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RESEARCH ARTICLE

Performance of *Hevea brasiliensis* under drought conditions on osmoregulation and antioxidant activity through evaluation of vacuolar invertase and reducing sugars

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ABSTRACT

Rubber tree cultivation is limited in many regions by abiotic factors such as drought. We investigated the biochemical mechanisms responsible for responses to, and recovery from, drought conditions during the establishment phase of four high latex producing rubber tree clones (RRIM600, IAC40, PR255 and GT1). Five-month-old plants were exposed to 32 days of water restriction, followed by 15 days of soil rehydration. Leaf area, as well as their osmolyte accumulations, saccharolytic enzyme activity and oxidative stress markers, were accompanied. Although clones IAC40 and PR255 responded more precociously to drought conditions, halting leaf expansion before clones GT1 and RRIM600, they demonstrated slow recuperation after re-establishing irrigation. The greater tolerances of clones RRIM600 and GT1 to drought conditions were related to greater vacuolar invertase (VINV) activity in their leaves, which guaranteed more significant accumulations of vacuolar reducing sugars (RS). Similar to RS, glycine betaine accumulations were related to osmoprotection and to reducing oxidative damage (lipidic peroxidation) caused by water deficit conditions. The observed decreases in cytosol neutral invertase (AINV) and cell wall insoluble invertase (CWINV) activities, which resulted in cytosol hexose decreases, may be related to increases in antioxidant enzyme (superoxide dismutase and ascorbate peroxidase) activities in the leaves in response to water deficit conditions. As such, the introduction of specific sugars (RS) and the modulation of key carbon metabolism enzymes, such as VINV, are promising strategies for promoting drought tolerance in rubber tree clones.

Introduction

Water stress is a common problem for many crops due to regular occurrences of long periods without rainfall aggravated by climate change (1). Severe drought events have become more frequent globally in recent decades (2, 3), resulting in predictions of future decreases in areas amenable to food and forest resource production (2, 4). Not even forest plants moderately tolerant to water deficits, such as rubber trees, have been able to escape the deleterious effects of drought (5), making water stress control a challenge for their cultivation.

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The cultivation of *Hevea brasiliensis* (Willd. ex A.Juss.) Müll. Arg., a species native to the Amazon region, has spread to many parts of the world as a major commercial source of natural latex. Rubber trees are cultivated in the southeastern and central-western regions of Brazil to attend to high commercial demands for natural rubber, but also to escape from the fungal disease known as South American leaf blight caused by *Microcyclus ulei* (Henn.) Arx, which attacks plants in the Amazon region (6). One of the problems linked to the cultivation of *H. brasiliensis* (7, 8) in these new areas (known as escape zones from the leaf fungus), however, is the presence of regions with warm frontand/or irregular rainfall regimes that impose water stress conditions (5). The selection of rubber tree clones adapted to dry conditions has been an important strategy adopted against growth and latex production limitations imposed by dry microclimates (9).

Drought conditions can directly interfere with the physiological processes related to carbohydrate metabolism (10). Carbohydrates play important roles in membrane protection and in the elimination of free radicals, thus preventing oxidative damage to cells (9, 10). Soluble sugars are also responsible for maintaining cell turgor and avoiding leaf dehydration (13). Accumulations of soluble and reducing sugars will vary among genotypes of the same species depending on environmental conditions (14) and it is well established that cultivars that demonstrate greater accumulations of sugars in their leaves or roots also demonstrate greater tolerance to dehydration (13, 14).

Carbohydrates are involved in the physiological processes of osmoregulation, acting to maintain the turgor pressure necessary for cell expansion, and counteracting the effects of water stress (10, 15). During conditions of water stress, carbohydrates also participate in the formation of transduction signalling networks in which invertase enzymes interact with enzymatic and non-enzymatic antioxidant systems (18–20). Within that network, equilibria between the production and utilisation of carbon (21) and reactive oxygen species (ROS) (22) are rigidly coordinated by hormones, sugars and enzymes intrinsically linked to adaptive processes and plant development under stress conditions (2).

The genetic expression of the enzyme sucrose synthase (SuSy) is influenced by temporal, metabolic, and environmental factors (such as temperature, photoperiod, light and water availability, nutrient concentration, pH, among others.), and it is inhibited explicitly by conditions that result in fructose accumulation (23). Modifications on sugar metabolism (hexoses and sucrose) and starch (24) and the activities of saccharolytic metabolic enzymes such as sucrose phosphate synthase (SPS) (25), invertases (INV) and SUSY (26) are intimately related to photosynthetic efficiency (27) which is typically reduced under drought conditions (28). Similarly, the activities of antioxidant systems are responsible for protecting against damage caused by ROS and, principally, for maintaining photosystem integrity under water stress conditions (29). Therefore, the regulation of carbon and oxidative metabolism is related to plant tolerance to drought stress conditions (13, 14). Studies of the combined actions of carbon and oxidative metabolism within the network of transduction signals responding to stress conditions will not only allow a better understanding of those molecular signalling processes but could aid in perfecting biotechnological strategies (such as tissue-culture, somatic embryogenesis, culture of protoplast) designed to increase plant productivity under otherwise unfavourable abiotic conditions.

Although the GT1, IAC40, PR255, and RRIM600 rubber tree clones are considered elite options for increasing latex productivity in Brazil (30–32), there have been no comparative studies evaluating the effects of water deficits on their oxidative and carbohydrate metabolisms during seedling establishment. The establishment phase is the costliest in terms of seedling maintenance and is critical to latex production. A better comprehension of the metabolic responses of those clones to water stress would allow the selection of drought tolerance physiological markers that could be used (together with other criteria) to indicate varieties with the greatest potential for successful cultivation in non-traditional areas that experience Indian summers and/or lack irrigation infrastructures.

We examined here the biochemical responses of four high latex producing rubber tree clones to drought conditions during their implantation phase, in order to elucidate the roles of carbon and oxidative metabolism and osmolyte accumulation in water deficit tolerance. We also sought to understand better how their innate metabolic divergence could be related to different degrees of drought tolerance and subsequent rehydration.

Materials and Methods

Genotypes evaluated and water stress conditions

We evaluated rubber tree genotypes selected for high latex production currently cultivated in most plantations in southeastern Brazil and on large scales in other regions of that country (29-31). GT1, IAC40, PR255 and RRIM600 genotype seedlings were procured from the Fiorese nursery, located in Sales Oliveira, São Paulo State, Brazil in 35 cm × 15 cm tubes containing a single seedling at the second leaf stage (in a pine bark substrate).

We transplanted the seedlings to PVC tubes (20 cm diameter and 40 cm tall) containing 12.6 dm^3 of the substrate (a mixture of soil and sand; 80:20 v/v) and subsequently cultivated them under greenhouse conditions with mean daily temperatures between 18 and 31 ºC and a mean illumination level of 825 µmol of photons m^{-2} s^{-1}.

During the substrate acclimation phase (20 d), we irrigated all of the clones every two days with 700 ml of water, to maintain the soil moisture at 90% of its field capacity. One visible sign of acclimation was the sprouting of new leaves. Soon after that step, we initiated all treatments by suspending irrigation. The plants demonstrated visible symptoms of senescence

and leaf fall thirty-two days after suspending irrigation, and we considered that situation to mark the end of the experimental dry period. Irrigation was then reinitiated (after 32 d), and the substrate again moistened to 90% of its field capacity (700 ml of water) for 15 days, totalling a 47 days experimental period.

The experimental design was randomised, using a double 4 x 2 factorial scheme (four genotypes and two water status regimes with or without substrate irrigation). Biochemical evaluations were made after 32 d without irrigation (DWI) and again at 15 d after soil rehydration (DSR), totalling two treatments with five repetitions per clone. Each experimental block was therefore composed of one plant in each of 40 containers. All biochemical evaluations were performed on samples of the third leaf (the first fully expanded leaf below the apex).

Leaf fresh mass and leaf area

Leaf fresh mass was evaluated at the beginning (0 d) of the dry period, at 16 days and at the end (32 d) of that water deprivation regime, as well as 15 days after soil rehydration (corresponding to the 47th day of treatment). To that end, we collected all of the leaves of selected plants (leaves at all stages of maturation, including the smallest leaves and buds).

The overrated leaf area (OLA) was evaluated at the beginning (0 d) of the dry period, at 8, 16 and 24 days, and then at the end of the dry period (32 d), as well as at eight and 15 days after soil rehydration (which corresponded to 40 and 47 days of treatment respectively). The length and width of each leaflet of the third set of leaves of all of the plants (expressed in cm²) were measured from the beginning of the dry period. If there was a loss of any leaflet or leaf, those measurements were discarded. The data from at least five leaves/clone/treatment (without any loss of leaflets) were considered in the statistical evaluations.

Soluble carbohydrates, starch, soluble proteins, amino acids and glycine betaine

Leaf macromolecule contents were quantified by homogenising 200 mg of dry leaf in 5 ml of 100 mM potassium phosphate buffer (pH 7.0), and then holding the extract in a 40 °C water bath for 30 minutes. The homogenate was then centrifuged in refrigerated centrifuge Sorvall ST16-R (Thermo Scientific, USA) at 10000 x *g* for 20 minutes, and the supernatant collected. That process was repeated twice, and the supernatants combined (34). Starch was extracted by re-suspending the pellet in 8 ml of 200 mM potassium acetate buffer, pH 4.8; 2 ml of the enzyme amyloglucosidase was then added, and the slurry was incubated in a water bath at 40 °C for two hours. After centrifuging at 10000 x *g* for 20 min, the supernatant was collected and completed to a volume of 15 ml with distilled water. The Antrona method (35) was used to quantify starch and total soluble sugars. Reducing sugars were quantified following Miller (36); amino acids were quantified using the ninhydrin method (37); proteins were quantified following Bradford (38); and glycine betaine (GB) was quantified using 0.05 gm of dry leaf material, following Grieve (39).

Activities of saccharolytic enzymes

The activities of invertase (EC 3.2.1.26) isoforms were evaluated by macerating samples of 0.5 gm of fresh leaf in liquid nitrogen. The extractions and incubations of soluble invertases (AINV: neutral cytosol invertase and VINV: vacuole acidic invertase) were performed (40) and the isolation and incubation of insoluble invertase (CWIN: cell wall acidic invertase) were performed following standard procedure (41). Their enzymatic activities were quantified by collecting aliquots after 10 and 70 min. of incubation and subsequently determining the amounts of reducing sugars produced following the DNS method (36).

Sucrose synthase (E.C. 2.4.1.13) enzyme activity was evaluated following the standard methodology (41), homogenising 0.2 gm of leaf tissue in 2 ml of 50 mM buffer, pH 7.0, 2 mM $MgCl_2$, 2 mM DTT, and 1 mM EDTA. Enzymatic activity was measured after 40 min. of incubation (subtracting the zero time values) using the DNS method for quantifying reducing sugars following standard method (36).

The activity of sucrose phosphate synthetase (SPS; E.C. 2.421.14) was evaluated by homogenising 0.25 gm of fresh leaf material in 1.5 ml of 100 mM Hepes extraction buffer (pH 7.0), 200 mM $MgCl_2$, 50 mM disodium EDTA, 10 mM 2-mercaptoethanol and 2% ascorbic acid. Extraction and enzymatic incubation were carried out as per the procedure (42).

Oxidative markers

Antioxidant enzymes were evaluated by macerating 0.2 g of fresh leaves in 1.5 ml of an extraction buffer solution containing 400 mM potassium buffer (pH 7.8), 10 mM EDTA, and 200 mM l-ascorbic acid. The protein contents of the samples were determined using the Bradford method. Superoxide dismutase (SOD; EC1.15.1.1, (43), catalase (CAT; EC1.11.1.6 (44)), and ascorbate peroxidase (APX; EC 1.11.1.11 (45) activities were measured using standard procedures. Lipidic peroxidation was determined by estimating the malondialdehyde (MDA) concentration in 0.5 gm of fresh plant material (46).

Statistical evaluations

The data obtained were first submitted to evaluations of normality using the Shapiro-Wilk ($P \geq 0.05$) and Bartlett ($P \geq 0.05$) tests (47, 48) to verify the homoscedasticity of the variances. The data considered normal and demonstrating homogeneity of variance were submitted to two-way analysis of variance (ANOVA). Interactions between treatments (control and drought) and clones (GT1, IAC40, PR255 and RRIM600) were included in the model. The model was used within each evaluation time. When differences were detected by ANOVA, the means were compared by the Scott-Knott test ($P < 0.05$), using R software (49) and the ExpeDes (50) and ggplot packages (51).

Multivariate methods have numerous appealing properties for data integration. The multivariate

analysis consists of a set of statistical methods used in situations where several variables are measured simultaneously, correlated with each other in each sample element, in order to reduce the dimensionality of the data (52). To verify the overall behaviours of the clones in relation to the full set of variables, we used the reduction of data dimensionality in principal components analysis (PCA). We elaborated a graphic biplot, using the factoextra (53) and ggfortify packages (54).

Results

Leaf fresh mass and leaf area

Leaf fresh mass accumulations (FW; F=0.28) and overrated leaf area (OLA; F=1.08) did not significantly differ (P<0.05) between the controls of the four clones examined (Fig. 1A & 2A). In all of the clones, the fresh weight (FW) and OLA under drought conditions did not differ significantly (P<0.05) between them on the 16th and 32nd day after initiation (Fig. 1B & 2B). Although the OLA significantly diminished in all of the clones (P<0.05) after 16 days of water deficit in relation to their respective controls (Fig. 2 C–F), FW losses at 16 days were only observed in clones IAC40 and PR255; clones GT1 and RRIM600 showed decreased FW among plants submitted to drought conditions only after 16 days in relation to the controls (Fig. 1C–F). After 15 days of rehydration (day 47 of the experiment), all of the clones demonstrated FW and OLA increases, although greater FW (F=4.49) and OLA (F=3.72) gains were observed in clones RRIM600 and GT1 (P<0.05; Fig. 1B & 2B). Sprouting of new leaves was observed in all plants of all clones after reinitiating irrigation.

Total leaf soluble sugars (TSS), reducing sugars (RS), amino acids (Aas), total protein (TSPs) and starch concentrations

There were significant (P<0.05) accumulations of TSS (F=125.23), RS (F=84.41), and Aas (F=165.89), but decreased concentrations of starch (F= 48.17) and TSPs (F=115.44) in the leaves of all of the clones during the suspension of irrigation (Fig. 3). After reinitiating irrigation, leaf concentrations of TSS and RS were significantly greater (P<0.05) than those of their respective controls for clones GT1 and PR255; TSS and RS concentrations did not statistically (P>0.05) differ from the other clones (IAC40 and RRIM600). Starch concentrations in the leaves became reduced under water deficit conditions; those concentrations increased after re-establishing irrigation, although still remaining below values observed in the controls (P<0.05).

Leaf concentrations of TSS (F=5.74) and RS (F= 7.76) were different among the different clones in response to drought conditions as well as after re-establishing irrigation (P<0.05; Fig. 3 A & B). High concentrations of TSS were observed in the leaves of clones GT1 and RRIM600 during water stress conditions and in clones GT1 and IAC40 after re-establishing irrigation. Although clone RRIM600 demonstrated the highest leaf RS concentrations during water stress, that clone demonstrated the lowest leaf RS concentrations in relation to the other clones after re-establishing irrigation. Starch (F=1.03), Aas (F=0.79) and TSPs (F=1.76) concentrations in the leaves did not significantly differ (P>0.05) among the clones within each treatment (Fig. 3).

Saccharolytic enzymes

Cell wall invertase (CWINV; F=60.48), neutral cytosol invertase (AINV; F=99.55) and sucrose synthase (SuSy; F=19.78) activities significantly diminished (P<0.05) in the leaves of all clones after suspending irrigation (Fig. 4). Vacuolar invertase (VINV) activity increased significantly in the leaves of clones GT1 and RRIM600 (F=7.78,) under water deficit conditions and after reinitiating irrigation. Sucrose phosphate synthase (SPS; F=10.26; P<0.05) activity significantly diminished under water deficit conditions only in clones IAC40 and PR255 (Fig. 4). After rehydration, the activities of the enzymes CWINV, AINV and SuSy did not significantly differ from those of their respective controls (P>0.05). Similarly, SPS activity in the leaves of clones IAC40 and PR255 did not differ from their respective controls after reestablishing irrigation (P>0.05). The activities of the enzymes CWINV (F=0.80), AINV (F= 0.54), and SuSy (F= 0.248) did not differ among the clones in any of the treatments; SPS activity under water stress conditions was significantly lower (P<0.05) in the leaves of clones IAC40 and PR255 in relation to the others (F=8.65; Fig. 4). VINV activity was greater in clones GT1 and RRIM600 in relation to the others under drought conditions and after the reestablishment of irrigation.

Oxidative stress markers

Superoxide dismutase (SOD; F=89.215), ascorbate peroxidase (APX; F=158.53), and catalase (CAT; F=76.17) enzyme activities, as well as malondialdehyde (MDA; F=173.97) concentrations significantly increased (P<0.05) under water stress conditions, but did not differ (P>0.05) from control values after rehydration (Fig. 5). While SOD (F=0.79) and CAT (F=0.81) activities did not significantly differ (P>0.05) among the clones in any of the treatments, APX (F=3.04) activity was higher in clone RRIM600 after suspending irrigation (Fig. 5). MDA (F=3.72) concentrations were significantly (P<0.05) higher in clone PR255 after water deficit conditions in relation to the other clones (Fig. 5).

There were significant accumulations of glycine betaine (GB) (F=233.12) in the leaves of all of the clones during the suspension of irrigation (Fig. 5E). Under conditions of water deficit, glycine betaine (F=2.53) concentrations were lower in clone PR255 than in the others (Fig. 5). No significant differences between controls and treated clones, or among the clones themselves, were observed after re-establishing irrigation.

Multivariate analysis

The estimated values at plot level for each clone and variable were used to generate principal component analysis (PCA), to reduce the data dimensionality into uncorrelated principal components (eigenvalues) that capture all the variance contained in the original set. The first two main components were used to build biplots, where length (eingvalues) and angles of the

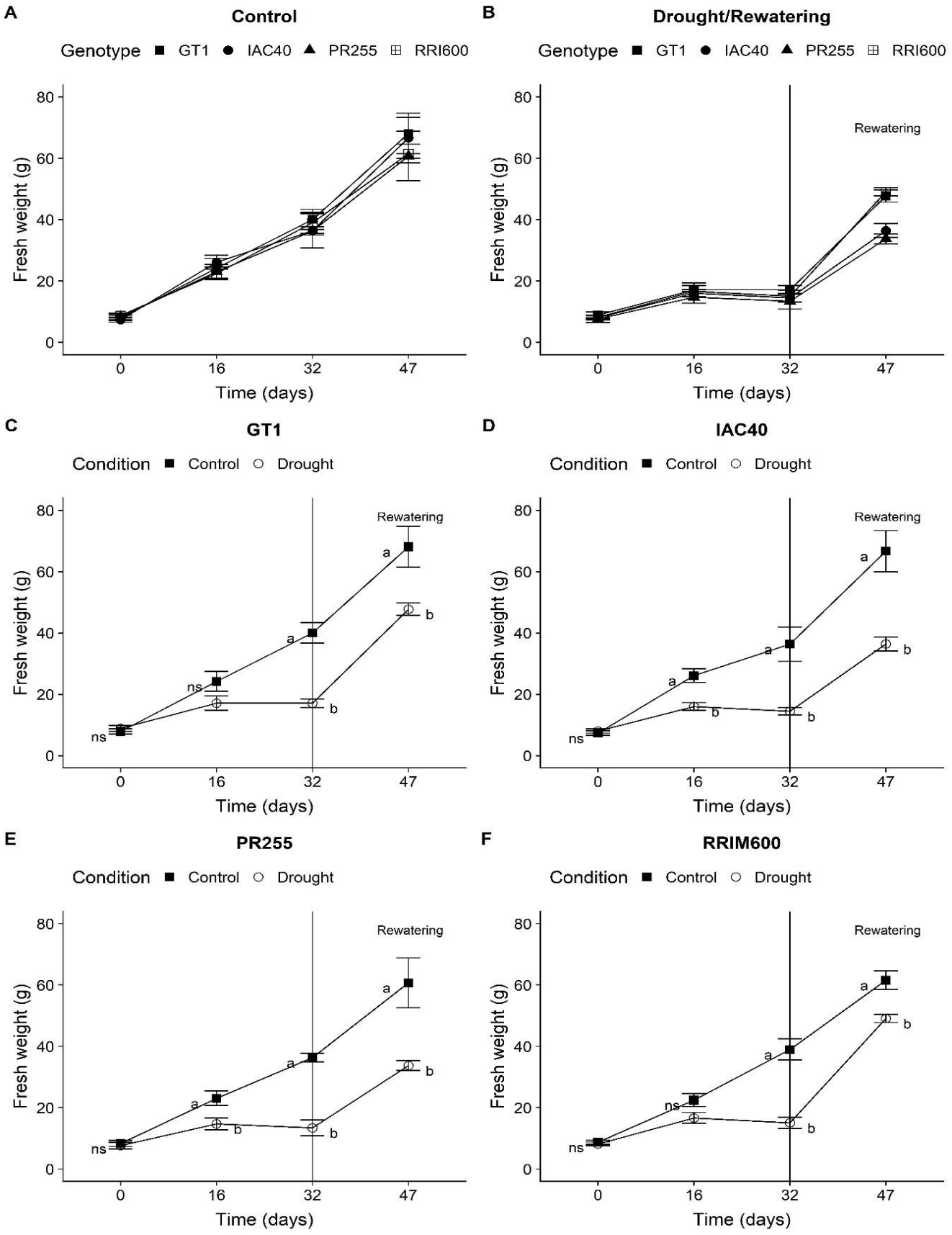

Fig. 1. Fresh masses of the leaves of irrigated *H. brasiliensis* plants (A), leaves after the suspension of irrigation and after re-irrigation (B), and within each clone under the two irrigation regimes (C, D, E and F). Values are presented as the mean ± standard deviation of five repetitions. Different letters indicate significant differences (P<0.05) between the control and the different treatments (dry and re-irrigated) within each clone by the Scott and Knott test.

variables represent variance and covariance respectively, and the relation between clones and variables must be understood as dots products, that is, the projection of the clones over the variables.

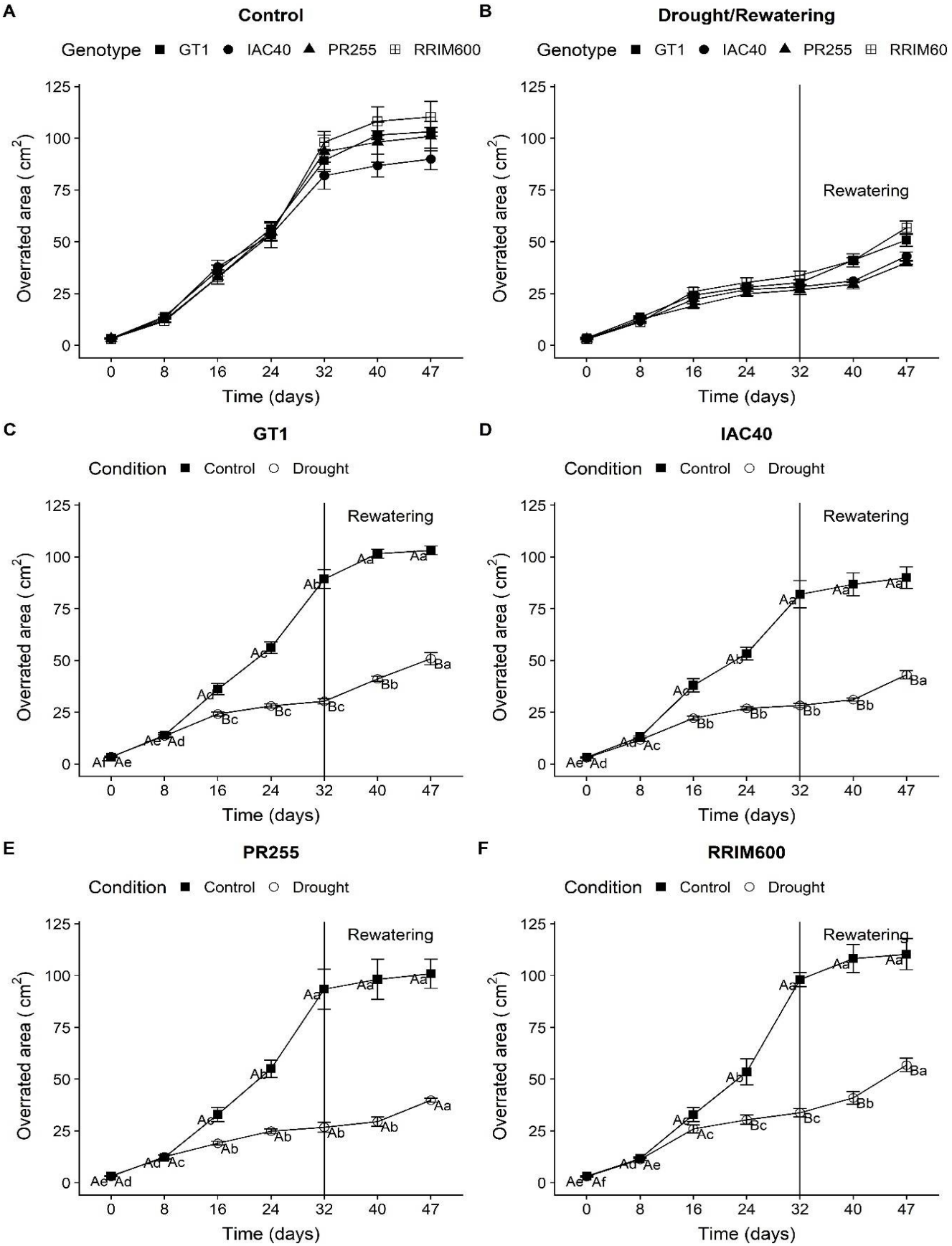

Fig. 2. Observed leaf areas of irrigated *H. brasiliensis* plants (A), and plants after the suspension of irrigation and after re-irrigation (B) and within each clone under the two irrigation regimes (C, D, E and F). Values are presented as the mean ± standard deviation of five repetitions. Different letters indicate significant differences (P<0.05) between the controls and the different treatments (dry and re-irrigated) within each clone by the Scott and Knott test.

The main trends of the metabolic responses of leaves were visualised by principal component analysis (PCA). PCA evaluates variations in the values

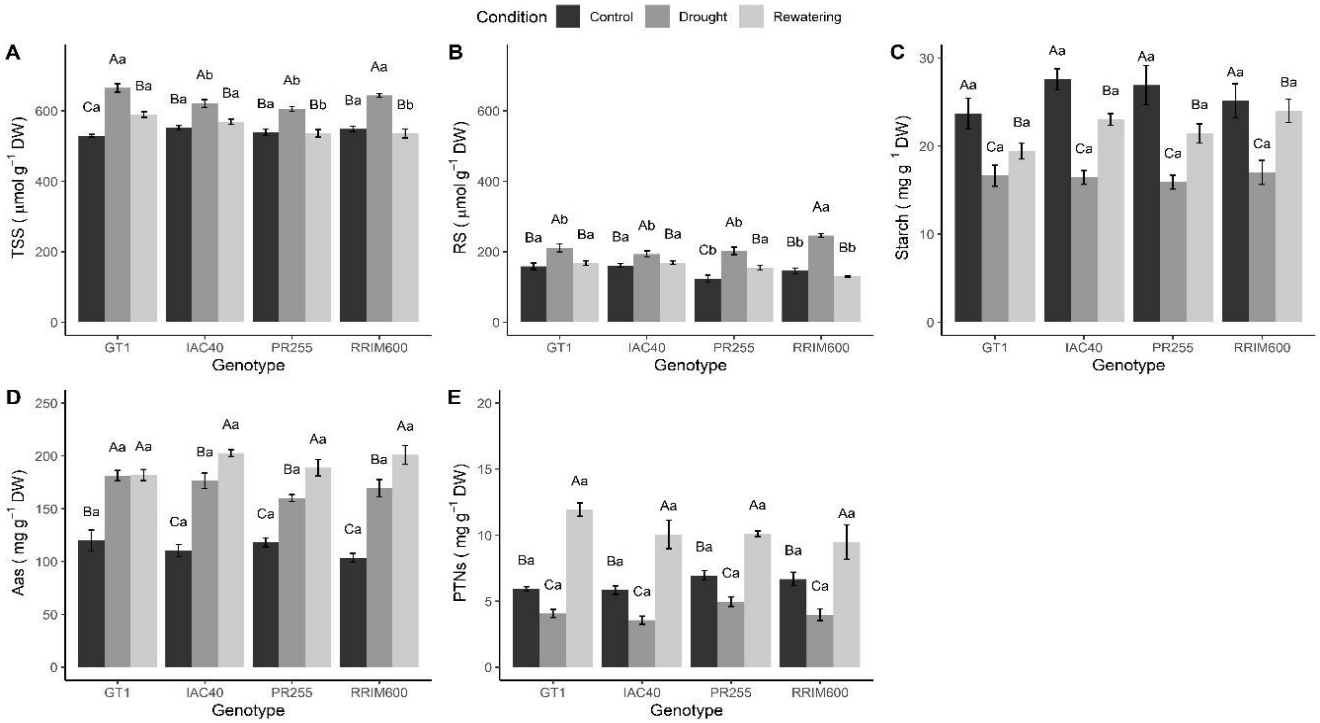

Fig. 3. Concentrations of total soluble sugars (TSS) (A); reducing sugars (RS) (B); starch (C); amino acids (Aas) (D), and total soluble proteins (TSPs) (E) in the leaf tissues of irrigated *H. brasiliensis* plants, after the suspension of irrigation, and then re-irrigation. The bars represent the mean ± standard deviation of five repetitions. Uppercase letters compare control, water deficit and re-irrigation treatments within each clone; lowercase letters compare the same treatments (control, water deficit and re-irrigation) between clones. Different letters indicate significant differences at $P < 0.05$ (Scott and Knott test).

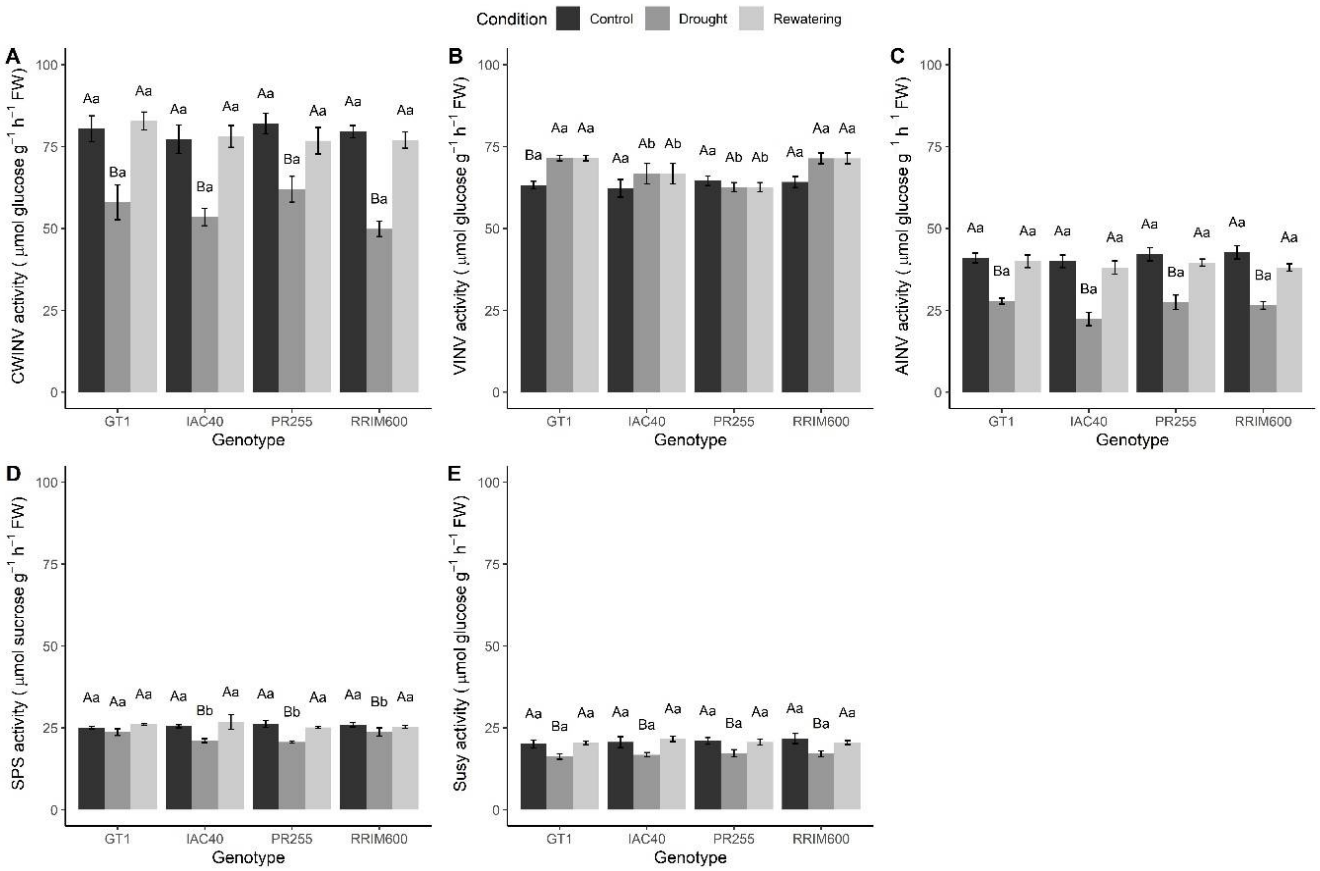

Fig. 4. Cell wall invertase CWINV (A), vacuolar invertase (VINV) (B), neutral cytosol invertase (AINV) (C), sucrose phosphate synthetase (SPS) (D), and sucrose synthase (SuSy) (E) activities in the leaf tissues of irrigated H. brasiliensis plants, after the suspension of irrigation, and then re-irrigation. The bars represent the mean ± standard deviation of five repetitions. Uppercase letters compare control, water deficit, and re-irrigation treatments within each clone; lowercase letters compare the same treatments (control, water deficit and re-irrigation) between clones. Different letters indicate significant differences at $P < 0.05$ (Scott and Knott test).

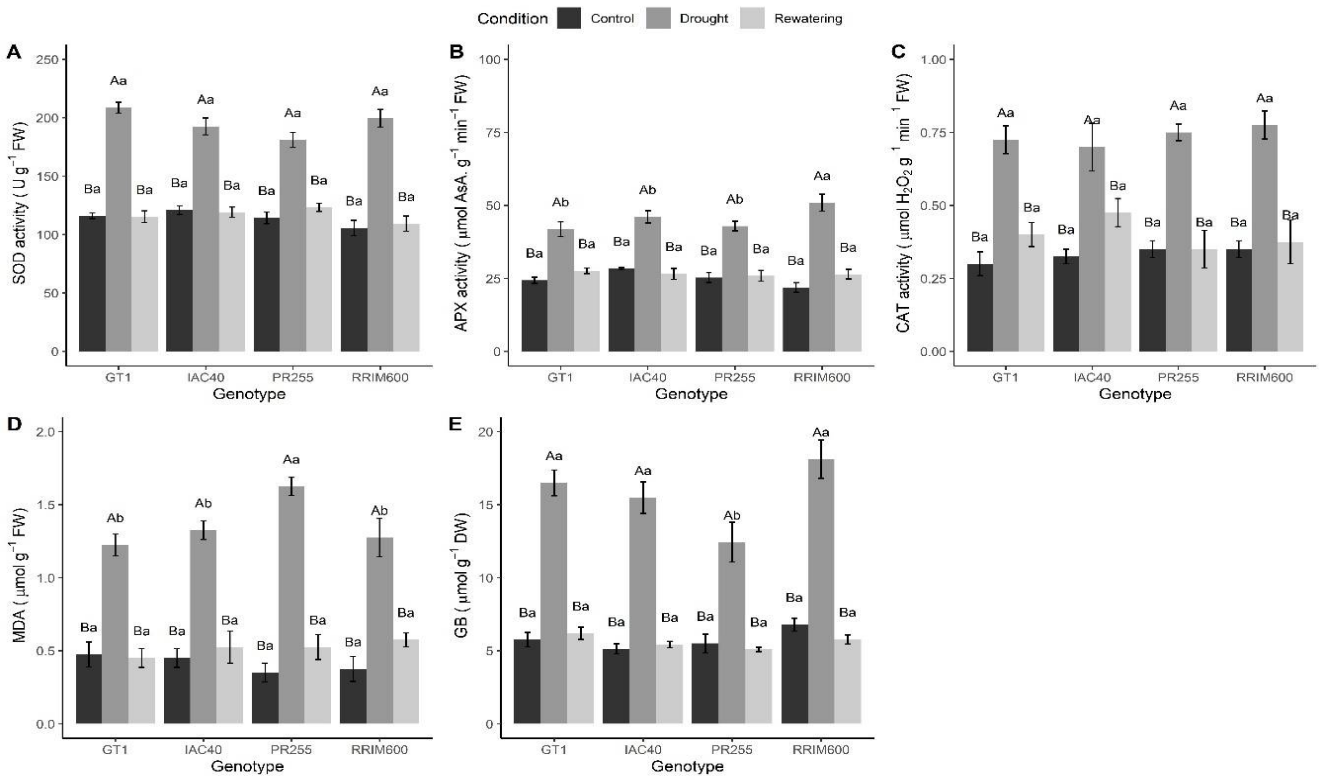

Fig. 5. Superoxide dismutase (SOD) (A), Ascorbate peroxidase (APX) (B), and Catalase (CAT) (C) activities and malondialdehyde (MDA) (D) and glycine betaine (GB) (E) concentrations in the leaf tissues of irrigated *H. brasiliensis* plants, after the suspension of irrigation and then re-irrigation. The bars represent the mean ± standard deviation of five repetitions. Uppercase letters compare control, water deficit and re-irrigation treatments within each clone; lowercase letters compare the same treatments (control, water deficit and re-irrigation) between clones. Different letters indicate significant differences at $P < 0.05$ (Scott and Knott test).

of experimental parameters and derives new complex variables from them that reflect maximal changes in the parameter data set. The PCA analysis of our data showed that the two main axes explained 54.30% and 27.21%, 52.38% and 31.11% and 50.02% and 26.07% of the data variability for control, drought and the reinitiation of irrigation respectively (Fig. 6). It was possible to see that under control conditions, Axis 1 ordered the clones mainly in accordance with their saccharolytic enzyme activities, with clones PR255 and RRIM600 being at one end of the gradient and GT1 and IAC40 at the other. Under drought conditions, the invertase isoforms altered their behaviour, with VINV separating PR255 at one end of the gradient and the other clones at the opposing end. Once again, during rewatering, saccharolytic enzymes (except for SuSy) ordered the clones into two groups, with clone GT1 composing one group. Under those three scenarios, the metabolic responses of GT1 were the most stable, while the changes in clone RRIM600 were more noticeable.

Discussion

Important alterations of oxidative and carbohydrate metabolism occurred under water deficit conditions had affected plant growth and productivity. Elevated antioxidant capacities (55) and modifications of

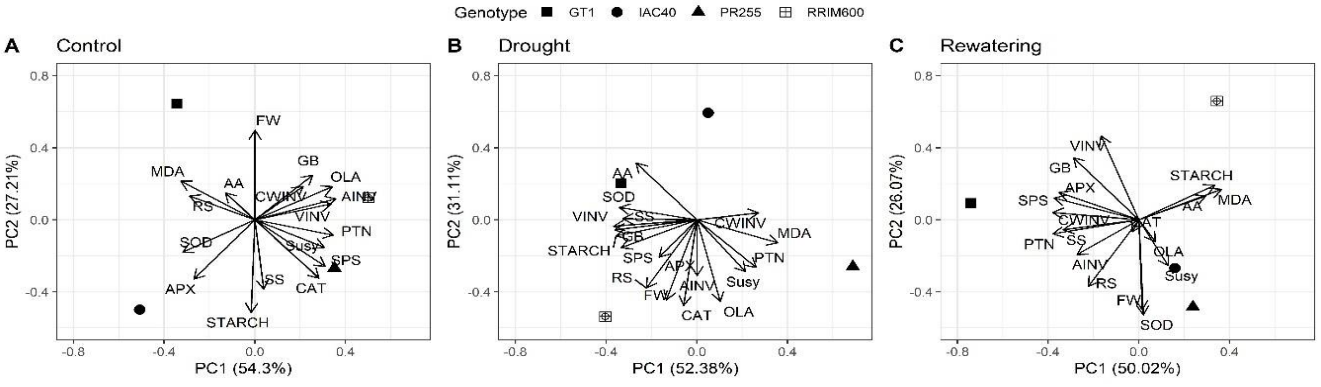

Fig. 6. Principal component analysis of the concentrations of total soluble sugars (SS), reducing sugars (RS), starch, total soluble proteins (TSPs), total amino acids (Aas), antioxidant system enzymes: Superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), saccharolytic enzymes; cell wall invertase (CWINV), neutral cytosol invertase (AINV), acidic vacuolar invertase (VINV), malondialdehyde concentration (MDA), glycine betaine concentration (GB), leaf fresh mass (FW) and observed leaf area (OLA) in clones of *H. brasiliensis*, in the leaf tissues of irrigated plants (A), plants subjected to the suspension of irrigation (B) and re-irrigated plants (C).

metabolic pathways can result in accumulations of reducing sugars in plant tissues (56) that can, for example, guarantee greater tolerance to water stress. However, those metabolic responses will vary depending on the developmental states of the rubber tree plants (57). As such, a better understanding of the metabolic responses of different rubber tree clones to drought conditions during their implantation phase could provide subsidies for identifying varieties demonstrating optimal performances and production in non-traditional cultivation regions.

Fresh mass and leaf area are two growth parameters intimately related to drought tolerance (58). Stoppage of leaf expansion (leaf area) is a common plant response to water stress conditions, as it reduces water losses by reducing transpiration surfaces. Although clones IAC40 and PR255 responded more precociously to drought conditions, halting their leaf expansion before clones GT1 and RRIM600, they demonstrated slow recuperation after re-establishing irrigation, with smaller OLA and FW gains after rehydration. The slower stoppage of leaf expansion in OLA of clones GT1 and RRIM600 during drought, on the other hand, and their more rapid recuperation of OLA and FW after re-irrigation indicated greater tolerance to water stress conditions. Within that context, we became interested in identifying the metabolic responses that generated the observed differences in drought tolerance.

Evaluations of the activities of various invertase isoenzymes pointed to the importance of vacuolar invertase (VINV) in all of the rubber tree clones tested. Different from the other isoenzymes examined, VINV activity did not diminish under drought conditions and even demonstrated increased activity in clones GT1 and RRIM600 (Fig. 4). The role of VINV in drought tolerance was investigated earlier (55, 56) and increased vacuolar invertase activity (and concomitant hexose accumulation) in mature corn leaves was reported (56) as being related to drought tolerance. Under conditions of low water availability, the maintenance/increase of vacuolar hexose concentrations may aid in the osmotic adjustment and expansion of plant leaves (60). The diminishing activities of CWINV and AINV, as well as that of SuSy (which catalyses both the synthesis and degradation of sucrose (61) and SPS, together with the maintenance/increase in VINV activity (Fig. 4) indicates a redirection of carbon metabolism towards hexose accumulation in plant vacuoles under drought conditions. That hypothesis is supported not only by the observed increases in RS concentrations (Fig. 3B) during water stress conditions (as well as its high co-linearity with VINV; Fig. 6) but also by concomitant increases in TSS (Fig. 3A) and decreases in starch (which increase glycose/maltose availability; Fig. 3C).

Soluble sugar accumulations are typically observed under stress conditions that involve growth restrictions (58, 59). For example, RS accumulation in plant vacuoles allows for osmotic adjustments during water stress, as the diminishing osmotic potential of that organelle guarantees the influx of water and the maintenance of cell turgor and cell expansion (55).

As with soluble sugars, amino acid accumulation is a common response to water stress, facilitating osmotic adjustments without interfering with normal cell metabolism (64). Although amino acid accumulations have been observed during exposure to drought conditions in numerous plants, their concentrations did not differ among the different clones studied here (Fig. 3D), indicating the primary role of RS in water restriction responses in rubber tree clones. The higher VINV activity observed in clones GT1 and RRIM600 after a period of water stress (Fig. 2, 3, 4) resulted in greater vacuolar RS accumulations that allowed greater subsequent leaf expansion, thus demonstrating the intrinsic relationship of VINV activity to leaf expansion and drought tolerance. It is important, however, to emphasise that the amino acid GB was observed to accumulate in all of the clones exposed to water stress (Fig. 5E). That accumulation is considered a precocious response to water stress (65) and results in greater tolerance to its effects (66, 67). GB has recognised roles in osmoregulation and osmoprotection, in addition to being involved in protection against oxidative damage as an antioxidant agent (68). Increased lipid peroxidation (measured by MDA concentration) was observed in clones demonstrating the lowest leaf GB concentrations (Fig. 5). Additionally, the PCA indicated opposite positions of the MDA and GB vectors in the plants during the water stress period (Fig. 6), which reinforces the role of GB as an antioxidant molecule under drought conditions.

Similarly, in addition to their osmotic control roles, vacuolar RS can serve as protective agents by removing reactive oxygen species (ROS) that are commonly formed in plants exposed to water stress conditions (69). The importance of RS as antioxidant agents was also demonstrated by the opposing positions of the MDA and RS vectors in the PCA under drought conditions (Fig. 6). The high levels of H_2O_2 present in the cytoplasm, derived from ROS production in chloroplasts, mitochondria and other cellular compartments under stress conditions, is directed toward the vacuoles (as plant cell “detoxification and dumpsites”). The vacuolar sugars there have important roles in removing ROS and thus preventing lipid peroxidation (70). The increase in antioxidant enzyme activity and MDA (peroxidised lipids), observed in rubber tree plants during drought conditions (Fig. 5) attest to the fact that water deficits induce oxidative stress due to the overproduction of ROS. However, one can argue that the observed lipidic peroxidation (increasing concentrations of MDA) would indicate that the RS were not efficient in removing all ROS, as oxidative damage was observed.

Nonetheless, the leaves reinitiated expansion after rehydration. As was noted above, leaf expansion depends on the entrance of water into the vacuoles to maintain turgor pressure and guarantee cell division and growth. Therefore, the tonoplast of the plant cells must not have suffered any significant drought induced damage due to the protection provided by vacuolar RS, as leaf growth rapidly

resumed after the water became available. Sugars can substitute water in cell membranes under drought conditions and maintain the space between phospholipid molecules (71), resulting in the formation of a solid but amorphous structure that prevents membrane fusion and leaves it "hydrated" in a process known as sugar vitrification (71). The largest increases in leaf area after rehydration were observed in clones GT1 and RRIM600, which demonstrated the greatest accumulations of vacuolar RS during water stress conditions which corroborates that hypothesis. The differential tolerance of those rubber tree clones to drought conditions is related to VINV activity and the consequent accumulation of RS in plant vacuoles.

Although the details of the crosstalk between ROS and sugar signalling pathways still needs to be intensively studied, the signalling role of sucrose has been well established in many plants (68, 69). Acid/neutral INVs (A/NINVs) compose part of the antioxidant system involved in cellular homeostasis of ROS (74). Interestingly, increased A/NINV activities were observed in *Pisum sativum* L., *Nicotiana tabacum* L. and *Arabidopsis thaliana* (L.) Heynh. during infection by powdery mildew (75), oomycetes (76) and beet curly top virus (77) respectively. On the other hand, tobacco plants affected by *Phytophthora nicorianae* Breda de Haan did not show altered VINV activity (76), suggesting that the enzyme is not directly involved in plant defences against pathogens, even though ROS production is induced in plant tissue once the infections are established, resulting in programmed cell death (PCD) (78). The roles of carbohydrates in controlling cell ROS production during infectious processes therefore appear to involve increases in A/NINVs activities. Those enzyme activities result in diminishing concentrations of sucrose and increasing concentrations of hexoses in the cytosol that signal genetic suppression and antioxidant enzyme activity, causing ROS accumulation in the cytosol and induced PCD. Similarly, the diminishing A/NINVs activities observed in rubber tree plants under drought conditions (Fig. 4) resulted in sucrose accumulation and diminishing concentrations of hexoses in the cytosol, leading to greater genetic expression and increased activities of antioxidant enzymes such as SOD, CAT and APX (Fig. 5). Rats fed on diets rich in sucrose and that demonstrated high cellular levels of that carbohydrate, also demonstrated higher Cu-Zn-SOD mRNA levels as compared to animals fed with starch. In the case of the rubber tree seedlings, A/CWINV activities (and TSS levels) increased after re-irrigation (Fig. 3) and returned to control levels (Fig. 4), as did SOD and APX antioxidant enzyme levels (Fig. 5). The crosstalk between carbohydrate and oxidative metabolism in plants still needs to be better understood.

Conclusion

One of the greatest limitations of rubber tree production in non-conventional growing areas, such as those experiencing long periods of drought, is seedlings sensitivity to severe climatic conditions. Among the metabolic characteristics evaluated here, greater vacuolar invertase activity resulted in vacuolar accumulations of reducing sugars and greater tolerance to water stress conditions. Those vacuolar sugars function as osmoregulators and help protect the tonoplast against oxidative damage caused by ROS accumulations due to water stress; their protective roles also allow rapid recovery of growth (leaf expansion) when water becomes available. In general, all four rubber tree clones studied here demonstrated reasonable tolerance to drought conditions. With the re-establishment of irrigation after 32 days of water deprivation, all clones reassumed metabolic characteristics similar to those of control plants and, significantly, increased their leaf areas. However, clones GTI and RRIM600, demonstrated greater drought tolerance and more accelerated leaf area gains after rehydration (Fig. 2B). Those more rapid responses were principally due to their initial metabolic responses to drought, especially those related to carbon metabolism and greater VINV activity. Consequently, greater accumulations of vacuolar RS guaranteed greater protection against osmotic stress and oxidation. As such, the introduction of specific sugars (RS) and the modulation of key carbon metabolism enzymes, such as VINV, appear to be promising strategies for promoting drought tolerance in rubber tree clones. Additionally, RS concentrations and VINV activities can serve as markers of drought tolerance during the selection of high productivity rubber tree clones.

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Authors' contributions

JOS: Conceptualisation, methodology, investigation, formal analysis and writing. LEMO: Conceptualisation, supervision, funding acquisition, project administration. VTC, GML, TS, RBM, JMSL and CSR: Investigation. ACMP: Statistical analysis. MPG: formal analysis, writing, critical review and editing.

Conflict of interests

Authors do not have any conflict of interests to declare.

References

1. Debaeke P, Bedoussac L, Bonnet C, Bret-Mestries E, Seassau C, Gavaland A, *et al.* Sunflower crop: environmental-friendly and agroecological. OCL [Internet]. 2016;24(3):304. Available from: https://pdfs.semanticscholar.org/253b/18b55a431c-cb1094bca40524709e1b3af451.pdf

2. Farooq M, Hussain M, Wahid A, Siddique KHM. Drought stress in plants: An overview. In: Aroca R, editor. Plant responses to drought stress: From morphological to molecular features. 2012.
3. Miyan MA. Droughts in Asian Least Developed Countries: vulnerability and sustainability. (Special Issue: International Geosphere-Biosphere Programme: Asia-Pacific Network for Global Change Research.). Weather Clim Extrem. 2015;7:8-23.
4. Alexandratos N, Bruinsma J. World agriculture towards 2030/2050. Land use policy. 2012;
5. Santos JO, Oliveira LEMD, Souza TD, Lopes GM, Coelho VT, Gomes MP. Physiological mechanisms responsible for tolerance to, and recuperation from, drought conditions in four different rubber clones. Ind Crops Prod. 2019;141:111714.
6. Luiz Furtado E, Da Cunha AR, Alvares CA, Alberto J, Bevenuto Z, Passos JR. Ocorrência de epidemia do mal das folhas em regiões de “escape” do Brasil. Arq Inst Biol. 2015;82:1–6.
7. Rivano F, Mattos CRR, Cardoso SEA, Martinez M, Cevallos V, Le Guen V, *et al*. Breeding *Hevea brasiliensis* for yield, growth and SALB resistance for high disease environments. Industrial Crops and Products. 2013;44:659-70.
8. Silva CC, Mantello CC, Campos T, Souza LM, Gonçalves PS, Souza AP. Leaf-, panel- and latex-expressed sequenced tags from the rubber tree (*Hevea brasiliensis*) under cold stressed and suboptimal growing conditions: the development of gene-targeted functional markers for stress response. Mol Breed. 2014;34:1035-53.
9. Austin D, Rao GG, Rajogopal R, Sanjeeva RP, George MJ, Vijayakumar R, *et al*. Studies on soil-plant-atmosphere system in *Hevea*: II. Seasonal effects on water relations and yield. Indian J Nat Rubb Res. 1988;1:45-60.
10. Ashraf M, Harris PJC. Photosynthesis under stressful environments: An overview. Photosynthetica. 2013;51:163-90.
11. Krasensky J, Jonak C. Drought, salt and temperature stress-induced metabolic rearrangements and regulatory networks. J Exp Bot. 2012;63:1593–1608.
12. Sami F, Yusuf M, Faizan M, Faraz A, Hayat S. Role of sugars under abiotic stress Investigation of brassinosteroid mediated abiotic stress tolerance mechanism in crop plants. Artic Plant Physiol Biochem [Internet]. 2016;190:54-61. Available from: http://dx.doi.org/10.1016/j.plaphy.2016.09.005
13. Bartels D, Sunkar R. Drought and salt tolerance in plants. CRC Crit Rev Plant Sci. 2005;1:23-58.
14. Kameli A, Lolsel DM. Carbohydrates and water status in wheat plants under water stress. New Phytol. 1993;125:609-14.
15. Piaskowski JL, Brown D, Campbell KG. Near-infrared calibration of soluble stem carbohydrates for predicting drought tolerance in spring wheat. Agron J. 2016;108:285-93.
16. Adams HD, Germino MJ, Breshears DD, Barron-Gafford GA, Guardiola-Claramonte M, Zou CB, *et al*. Nonstructural leaf carbohydrate dynamics of Pinus edulis during drought induced tree mortality reveal role for carbon metabolism in mortality mechanism. New Phytol. 2013;197(4):1142-51.
17. Sami F, Yusuf M, Faizan M, Faraz A, Hayat S. Role of sugars under abiotic stress. Plant Physiol Biochem. 2016;109:54–61.
18. Griffiths CA, Paul MJ, Foyer CH. Metabolite transport and associated sugar signalling systems underpinning source/sink interactions. Biochim Biophys Acta-Bioenerg. 2016;1857(10):1715–25.
19. Liu S, Lan J, Zhou B, Qin Y, Zhou Y, Xiao X, *et al*. HbNIN2, a cytosolic alkaline/neutral-invertase, is responsible for sucrose catabolism in rubber-producing laticifers of *Hevea brasiliensis* (para rubber tree). New Phytol. 2015;206(2):709–25.
20. Nemati F, Ghanati F, Ahmadi Gavlighi H, Sharifi M. Comparison of sucrose metabolism in wheat seedlings during drought stress and subsequent recovery. Biol Plant. 2018;62(3):595-99.
21. Ruan YL. Sucrose Metabolism: Gateway to Diverse Carbon Use and Sugar Signaling. Annu Rev Plant Biol. 2014;65:33-67.
22. Gomes MP, Garcia QS. Reactive oxygen species and seed germination. Biologia (Bratisl) [Internet]. 2013;68(3):351–57. Available from: http://link.springer.com/10.2478/s11756-013-0161-y
23. Everard JD, Loescher WH. Primary products of photosynthesis, sucrose and other soluble carbohydrates. In: Encyclopedia of Applied Plant Sciences. 2016;96–104.
24. Cavatte PC, Oliveira ÁAG, Morais LE, Martins SCV, Sanglard LMVP, Damatta FM. Could shading reduce the negative impacts of drought on coffee? A morphophysiological analysis. Physiol Plant. 2012;144(2):111-22.
25. Basu PS, Sharma A, Garg ID, Sukumaran NP. Tuber sink modifies photosynthetic response in potato under water stress. Environ Exp Bot. 1999;42(1):25–39.
26. Lawlor DW, Cornic G. Photosynthetic carbon assimilation and associated metabolism in relation to water deficits in higher plants. Plant Cell Environ. 2002;25(2):275-94.
27. Loggini B, Scartazza A, Brugnoli E, Navari-Izzo F. Antioxidative defense system, pigment composition, and photosynthetic efficiency in two wheat cultivars subjected to drought. Plant Physiology. 1999;119(3):1100-99. Available from: https://doi.org/10.1104/pp.119.3.1091
28. Yang J, Zhang J, Li C, Zhang Z, Ma F, Li M. Response of sugar metabolism in apple leaves subjected to short-term drought stress. Plant Physiol Biochem [Internet]. 2019;141:164–71.
29. Silva PEM, Cavatte PC, Morais LE, Medina EF, DaMatta FM. The functional divergence of biomass partitioning, carbon gain and water use in *Coffea canephora* in response to the water supply: Implications for breeding aimed at improving drought tolerance. Environ. Exp. Bot. 2013;87:49-57.
30. Cavalcante JR, De Cássia CE. Desempenho de cinco clones jovens de seringueira na região do Planalto Ocidental Paulista. Bragantia. 2002;61(3):237-45.
31. Gonçalves PS, Aguiar ATE, Gouvêa LRL, Souza Gonçalves P, Tosoni EAA, Gouvêa LRL. Expressão fenotípica de clones de seringueira na região noroeste do estado de São Paulo. Bragantia. 2006;65(3):389–98.
32. Gonçalves PS, Bortoletto N, Sambugaro R, Furtado EL, Bataglia OC, Ortolani AA, Gentil GJ. Desempenho de clones de seringueira de origem amazônica no planalto do Estado de São Paulo. Pesqui. Agropecuária Bras. 2001;36(12):1469–77.
33. Gonçalves PS, Marques JRB. Clones de seringueira: Influência dos fatores ambientais na produção e recomendação para o plantio. In: Seringueira. Viçosa: EPAMIG; 2008;179–48.
34. Zanandrea I, Alves JD, Deuner S, Goulart PDFP, Henrique PDC, Silveira NM. Tolerance of *Sesbania virgata* plants to flooding. Aust J Bot. 2010;57(8):661-69.
35. Dische Z. General color reactions. Carbohydr Chem. 1962;477–512.
36. Miller GL. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. Anal. Chem. 1959;31(3):426-28.
37. Yemm EW, Cocking EC, Ricketts RE. The determination of amino-acids with ninhydrin. Analyst. 1955;80(948):209-14.
38. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Bio. 1976;72(1-2):248–54.
39. Grieve CM, Grattan SR. Rapid assay for determination of water soluble quaternary ammonium compounds. Plant Soil. 1983;70(2):303-07.
40. Zeng Y, Wu Y, Avigne WT, Koch KE. Rapid repression of Maize Invertases by low oxygen. invertase/sucrose synthase balance, sugar signaling potential, and seedling survival. Plant Physiol. 1999;121(2):599–608.
41. Cazetta J, Seebauer JR, Bellow F. Sucrose and nitrogen supplies regulate growth of maize kernels. Ann Bot. 1999;84(6):747–54. Available from: https://doi.org/10.1006/anbo.1999.0976
42. Lowell CA, Tomlinson PT, Koch KE. Sucrose-metabolizing enzymes in transport tissues and adjacent sink structures in developing citrus fruit. Plant Physiol. 1989;90(4):1394–1402. Available from: https://doi.org/10.1104/pp.90.4.1394
43. Giannopolitis CN, Ries SK. Superoxide dismutases: I. Occurrence in higher plants. Plant Physiol. 1977;59(2):309-14.Available from: https://doi.org/10.1104/pp.59.2.309
44. Havir EA, McHale NA. Biochemical and developmental characterization of multiple forms of catalase in tobacco leaves. Plant Physiol. 1987;84(2):450-55. Available from https://doi.org/10.1104/pp.84.2.450
45. Nakano Y, Asada K. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell

Physiol. 1981;22(5):867–80. Available from: https://doi.org/10.1093/oxfordjournals.pcp.a076232

46. Buege JA, Aust SD. [30] Microsomal lipid peroxidation. Methods Enzymol. 1978;52:302–10. Available from: https://doi.org/10.1016/S0076-6879(78)52032-6

47. Shapiro SS, Wilk MB. An analysis of variance test for normality (complete samples). Biometrika. 1965;523(3):591-611. Available from: https://doi.org/10.1093/biomet/52.3-4.591

48. Bergman ET, Roediger HL. Can Bartlett's repeated reproduction experiments be replicated?. Mem. Cogn. 199;27:937–47. Available from: https://doi.org/10.3758/BF03201224

49. R core team. R: A Language and environment for statistical computing. R Foundation for Statistical Computing. 2019.

50. Ferreira EB, Cavalcanti PP, Nogueira DA. ExpDes.pt: pacote experimental designs (Portuguese). 2018. Available from: https://cran.r-project.org/package=ExpDes.pt

51. Ginestet, C. ggplot2: Elegant graphics for data analysis. Journal of the Royal Statistical Society. 2011;174:245-46. Available from: https://doi.org/10.1111/j.1467-985X.2010.00676_9.x

52. Groth, D., Hartmann, S., Klie, S., Selbig, J. Principal components analysis. In: Reisfeld B., Mayeno A. (eds) Computational Toxicology. Methods in Molecular Biology (Methods and Protocols) Humana Press, Totowa. 2013;930:527-47. Available from: https://doi.org/10.1007/978-1-62703-059-5_22

53. Kassambara A, Mundt F. Package "factoextra" for R: Extract and visualize the results of multivariate data analyses. R Packag version. 2017;1(5):337-54.

54. Tang Y, Horikoshi M, Li W. Ggfortify: Unified interface to visualize statistical results of popular R packages. The R Journal. 2016;8(2):474.

55. Keunen E, Peshev D, Vangronsveld J, Van Den Ende W, Cuypers A. Plant sugars are crucial players in the oxidative challenge during abiotic stress: Extending the traditional concept. Plant Cell Environ. 2013;36(7):1242-55. Available from: http//dx.-doi.org/ 10.1111/pce.12061

56. Trouverie J, Chateau-Joubert S, Thévenot C, Jacquemot MP, Prioul JL. Regulation of vacuolar invertase by abscisic acid or glucose in leaves and roots from maize plantlets. Planta. 2004;219(5):894-905. Available from: https://doi.org/10.1007/s00425-004-1289-3

57. Devakumar A. Photosynthesis in mature trees of *Hevea brasiliensis* experiencing drought and cold stresses concomitant with high light in the Field. Indian J Nat Rubber Res. 2002;15:1–13.

58. Wellstein C, Poschlod P, Gohlke A, Chelli S, Campetella G, Rosbakh S, *et al*. Effects of extreme drought on specific leaf area of grassland species: A meta-analysis of experimental studies in temperate and sub-Mediterranean systems. Glob Chang Biol. 2017;23(6):2473-81. Available from: https://doi.org/10.1111/gcb.13662

59. Bonfig KB, Gabler A, Simon UK, Luschin-Ebengreuth N, Hatz M, Berger S, Muhammad N, Zeier J, Sinha AK, Roitsch T. Post-translational derepression of invertase activity in source leaves via down-regulation of invertase inhibitor expression is part of the plant defense response. Mol Plant. 2010;3(6):1037-48. Available from: https://doi.org/10.1093/mp/ssq053

60. Roitsch T, González MC. Function and regulation of plant invertases: Sweet sensations. Trends in Plant Science 2004;9(12):606–13. Available from https://doi.org/10.1016/j.tplants.2004.10.009

61. Geigenberger P, Stitt M. Sucrose synthase catalyses a readily reversible reaction *in vivo* in developing potato tubers and other plant tissues. Planta. 1993;189(3):329-39. Available from: https://doi.org/10.1007/BF00194429

62. Skirycz A, Inzé D. More from less: Plant growth under limited water. Current Opinion in Biotechnology. 2010;21(2):197-203. Available from: https://doi.org/10.1016/j.copbio.2010.03.002

63. Muller B, Pantin F, Génard M, Turc O, Freixes S, Piques M, *et al*. Water deficits uncouple growth from photosynthesis, increase C content and modify the relationships between C and growth in sink organs. J Exp Bot. 2011;62(6):1715-29. Available from: https://doi.org/10.1093/jxb/erq438

64. Parvanova D, Popova A, Zaharieva I, Lambrev P, Konstantinova T, Taneva S, *et al*. Low temperature tolerance of tobacco plants transformed to accumulate proline, fructans or glycine betaine. Variable chlorophyll fluorescence evidence. Photosynthetica. 2004;42(2):179-85. Available from: https://doi.org/10.1023/B:PHOT.0000040588.31318.0f

65. Quan R, Shang M, Zhang H, Zhao Y, Zhang J. Engineering of enhanced glycine betaine synthesis improves drought tolerance in maize. Plant Biotechnol J. 2004;2(6):477–86. Available from: https://doi.org/10.1111/j.1467-7652.2004.00093.x

66. He C, Zhang W, Gao Q, Yang A, Hu X, Zhang J. Enhancement of drought resistance and biomass by increasing the amount of glycine betaine in wheat seedlings. Euphytica. 2011;177(2):151–67. Available from: https://doi.org/10.1007/s10681-010-0263-3

67. Wang M, Tang JC. Research of antibiotics pollution in soil environments and its ecological toxicity. J Agric Environ Sci. 2010;29:261–66.

68. Giri J. Glycine betaine and abiotic stress tolerance in plants. Plant Signal Behav. 2011;6(11):1746-51. Available from: https://doi.org/10.4161/psb.6.11.17801

69. Van den Ende W, Valluru R. Sucrose, sucrosyl oligosaccharides and oxidative stress: scavenging and salvaging? J Exp Bot. 2009;60(1):9–18. Available from: https://doi.org/10.1093/jxb/ern297

70. Peshev D, Vergauwen R, Moglia A, Hideg E, Van den Ende W. Towards understanding vacuolar antioxidant mechanisms: a role for fructans? J Exp Bot. 2013;64(4):1025–38. Available from: https://doi.org/10.1093/jxb/ers377

71. Valluru R, Van Den Ende W. Plant fructans in stress environments: Emerging concepts and future prospects. Journal of Experimental Botany. 2008;59(11):2095-16. Available from: https://doi.org/10.1093/jxb/ern164

72. Tognetti JA, Pontis HG, Martínez-Noël GMA. Sucrose signaling in plants: A world yet to be explored. Plant Signaling and Behavior. 2013;8(3):e23316. Available from: https://doi.org/10.4161/psb.23316

73. Wind J, Smeekens S, Hanson J. Sucrose: Metabolite and signaling molecule. Phytochemistry. 2010;71:1610–14. Available from: https://doi.org/10.1016/j.phytochem.2010.07.007

74. Xiang L, Li Y, Rolland F, Van den Ende W. Neutral invertase, hexokinase and mitochondrial ROS homeostasis: emerging links between sugar metabolism, sugar signaling and ascorbate synthesis. Plant Signal Behav. 2011;6(10):1567–73. Available from: https://doi.org/10.4161/psb.6.10.17036

75. Storr T, Hall JL. The effect of infection by Erysiphe pisi DC on acid and alkaline invertase activities and aspects of starch biochemistry in leaves of *Pisum sativum* L. New Phytol. 1992;121(4):535–43. Available from: https://doi.org/10.1111/j.1469-8137.1992.tb01123.x

76. Essmann J, Bones P, Weis E, Scharte J. Leaf carbohydrate metabolism during defense. Plant Signal Behav. 2008;3(10):885–87. Available from: https://doi.org/10.4161/psb.3.10.6501

77. Park J, Kim S, Choi E, Auh C-K, Park J-B, Kim D, Chung YJ, Lee TK, Lee S. Altered invertase activities of symptomatic tissues on Beet severe curly top virus (BSCTV) infected *Arabidopsis thaliana*. J Plant Res. 2013;126(5):743-52. Available from: https://10.1007/s10265-013-0562-6

78. Tripathy BC, Oelmüller R. Reactive oxygen species generation and signaling in plants. Plant Signal Behav. 2012;7(12):1621–33. Available from: https://doi.org/10.4161/psb.22455