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Influence of Water Deficit on the Physiological and Biochemical Parameters of in vitro *Plants from* Hevea brasiliensis *Clone PB 260*

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The influence of a water deficit was investigated on in vitro *plantlets from the rubber tree (*Hevea brasiliensis*) clone PB 260. The Fraction of Transpirable Soil Water (FTSW) was used to identify three levels of water deficit. Transpiration and stomatal conductance decreased for a FTSW of 0.4 while fluorescence appeared more stable. Proline and ascorbate contents increased from the same level of water deficit. Antioxidant enzyme activities were more stable than glutathione reductase activity, which dramatically increased in line with the water deficit to peak at 0.12 UI/mg for a FTSW of 0.2. When plants were rehydrated, enzyme activities decreased. The accumulation of proline for the lowest FTSW suggested water stress. Antioxidant systems did not play a predominant role for this genotype.*

Keywords: abiotic stress; ascorbate peroxidase; water deficit; glutathione reductase; oxidative stress; proline; rubber tree

Global warming and climate change involve significant changes in temperature, rainfall and wind. In addition to these changes occurring in traditional growing areas, rubber planting material needs to be adapted to new growing areas. Nontraditional areas can be found in several countries, such as northeastern India, northern and northeastern Thailand, East Java in Indonesia, Yunnan in China or Mato Grosso in Brazil. Earlier studies have highlighted the importance of environmental factors[1]. Of these, rainfall is considered as a major factor that can influence tree productivity[2] by affecting physiological and biochemical processes in plants. Soil moisture and thereby water uptake by roots is essential. Fast adaptation of rubber clones to abiotic stresses remains difficult and requires a better understanding of physiological and molecular defence mechanisms, in order to identify key factors to be used in phenotyping new progenies for their tolerance of abiotic stresses.

Among the physiological responses, stomatal closure to control water losses is dominant and among the earliest responses identified[3]. However, this limitation reduces carbon assimilation at the chloroplast level and photosynthesis is impaired[4,5]. It has been reported that photosystem II (PSII) plays an

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important role in the photosynthesis response to environmental stresses in higher plants[6]. Consequently, chlorophyll, a fluorescence emitted by PSII, can be used as a tool to screen the physiological status of plants[7]. The PSI and PSII reaction centres are also the major generation sites for reactive oxygen species (ROS) in chloroplast[8]. Environmental stress such as drought can trigger the formation of free radicals and reactive oxygen species (ROS), which can act as signalling molecules or result in cellular damage[9–11]. Oxidative stress occurs if there is an imbalance between ROS and antioxidants[12]. The antioxidative system provides essential protection against such oxidative damage[13]. It consists of two mechanisms, one enzymatic and the other non enzymatic[14]. The enzymatic mechanism increases or activates antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR) and catalase. The non enzymatic mechanism involves the synthesis of antioxidant compounds such as ascorbate, glutathione, α-tocopherol or carotenoids to react directly with ROS.

In order to gain a clearer understanding of rubber tree responses to different levels of water deficit, we analysed a set of selected ecophysiological and biochemical parameters, particularly the antioxidant system. The fraction of transpirable soil water (FTSW) is defined by the extractable water remaining in the pot which is used to estimate water use and plant transpiration[15]. FTSW is useful for comparing plant responses to water stress, but the root system has to occupy the entire soil volume. Commercial clone PB 260 is produced by somatic embryogenesis[16,17] and used for genetic transformation in our laboratory[18–20]. This study was thus conducted on self-rooted *in vitro* plantlets from clone PB 260 in order to avoid any effects of the non clonal root system. Some discriminating parameters were suggested for further studies designed to analyse the diversity of plant defence mechanisms under drought conditions within a rubber tree stand.

MATERIALS AND METHODS

Plant Material

In vitro plantlets of clone PB 260 were regenerated by somatic embryogenesis after cryopreservation[21]. The plantlets were acclimatised in a greenhouse at 28ºC with 60% relative humidity. After seven months, the plants were potted in 1 L pots with the same weight of potting mixture and grown under controlled conditions (air temperature from 21 to 31ºC, relative humidity 45%, daily PAR around 250 µmol/m^2/s). We waited until the root system occupied all the segments of the available soil volume. A water deficit was imposed by withholding watering until FTSW reached different levels: 0.6 / 0.4 / 0.2. Some plants reaching a FTSW of 0.2 were then rehydrated. For each treatment, four plants were watered to maintain a FTSW of 0.9 (control) and four plants were subjected to various water deficit levels. Each pot subjected to treatment was enclosed in a plastic bag tied at the stem to prevent evaporation from the soil. The water deficit level was maintained one week before sample collection. FTSW was calculated according to Luquet *et al*.[22] as the ratio: FTSW = ASW : TTSW.

ASW is the available soil water content (difference between the actual soil water content weight and the wilting point weight). TTSW is the total transpirable soil water content (difference between the full capacity weight and the wilting point weight). The wilting point is defined as the moisture content at which no soil water is available to the plant. The wilting point was determined in a preliminary study. Full capacity weight was determined for each plant before dehydration at the beginning of the experiment.

Transpiration, Stomatal Conductance and Chlorophyll Fluorescence Measurements

Transpiration was estimated daily by weighing pots on a balance. The relative transpiration rate was calculated as the ratio between the daily transpiration rate of the drying pot and the mean transpiration rate of the control pots, to minimise variations across days. In order to take into account the different plant sizes, a normalised transpiration rate (NTR) was calculated as the ratio between the relative transpiration rate and the mean relative transpiration rate for that plant when the soil was watered to maintain a FTSW of 0.9 before starting the dehydration treatment[23].

Both stomatal conductance (g_s) and chlorophyll fluorescence were measured *in situ* around midday, using an SC-1 Decagon Devices leaf porometer (Pullman, USA) and a Handy PEA portable fluorimeter (Handy-Plant Efficiency Analyser, Hansatech Instruments, King's Lynn, Norfolk, UK) on dark adapted attached leaves, respectively. Readings were taken on the abaxial side of mature leaves after a dark adaptation period of 30 min using leaf clips. After illumination (1 s at 3000 µmol (photons)/m^2/s with an array of six light emitting diodes) OJIP fluorescence transients were analysed. Fast fluorescence kinetics (F_0 to F_m) were monitored from 10 µs to 1 s. Fluorescence intensity at 50 µs was considered as F_0[24]. The performance index (PI) plant vitality indicator[7,25] was calculated automatically. Each measurement was taken on apparently healthy leaves.

Colorimetric Measurement of Antioxidant and Protective Compounds

For all measurements of antioxidant and protective compounds, a total extract was obtained from 0.5 to 0.9 grams of leaf ground in liquid nitrogen. Powder samples were mixed with 10 mL of 3% sulphosalicylic acid at 4ºC. Samples were centrifuged twice at 11,000 g for 15 min at 4ºC. Leaves of control plants and water-stressed plants were collected at the same time at each FTSW level.

Ascorbic acid content was measured as described by Okamura[26]. Briefly, a standard curve was established with a range of ascorbic acid (AA) concentrations: 0, 20, 40, 60, 80 and 100 nmol. For AA content determination in leaves, 50 µL of total extract was mixed with 1.8 mL of freshly prepared buffer A (10 mL of 0.15 M potassium phosphate buffer pH= 7.4, 25 mL of 10% TCA, 15 mL H_2O mQ, 20 mL of 50% phosphoric acid, 20 mL of 4% 2.2'dipyridyl dissolved in absolute ethanol). Then 200 µL of buffer B was added (3% $FeCl_3$). After 30 min, absorbance was measured with a spectrophotometer at 525 nm.

Thiol contents were measured as described by Ellman[27]. A standard curve was established with a range of reduced glutathione (GSH) concentrations: 0, 20, 40, 60, 80, and 100 nmol. Briefly, 1 mL of calibration range GSH or 500 µL of total extract mixed with 500 µL of 3% sulphosalicylic acid for GSH content determination in leaves, was supplemented with 50 µL DTNB (10 mM DTNB and 20 mM EDTA) and 1 mL 0.5 M Tris. Absorbance was measured before 30 min with a spectrophotometer at 412 nm.

Proline content was measured as described by Bates[28]. Briefly, a standard curve was established with a range of proline concentrations in 1 mL (0, 50, 100, 150, 200, 250 nmol). For proline content determination in leaves, 1 mL of total extract was mixed with 1 mL of ninhydrin (1.25 g of ninhydrin dissolved in 30 mL glacial acetic acid and 20 mL of 6M phosphoric acid) and 1 mL glacial acetic acid. After heating for 1 h at 100ºC, samples were placed on ice and

2 mL of toluene was added. Absorbance was measured on the upper phase at 520 nm.

Antioxidant Enzyme Activities

An extract from leaves frozen in nitrogen was prepared in potassium phosphate buffer 0.1M pH 7 with EDTA (0.1 mM), ascorbate (0.1 mM) and PVP (10% w/w). Protein concentration was determined according to the Bradford method. Ascorbate peroxidase, glutathione reductase, peroxidase and catalase activities were assayed according to the protocol modified by Joët *et al*.[29].

RESULTS

Changes in Physiological Parameters for Various Water Deficit Levels Monitored by the FTSW

We used the fraction of transpirable soil water to monitor the water deficit imposed on plants. This parameter enabled us to compare plant transpiration at the same level of water deficit. The normalised transpiration ratio did not fluctuate very much (0.8-0.7) until FTSW reached ≈ 0.45 (*Figure 1A*), then the transpiration ratio decreased to 0.3 indicating significant transpiration rates for plants subjected to water deficit compared to control plants (FTSW = 0.9). This decrease in transpiration corresponded to plant dehydration kinetics.

Stomatal conductance was measured after one week at the required FTSW level. g_s held steady between 115 and 92 mmol/m^2/s until FTSW decreased below a mean value of 0.34, when g_s fell substantially to 13 mmol/m^2/s (*Figure 1B*).

The chlorophyll fluorescence parameter, like the maximum efficiency of PSII photochemistry F_v:F_m remained almost unchanged throughout water stress (*Figure 1D*). The performance index calculated from the JIP test seemed to be more accurate (*Figure 1C*). PI was equal to or over 10 until FTSW reached 0.34, after which that value decreased to 6.3. Even variability was important as the difference was significant with $P < 0.008$ between 15.6 and 6.3 for FTSW levels of 0.55 and 0.15, respectively.

Some visual drought symptoms, such as yellowing of the leaf lamina, were observed for FTSW 0.2, but not for all the water-stressed plants (*Figure 2A*). At FTSW 0.4, only some leaf scorching was observed on a few plants (*Figure 2B*).

Osmoprotectant and Water Soluble Antioxidants

Proline content evolved with water availability (*Figure 3A*). The free proline content increased when soil water depletion was high; the increase seemed to begin from FTSW 0.4. The proline concentration was enhanced 5- to 6-fold (6.12 µmol/g DM) in water-stressed plants with FTSW 0.2 compared to the average concentration of controls or stressed plants (1.5 µmol/g DM) at lower FTSW values. Interestingly, the proline content returned to baseline after re-watering of water-stressed plants (1.05 µmol/g DM) and only this difference was significant.

GSH concentrations did not show significant changes between stressed and control plants during water stress (*Figure 3B*). The only significant value was also observed for re-watered stressed plants towards their control values.

AA concentrations tended to increase in stressed plants (nearly 0.14-0.15 µmol/g DM) in line with drought intensity, but differences

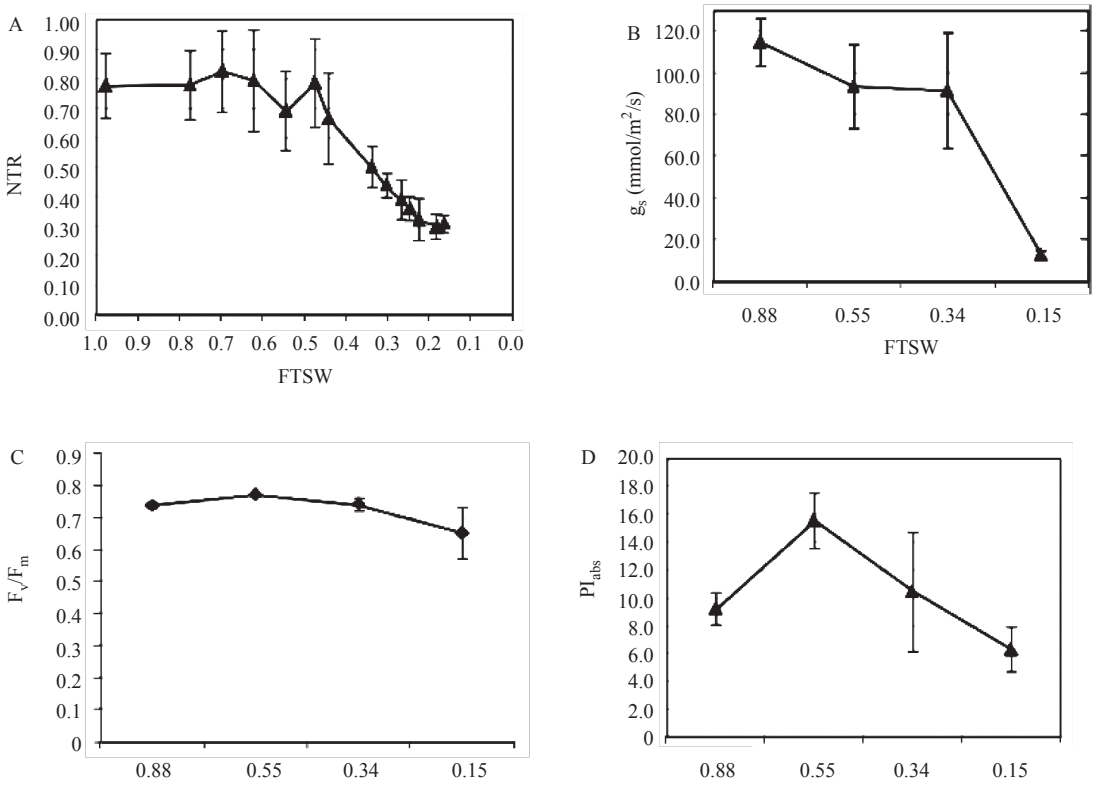

Figure 1. Normalised daily transpiration rate (NTR) (A), stomatal conductance (g_s) (B) performance index (PI_{abs}) (C) and F_v:F_m (D) in response to water deficit (fraction of transpirable soil water, FTSW). For panel A, values are means of four plants allowed to dry up to FTSW 0.2, compared to their well watered controls. For panels B, C and D, values are means of four plants. Results were obtained after one week at different water stress levels, except for FTSW 0.88 where values are means of the fourteen well watered control plants. Error bars indicate standard error.

were not statistically significant (*Figure 3C*). However, the AA concentration decreased significantly for stressed plants after re-watering (0.06 µmol/g DM), confirming the tendency of an increase in line with drought stress.

Antioxidant Enzyme Activities

The glutathione reductase (GR) activity of control plants (≈ 0.07 UI/mg protein) was stable over the water stress period. Water-stressed plants displayed a high GR activity (0.12 UI/mg) when the water stress level was high with FTSW at 0.2 (*Figure 4A*). The p-value obtained by Fisher LSD tests was 0.09. After re-watering, the GR activity of stressed plants decreased (0.05 UI/mg).

The ascorbate peroxidase (APX) activity of control plants was also quite stable over the water stress phase (≈ 1.66 UI/mg). The activity of water-stressed plants slightly increased

Figure 2. Water-stressed plant and control under a severe water deficit FTSW 0.2 (A) and under a moderate deficit FTSW 0.4 (B). In (A) yellowing of the leaf lamina and leaf scorching can be seen on the water-stressed plant, no symptoms on the control plant. In (B), slight leaf scorching can be seen on the water-stressed plant, no symptoms on the control plant.

(1.73 UI/mg) at FTSW 0.2 (*Figure 4B*). No significant difference was observed between control and stressed plants.

The peroxidase (PX) activity of control plants fluctuated over the water stress period. Water-stressed plants showed less activity than control plants (*Figure 4C*).

The catalase (CAT) activity of control plants varied over the water stress period. The activity of water-stressed plants kept a weak level during the drought period (*Figure 4D*).

DISCUSSION

Physiological Markers of Stress

FTSW is a crucial parameter for controlling the water deficit under growing conditions in pots. The FTSW parameter was used to compare different plants in the same physiological state even when there were differences in their growth. According to Sinclair *et al*.[30] the level of water stress for each plant is expressed as a function of water availability in each pot. The intensity of water stress is estimated by the degree of pot drying. The FTSW parameter, which was initially developed in annual crops, but already used as an indicator of water deficits in vineyards[31], could be extended to a tropical crop such as *H. brasiliensis* as it is related to transpiration or decreases in stomatal conductance.

Transpiration was the first parameter showing changes in the physiological status of our *in vitro* plants after withholding water. It was closely linked to stomatal closure. Plants maintained a stable NTR until the water concentration in the soil reached 50 – 45% of

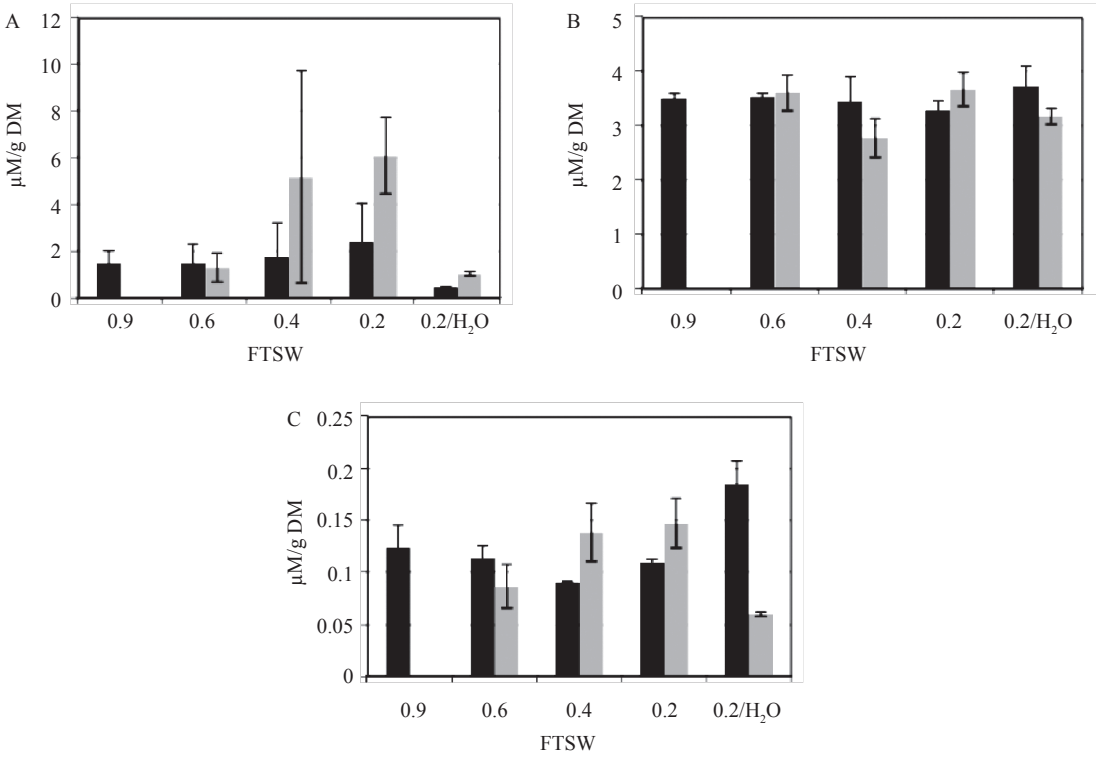

*Figure 3. Effect of water deficit on free proline content (A), thiol content (B) and ascorbate content (C), (black columns, control plants; grey columns, water-stressed plants). Values are means of four plants. Results were obtained after one week at different water stress levels, except for FTSW 0.9 where values are means of the fourteen well watered control plants. Rewatered plants were analysed after one watering week following one week at FTSW 0.2. FTSW values correspond to theoretical values. Error bars indicate standard error. * p-value < 0.05 obtained by Fisher LSD tests.*

total soil water availability (FTSW 0.5 – 0.45). From that level, the transpiration rate fell steeply until the moment water deficit stress was halted. A two-phase response to soil water availability was thus observed. This two-phased decline in transpiration was consistent with a general description of woody plant transpiration responses to soil drying based on the fraction of extractable soil water[30]. The FTSW threshold value, before the dramatic decrease in NTR, was about 0.45 in *Hevea*, which is higher than in annual crops or other woody species such as *Acer rubrum, Robinia pseudoacacia, Hibiscus*, or *Ibex aquifolium*, for which values range from 0.23 to 0.32[30].

The determination of stomatal conductance indicated a FTSW threshold value (0.34) below the transpiration value (0.45). Given that g_s was maximum at FTSW 0.88, the ratio g_s:g_{max} remained steady at 80% during the plateau phase, then it was close to just 10% when FTSW reached 0.15. Surprisingly, the decrease in transpiration occurred before the

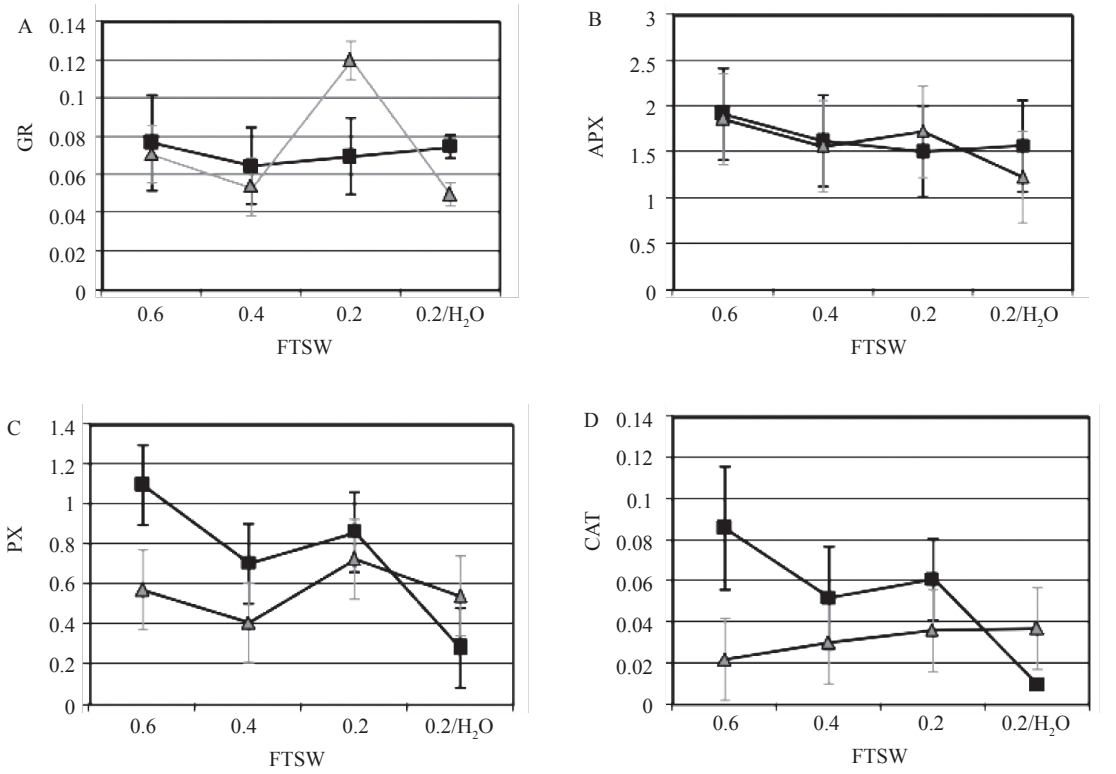

Figure 4. Effect of water stress on glutathione reductase (A), ascorbate peroxidase (B) and peroxidase (C) activities. Black squares: controls; grey triangles: water-stressed plants.
Activities in IU/mg protein. Symbols represent the mean ± S. E. of four plant results.
(*) p-value < 0.1 obtained by Fisher LSD tests.

decrease in g_s. Stomatal conductance and transpiration are closely linked, so the water stress level inducing physiological responses could range somewhere between FTSW values of 0.4 and 0.3 in *Hevea*. Dehydration avoidance involves minimising water loss and maximising water uptake[9]. In *Hevea*, water uptake could be maximised during the first stage of water deficit by increasing investment in the roots. Then water loss would be minimised by closing stomata, explaining the short time lag.

By adjusting stomatal closure, plants limit the entry of carbon dioxide (CO_2) inducing effects on net photosynthesis and on ROS production[4]. Changes in the primary photochemical reactions of the photosynthetic apparatus were monitored by chlorophyll fluorescence measurements. The F_v:F_m ratio and performance index were used to assess these changes.

The F_v:F_m ratio represents the maximum quantum yield of the primary photochemical reaction of photosystem II (PSII). The F_v:F_m ratio remained at around 0.75 up to a FTSW value of 0.34. When FTSW reached 0.15, the F_v:F_m ratio decreased to 0.65. The maximum efficiency of PSII photochemistry was

therefore reduced by 13.3% for the highest level of water deficit. For instance, a drought-susceptible olive tree cultivar presented a decrease in the F_v:F_m ratio (0.61) of 26.5% during a severe drought stress, compared to a more tolerant cultivar, which exhibited a 5% decrease in the F_v:F_m ratio[32]. Both clones PB 260 and RRII 105 have been reported to be drought susceptible based on their photosynthetic parameters. A decrease in photosynthetic oxygen, effective quantum yield of PSII, and the electron transport rate of PSII has been observed, accompanied by chlorophyll bleaching and leaf scorching when irrigation was withdrawn for 18 days[33]. In the field in a dry sub-humid climate, clone PB 260 is classed among the least drought tolerant clones based on its girth growth[34]. During cold stress, clone PB 260 was also one of the most sensitive, displaying weak optimum photochemical efficiency (F_v:F_m) and the highest foliar damage after 96 h at 10ºC, compared to other clones such as RRIM 600[35].

PI_{abs} reflects the functionality of both PSI and PSII and gives quantitative information on the current state of plant performance. Surprisingly, PI_{abs} increased from 9 to 15 between FTSW 0.88 (full capacity) and FTSW 0.55, then decreased steadily to 6 for a FTSW of 0.15. Oukkarroum *et al.*[36] and Zivcak *et al.*[37] considered that PI_{abs} was a more accurate parameter for detecting early changes in plant photosynthesis during water stress. However, inhibition of photosynthesis was not clearly observed until FTSW reached 0.15 and PI_{abs} dropped by 60% in comparison to its value at FTSW 0.55. In addition, leaf yellowing and scorching were only observed for FTSW 0.2. These results suggest that water stress actually occurred and could act on the growth capacities of plants. However, the chlorophyll fluorescence parameters did not enable us to detect any early signs of stress in *Hevea*.

Biochemical Markers of Stress

An increase in proline content was observed in *Hevea in vitro* plantlets as soon as the FTSW reached 0.4 and 0.2. This high proline content may explain the lack of cellular damage. Proline accumulation is well known to occur in plants in response to biotic or abiotic stresses. Proline can protect plants from osmotic stress by increasing cell potential and stabilising proteins, membranes and subcellular structures. This osmoprotectant role has been confirmed by genetic manipulation of the proline metabolism[38]. Another role is as a ROS-scavenger. Among various compatible solutes, proline is the only one that has been shown to protect plants against singlet oxygen and free-radical induced damage, by directly quenching singlet oxygen and hydroxyl radicals[39].

Environmental stresses, such as drought, can lead to the generation of reactive oxygen species. The reaction centres of PSI and PSII in chloroplast thylakoids are the major generation sites of ROS[8]. ROS production during the first steps of drought can be regulated by non enzyme antioxidant metabolites such as ascorbate, glutathione, α-tocopherol or carotenoids, or antioxidant enzymes[13] such as superoxide dismutase, catalase or ascorbate peroxidase and glutathione reductase of the glutathione-ascorbate cycle[14]. Consequently, antioxidants would play an essential role in the first stages of dehydration.

The ascorbate content increased when water stress was high (FTSW 0.4 – 0.2). In fact, a shuttle of ascorbic acid and H_2O_2 operates in the vacuole–cytosol–chloroplast network[40]. Ascorbic acid has a dual role. Firstly, as ROS molecule scavengers and secondly, as a reductant substrate for ascorbate peroxidase. Therefore, if ascorbate concentration is not sufficient, APX activity does not increase sufficiently despite its high affinity for H_2O_2. Changes in the ascorbate (AA) oxidative status

due to an accumulation of dehydroascorbate (DHA) need to be studied, the oxidative status is given as the DHA:AA + DHA ratio. Generally, plants susceptible to water deficit show a depletion of AA and an increase in this ratio[12]. The role of ROS as signalling molecules during the first stages of water deficit, prior to becoming toxic forms, might be considered for our kind of drought stress[41]. To confirm this hypothesis, lipid peroxidation should be investigated to determine at what drought level the scavenging system is overwhelmed and cells are damaged. At the same time, another major enzyme of the ascorbate-glutathione scavenging pathway, glutathione reductase (GR), displayed greatly increased activity at FTSW 0.2. GR is responsible for the conversion of GSSG to GSH to maintain the intracellular glutathione pool in a reduced state. Although the thiol content decreased slightly at FTSW 0.4 and then slightly increased at FTSW 0.2, thiols might function directly as an antioxidant molecule or indirectly as a reducing agent for dehydroascorbate reductase by recycling ascorbic acid. The ascorbate-glutathione cycle therefore seems to be activated, but not sufficiently so, because peroxidase activities (APX and total peroxidases) did not increase significantly. Catalase, which has a low affinity for H_2O_2 compared to APX, did not display any increase in activity either.

An increase in antioxidant enzyme activities is currently observed in response to drought, but this scavenging system is versatile. Contradictory results have been gathered over the years, probably due to plant age, tolerance of, or strategy towards water stress, or the duration and intensity of stress treatment[42]. For example, SOD activity increases in wheat, pea, rice and olive trees under water stress, but decreases in sunflower seedlings and in grass plants. APX and GR activities increase during drought stress in cotton, wheat seedlings, beans and rice, but these activities decrease in pea plants. In our system, detoxifying enzymes did not increase and no consequent foliar damage was observed until water stress levels reached a FTSW of 0.2, which could correspond to a low H_2O_2 concentration during the first level of water deficit. Antioxidant molecules might be sufficient to quench singlet oxygen, superoxide or even hydroxyl radicals during the lowest stage of water deficit. The hydrogen peroxide concentration might be restrictive, triggering SOD, catalase and APX activities. Consequently, either there was a low H_2O_2 concentration, due to no activation of SOD, which was not studied, or weak intrinsic APX and catalase activities. To answer these hypotheses, the analysis could be completed by SOD activity measurements, H_2O_2 estimations and transgenic studies. *Hevea* clone PB 260 has been described as susceptible to cold injury, without any change in SOD activity. By contrast, clone RRIM 600 has been described as being the most tolerant of cold injury, correlated with greater SOD activity and rapid stomatal closure under cold stress[35].

Use of Physiological Parameters in Breeding Programmes

A better understanding of drought tolerance mechanisms is required for early selection in rubber breeding programmes. Our study established that the FTSW is a crucial parameter for studying the response to water deficit in plants grown under controlled conditions. Proline is a simple and relevant factor for such analysis. Proline content is already used for the phenotyping of genotypes in Indonesia (*pers. comm*. Kuswanhadi). Interestingly, some studies on proline have confirmed its implication in defence mechanisms in *Hevea*[43]. Other foliar biochemical parameters (GR and APX activities, ascorbate, thiol contents) should be confirmed for further phenotyping of *H. brasiliensis* genotypes for drought tolerance. SOD is a crucial enzyme catalysing

the conversion of O_2^- into H_2O_2. Clone PB 260 transgenic plants carrying an additional copy of the *Hevea* cytosolic *CuZnSOD* gene presented an increase in APX and GR activities under water stress[44]. This increase suggests that the amount of H_2O_2 would be limiting to activate APX and catalase in our study. Furthermore, proline content increased substantially for over expressing lines under a water deficit, four times more than the transformed control line and eight times more than the wild type. This result suggests a possible interaction between the ROS signalling pathway and the ascorbate-glutathione cycle as well as the biosynthesis of proline and the water stress signalling pathway. SOD over expressing plants also exhibited lower stomatal conductance and a lower transpiration rate, suggesting better water use efficiency. For instance, clone PB 260 transgenic plants over expressing the *HbCuZnSOD* gene displayed an activation of the ROS scavenging system that could protect them from oxidative stress. Further studies using transgenic plants will enable a comprehensive approach to rubber tree defences through the crucial role of regulatory and functional proteins. This approach could lead to the identification of candidate genes to develop molecular breeding.

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