.8***	***0***	1.2	*0.53*
Others								
4-Aminobutyrate (GABA)	**2.3***	***0.03***	1.5	*0.2*	1.1	*0.65*	1.0	*0.91*
Adenosine	0.5	*0.08*	1.1	*0.56*	**0.6***	***0***	1.0	*0.88*
Glycerate-2-P	1.0	*0.84*	0.9	*0.39*	0.8	*0.12*	**1.3***	***0.05***
Glycerol-3-P	0.8	*0.25*	1.1	*0.82*	**0.5***	***0***	1.1	*0.74*
Myo-Inositol-P	1.2	*0.5*	0.7	*0.1*	**0.7***	***0.02***	1.3	*0.2*
Pantothenic acid	1.2	*0.31*	1.1	*0.71*	**0.7***	***0.02***	**2.4***	***0.01***

Values are means ± SE (n = 6) of the ratios between control and treated samples, namely as fold change (FC) of CD: control plants harvested simultaneously with the drought stressed samples; D: drought stress; CR: control plants harvested simultaneously with the rehydrated plants; R: rehydrated plants. *and bold indicate significant differences between treatments ($p < 0.05$) according to Students *t*-test.

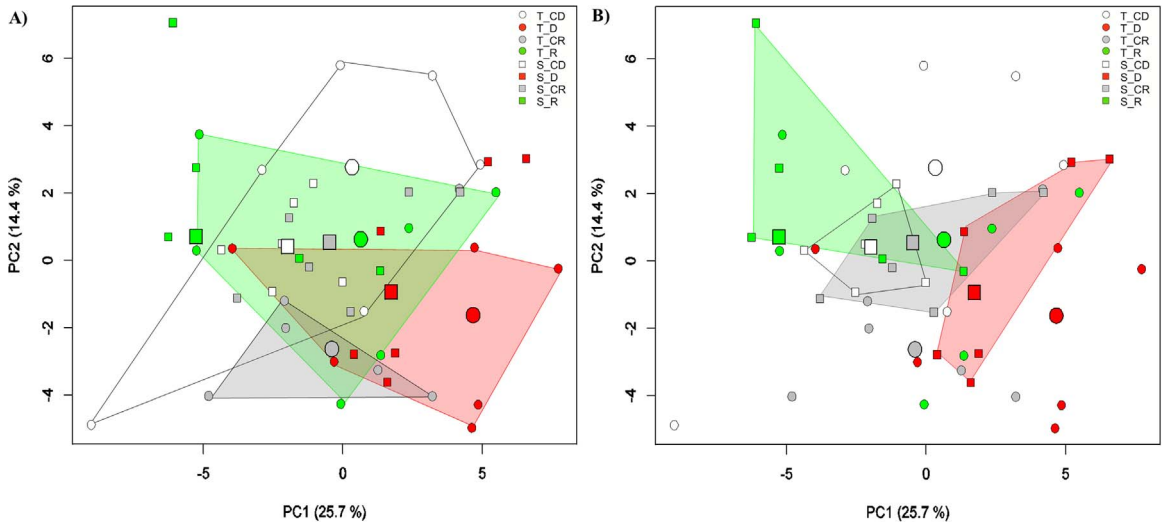

Fig. 3. Principal component analysis (PCA) of metabolites from peanut nodules from two cultivars with contrasting drought-stress tolerance and exposed to drought stress and rehydration. T: tolerant cultivar (EC-98); S: sensitive cultivar (Granoleico). CD: control plants harvested the same day as the drought-stressed plants; D: drought-stressed plants; CR: control plants harvested with rehydrated plants; R: rehydrated plants. The lines represent the convex hull for each treatment and cultivar: in panel A the convex hull for the cultivar EC-98 and in B for Granoleico. Bigger symbols are the median for each treatment and cultivar.

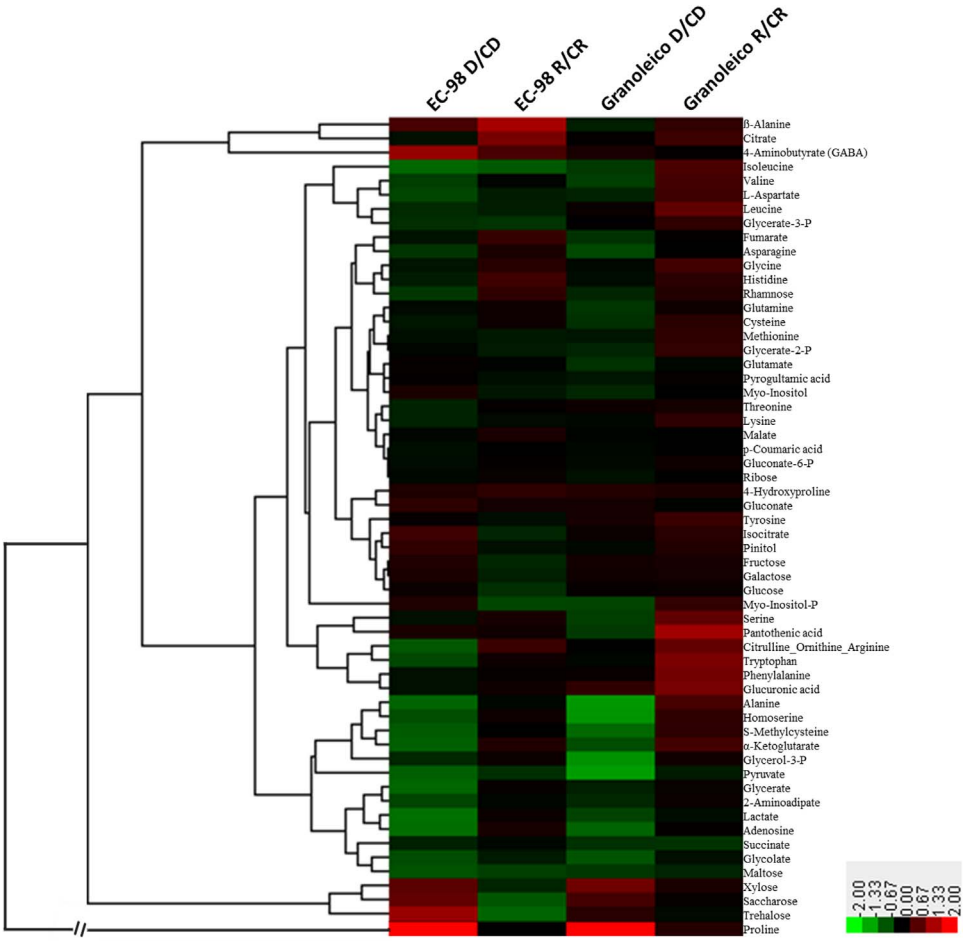

Fig. 4. Metabolite profiles of peanut nodules subjected to drought stress and rehydration. The hierarchical clustering analysis (HCL) presents the $\log_2$ fold change of the ratios between the treated and the control nodules from each cultivar and allowed to identify groups of metabolites that exhibited similar response patterns upon exposure to the different treatments.

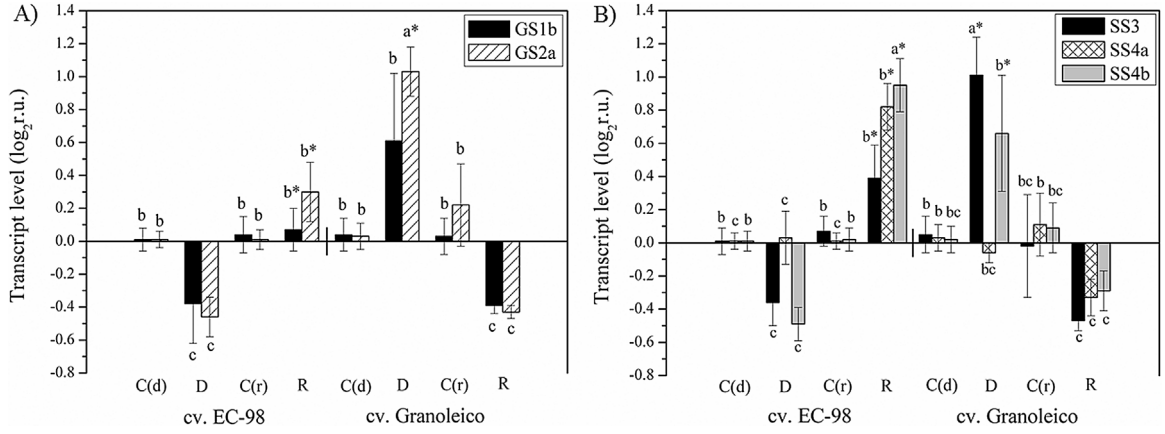

Fig. 5. Transcript levels of the ammonium assimilation enzyme glutamine synthetase (GS) (A) and the sucrose cleavage enzyme sucrose synthase (SS) (B) in peanut nodules from cultivars differing in drought stress tolerance during a drought stress and rehydration experiment. Data were obtained by qRT-PCR.
Values are means ± SE (n = 9). Different letters in columns indicate significant differences between treatments for each cultivar at $P < 0.05$ according to Duncan's test. Asterisks in columns indicate differences between cultivars for the same treatment at $P < 0.05$ according to Duncan's test.

4. Discussion

Drought stress causes severe effects on plant growth and impairs the process of nitrogen fixation in nodulated legumes. Several mechanisms have been described to explain this reduction. Among these, oxygen limitation, carbon shortage and nitrogen feedback were proposed as signals involved in nitrogen fixation depending on the legume studied [11]. More recently, sulfur and redox metabolism were suggested as mechanisms responsible of the decline in N-fixation [11]. Therefore, two peanut genotypes with contrasting drought-sensitivity were selected in order to elucidate the relative contribution of metabolic changes to impaired BNF efficiency. Low growth is commonly observed in plants exposed to drought stress. However cultivars with contrasting drought tolerance behave different based on genetically realized physiological mechanisms. Such contrasting tolerance to abiotic stresses is a rich resource to breed for increased yield [66,67]. Therefore, the study of the genetic diversity generates information to improve yield of economically important crops [68]. For peanut, efforts were made to identify varieties with increased tolerance to drought. In this regard, fifty peanut genotypes were used to analyze thiobarbituric acid reactive

Table 4
Specific activities of the ammonium assimilation enzyme glutamine synthetase (GS) and the sucrose cleavage enzyme sucrose synthase (SS) in peanut nodules from cultivars differing in drought stress tolerance during a drought stress and rehydration experiment.

Cultivar	Treatment	Glutamine synthetase (nmol γ - GH min^{-1} mg $protein^{-1}$)	Sucrose synthase (μmol NADPH min^{-1} mg $protein^{-1}$)
EC-98	**Control (D)**	9.21 ± 3.37 ab	21.33 ± 2.40 ab
	Drought stress	17.50 ± 5.02 a*	13.67 ± 1.20 b
	Control (R)	11.74 ± 1.80 ab	28.60 ± 4.26 ab
	Rehydration	7.31 ± 1.89 b	38.50 ± 12.29 a*
Granoleico	**Control (D)**	16.17 ± 2.43 a	41.60 ± 6.64 a*
	Drought stress	6.32 ± 0.65 b	38.25 ± 10.09 a*
	Control (R)	15.55 ± 3.69 a	35.67 ± 7.36 a
	Rehydration	7.68 ± 0.80 b	11.67 ± 2.91 b

Values are means ± SE (n = 9). Different letters in columns indicate significant differences between treatments for each cultivar at $P < 0.05$ according to Duncan's test. Asterisks in columns indicate differences between cultivars for the same treatment at $P < 0.05$ according to Duncan's test.

substances (TBARs) and chlorophyll content, harvest index and yield under drought stress [45]. The genotypes were classified as sensitive or tolerant to drought and two cultivars exhibiting a contrasting response (Granoleico and EC-98) were selected for the present study. In this work, the tolerant cultivar EC-98 sustained growth despite reductions in the water content and photosynthetic activity suggesting that this cultivar triggers a metabolic response that underlies the sustained biomass production. Similar results were reported for soybean and common bean plants exposed to drought stress where the cultivars showed a decrease in the water content despite absence of changes in the shoot biomass for the tolerant cultivars [69,70].

It is noteworthy that according to a review from Chaves et al. [71] a recovery in the photosynthetic machinery of about 50% within a day after rewatering indicates severe water stress. Then, a longer recovery period is needed to re-establish the photosynthetic machinery. The moderate recovery observed in this work in ΦPSII after 72 h of rewatering indicates that the plants had been exposed to a severe drought condition. A similar result was found by Iovieno et al. [72] in drought-stressed and rehydrated tomato plants. A conserved response among plants exposed to drought stress is the increased flow of assimilates from shoots to roots and an increase in root-to-shoot ratio [73]. The redistribution of photoassimilates is considered an efficient mechanism that diminishes the evaporative canopy surface area [74] and improves water uptake from the soil [73]. In this work, peanut plants increased the root biomass production under drought stress condition without significant differences between cultivars. The result is in line with published research that describes peanut as a tolerant species in comparison with other legumes [6,75].

The nodulation and nitrogen fixation processes were decreased in the sensitive cultivar Granoleico and unchanged in the tolerant one. The tolerant cultivar exhibited a decreased nodule number but maintained the nodule dry weight per plant. This result is in accordance with the findings of Moraes et al. [76] in cowpea who proposed that the shoot/root carbohydrate reserves were mobilized to fewer nodules, resulting in the maintenance of a constant total nodule dry weight in the cowpea-*Bradyrhizobium* sp. interaction grown in soils amended with sludge. Nodules are active sinks and carbohydrate reserves are needed to maintain their full functions [77,78]. In this regard, drought inhibited BNF in two soybean cultivars with contrasting tolerance to drought, but this inhibition occurred earlier and more severely in the sensitive cultivar [79]. Moreover, the study of two common bean cultivars with distinct sensitivity to mannitol-mediated osmotic stress revealed that the tolerant cultivar sustained a stable plant growth, nodule water status and symbiotic N_2 fixation process [80]. Previously, it was demonstrated that, upon exposure to drought stress, the sensitive cultivar had a lower nitrogenase activity and leghemoglobin content [46]. Both effects contributed to the lower BNF and were in accordance with the proposed mechanism of BNF impairment by imbalanced oxygen metabolism. The possibility that oxidative stress impairs the BNF was previously explored in the same experimental setup. The nodules of the sensitive peanut cultivar revealed symptoms of oxidative stress thus, reactive oxygen species and oxidatively damaged biomolecules (protein and lipids) accumulated despite the activation of the antioxidant system [46]. Furthermore peanut cv. EC-98 did not alter its H_2O_2 content although TBARs accumulated in leaves and nodules, probably due the severe stress imposed (unpublished data). Thus, the oxidative response of plants did not allow pinpointing the contrasting response of both genotypes. In the present work, both peanut cultivars exposed to drought stress had a lower water status and an impaired photosynthesis that could restrict the carbon supply to the nodules. However, they exhibited different growth rates and nodulation efficiencies suggesting that the tolerant cultivar had the ability to trigger a metabolic response which underlies the observed tolerance to drought. Therefore, the metabolite profiles of peanut nodules were investigated in order to elucidate the alterations induced by drought stress treatment in cultivars with contrasting drought sensitivity.

The metabolomics analysis revealed that the tolerant cultivar behaved in a more homeostatic manner since more than the 80% of the identified metabolites were unchanged. Besides, upon rehydration most responses triggered under drought stress were reversed reaching similar levels as control plants, indicating an almost fully restored metabolism. The tolerant cultivar accumulated metabolites with well-known functions as protectants against stress. In this sense, the sugar trehalose is involved in reactions ranging from osmoprotection to signaling in response to different environmental stresses in plants [81]. Tolerant varieties of wheat and cotton accumulate trehalose in response to water deprivation and reveal a higher trehalose phosphate synthase expression [82,83]. It is noticeable that the sugar trehalose was one of the metabolites contributing to the explanation of the total variance in principal component 2 (Fig. 3). This result reinforces the idea that trehalose accumulation is one of the traits that could explain the tolerance exhibited in the peanut cultivar EC-98.

Gamma-aminobutyric acid (GABA) was another metabolite with protective function that accumulated in drought-stressed plants from the tolerant cultivar, but not in the sensitive one. This non-proteineous aminoacid participates in plant metabolism in ways such as balancing the C:N-ratio, protecting against oxidative stress, osmoregulation and as a signaling molecule [84]. GABA has gained special interest due its rapid accumulation under stress conditions *via* the GABA shunt. Thus, GABA content was increased in soybean nodules upon exposure to drought stress [31,85]. In this legume, GABA concentrations in nodules contribute to the differentiation of drought-tolerant and drought-sensitive varieties [69]. Interestingly, the GABA accumulation is associated with the increase of other metabolite with protective functions namely proline, since GABA can yield proline through non-enzymatic reactions [86]. This pathway is not the unique source of proline in the cell, but it may support the tolerant cultivar in synthesizing proline in comparison with the sensitive one. Proline has multiple functions in the protection against oxidative stress, namely (i) as a molecular chaperone that protects the integrity of proteins and enhances the activities of enzymes, including antioxidant enzymes, (ii) as antioxidant in the detoxification of ROS, in particular of the hydroxyl ($^{\cdot}OH$) radical [87] and (iii) as valve to consume excess reducing power since proline biosynthesis in chloroplasts during stress lowers the $NADPH/NADP^+$ ratio [88]. Besides, the accumulation of the amino acid correlates positively with the level of tolerance in different cultivars of *Atriplex halimus* L., cotton and potato [89–91]. However both cultivars accumulated proline suggesting that proline is not the prime cause for distinct drought tolerance of EC-98 and Granoleico. Related to proline metabolism, the glutamate decrease in the sensitive cultivar, probably redirected to proline synthesis, can be associated with the low levels of glutamine, the principal molecule for ammonia transport after nitrogen fixation in

peanut. Thus, the impairment in nodule amide metabolism could have detrimental effects on peanut growth as revealed in the sensitive cultivar Granoleico.

An intriguing response is that the tolerant cultivar had the ability to sustain an active nitrogen fixation, despite a lower photosynthetic efficiency and the consequent depletion in organic acids such as pyruvate, lactate and α-ketoglutarate suggest the activation of anaplerotic pathways involved in carbon supply (malate) necessary for the highly demanding N-fixation process. Similar results were reported by Aranjuelo et al. [92] in alfalfa plants exposed to water withholding, revealing that under this detrimental condition the nodule metabolism reveals a compromise between the N-fixation demand and the lower C-provision.

Carbon assimilate import into the nodule is needed to support ammonium incorporation following nitrogen fixation. Sucrose breakdown is achieved by sucrose synthase (SS) in most, if not all, legumes studied so far [93]. Therefore, the SS activity and transcript levels were studied in peanut nodules. The findings revealed that peanut expressed three SS isoforms namely 3, 4a and 4b in comparison with other legumes such as *Lotus japonicus* where the prevailing forms are 1 and 3 [94] and *Pisum sativum* where the isoform 1 has the major contribution to the total activity [37]. Despite the potential lower carbon supply to the nodule due to impaired photosynthesis, the sucrose content was exacerbated in part probably by a lower SS transcription and activity in the tolerant peanut cultivar exposed to drought stress. Similar results were found in nodules from drought-stressed *P. sativum, Phaseolus vulgaris* and *Medicago truncatula* [10,24,80]. Depending on the legume, the photosynthetic activity can be maintained longer which allows to support BNF by providing sucrose during the first steps of drought [21]. Alternatively reserve starch could be hydrolyzed to supply sugar to the nodules [16,19,22]. Despite these considerations, the studies carried out in several legume species showed that the regulation of BNF during drought is species-specific and cannot be generalized [11]. Another aspect to notice is that the activity and transcript regulation, likely reflecting gene expression of SS can be modulated by redox modifications [37]. Supporting evidence for this finding was provided by a proteomic approach from Oger et al. [38] who identified sulfenylations in SS in *M. truncatula* during the initial steps of infection with *S. meliloti*, but also in mature nodules. In addition, previous work revealed that the sensitive peanut cultivar experience oxidative stress when exposed to drought stress [46] reinforcing the hypothesis that the SS might be oxidized if the internal redox homeostasis of the nodules is disturbed. Such mechanisms may explain the discrepancy between the increased transcript levels and the lack of changes in the SS activity in the sensitive peanut cultivar. On the other hand, in the tolerant peanut cultivar, the increased sucrose levels could be used for the synthesis of trehalose and might explain their high levels. Therefore, the nodule metabolism was directed to the synthesis of metabolites that improve drought stress tolerance. The changes in sugar metabolism redirected from respiration to osmoregulation and other defense strategies against stress were proposed by Aranjuelo et al. [92] in alfalfa plants. In our work, in response to rehydration the transcription of SS isoforms was induced and the specific activity reached the control levels. This activation was accompanied by a decreased sucrose and trehalose levels in comparison with well-watered plants. Thus, the tolerant peanut cultivar revealed the ability to restore metabolism after 72 h of rehydration to a state similar to well-watered plants.

The assimilatory enzyme GS was studied since it participates in N assimilation after N_2-fixation, particularly in amide-exporting legumes. For most legumes, two isoforms have been described with isoform 1 being responsible for the primary N assimilation [95]. In peanut nodules, two isoforms were identified (*GS1b* and *GS2a*) in comparison with other legumes such as *M. truncatula* where three isoforms were detected with *GS1a* being the most expressed (≈80%) [96]. However, the isoform *GS2b* can account for as much as 40% of the GS transcripts in *L. japonicus* [97]. In this work, the peanut cultivars exhibited two GS isoforms. It will be interesting to investigate their contribution to N-assimilation in peanut nodules in more detail. The changes in the metabolites Glu and Gln and in enzyme activity in plants exposed to drought stress showed a better correlation than the Glu and Gln and the transcript levels suggesting some posttranslational modification. This is supported by the report that GS is prone to be modified after transcription [96]. Upon rehydration, Glu and Gln reached the control levels in the sensitive cultivar but the enzyme activity remained below control levels despite the increased transcript level. In the case of glutamine, import from leaves or roots via phloem could provide the aminoacid due to the GS activity in other plant organs as suggested by Parsons et al. [98]. Taking into account that the water flow was restored after rehydration, the aminoacid supply may be enhanced. In the case of glutamate there are multiple pathways that could restore the endogenous pool, such as proline degradation which involves the sequential activity of the enzymes proline dehydrogenase and pyrroline-5-carboxylate [88]. Alternative pathways for refilling the glutamate pool may among others involve the activities of glutamate synthase or glutamate dehydrogenase [99]. Tightly correlated with the variation in aminoacid Glu and Gln levels was the response of Asn, the second most important metabolite involved in N export from peanut nodules [39]. The principal component analysis revealed that Asn was one of the metabolites that contributed to the explanation of the differences observed among genotypes (Fig. 3) and was also statistically decreased when analysed by *t*-test (Table 3) in the sensitive cultivar. Asn was proposed as compatible solute which contributes to stress tolerance [100,101]. However the protein content of the enzyme responsible of its synthesis was decreased in *Medicago truncatula* nodules exposed to drought stress [8]. The decreased content in Asn in the cultivar Granoleico can be considered as another explanation for the drought sensitivity phenotype. The tolerant cultivar exhibited a contrasting response, without variations in Glu, Gln and Asn and with the GS activity and transcript levels restored to control levels upon rehydration, reinforcing the conclusion that this cultivar readjusts its metabolism in a better way after suffering a drought stress episode.

5. Conclusion

Taken together the results revealed that in nodules from the sensitive cultivar Granoleico the metabolism of the amides is severely affected, as evidenced by the low content of the aminoacids Asn and Gln under drought stress. The lower content of these nitrogen compounds is in line with decreased BNF which coincides with impaired nitrogenase activity and low leghemoglobin content in the cultivar Granoleico, as previously reported [46]. The sensitive cultivar was unable to re-establish a metabolism similar to well-watered plants. This was revealed by the imbalanced levels of metabolites which were higher than in control plants, and the levels of transcript and activities of sucrose synthase and glutamine synthetase that were kept below the values of control plants. On the other hand, nodules from the tolerant cultivar EC-98 accumulated trehalose, proline and GABA, which are metabolites with known functions in protection against drought stress. This remarkable response, combined with the full restoration upon rehydration, together with smaller changes of drought stress-related metabolites in the nodules are proposed as traits that contribute to the effective control of BNF in the tolerant peanut cultivar EC-98.

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