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Selected Physiological Traits of *Hevea brasiliensis* Clonal Seedlings Influenced by Water Stress

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ABSTRACT

Water stress has been identified as one of the most critical environmental factors that will affect crop productivity in the coming years. Since the propagation of *Hevea brasiliensis* uses the bud-grafting method, selecting a good rootstock is crucial to withstand water stress conditions. This is due to the rootstock scion interaction accounting for about 20% contribution to the scion growth. In this study, four *Hevea* clonal seedlings namely RRIM 2002, RRIM 2020, RRIM 2023 and RRIM 2024, were observed for their intrinsic tolerance to water stress conditions. Two to three whorls of plants in polybags were grown in the glasshouse and subjected to two water treatments which were well-watered and water stress by withholding irrigation for 14 days. Physiological traits (chlorophyll pigments, membrane stability and epicuticular wax content), relative water content, and proline and sucrose contents were observed. After commencing the water stress condition for 14 days, all four clonal seedlings showed significantly decreased leaf relative water content. In addition, variation was discerned in leaf cell membrane stability that coincided with significantly lower membrane injury. An increasing trend in proline content from day seven to 14 and a comparable sucrose content in leaf tissue throughout the experiment were also observed. RRIM 2023 exhibited the highest membrane stability, whilst RRIM 2020 was severely affected, which reflected on their low ability to withstand water stress. A similar trend was observed for epicuticular wax content, chlorophyll b and total chlorophyll of RRIM 2023 compared to RRIM 2020. Hence, membrane stability supported by epicuticular wax content and chlorophyll levels revealed RRIM 2023 as the most resilient to water stress.

Keywords: *Hevea brasiliensis*; epicuticular wax; membrane stability; water stress.

INTRODUCTION

Water is becoming increasingly limited in many areas and water stress has been identified as one of the most critical environmental factors that limit crop productivity in the changing climate scenario (Raj et al., 2005). Water stress will cause the water soil availability to plants to decrease and thus further limit other traits as the severity is increased (da Silva et al., 2013). In *Hevea brasiliensis*, prolonged water stress conditions imposed on immature plants caused harm on the growth and physiological conditions such as hydraulic failure (Ahmad, 1999; Dey and Vijayakumar, 2005), chlorophyll instability and inhibition of photosynthesis (Sumesh et al., 2011; Thomas et al., 2015). In the case of rubber yield for the first, second and third year after tapping that were experiencing moderate to severe drought conditions, the yield reduced between 43 to 55% compared to the irrigated trees (Devkumar et al., 1998). Additionally, projection on drought frequency for 2024 showed that in the next 3 to 10 years would foresee about 27.6% of rubber planted areas being prone to drought (Mohd Hazir et al., 2018). Thus, screening and evaluation at the immature stage are important measures to gauge the level of adaptation of *Hevea* clonal seedlings to water stress.

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In *H. brasiliensis*, propagation of the rubber tree is carried out using seeds that are utilised as rootstock before being grafted with the desired bud patch, which is then represented as the clone. In the past, utilisation of rootstocks such as RRIM 623, PB 5/51 and GT1 were thought to have a significant impact on *Hevea* scion growth potential and yield productivity (Ng et al., 1981; MRB, 2019). In addition, RRIM 901, RRIM 712, RRIM 605, PB217 and PB 235 were the later additions of recommended rootstocks by MRB (MRB, 2019). However, the supply of the abovementioned rootstock seedlings is low due to limited number of their plantings nationwide. The current rootstocks were obtained mainly from RRIM 2000 series and RRIM 3001 clone that are widely planted in the field (MRB, 2019), but the information on these rootstocks in relation to water stress is lacking. Research on polybag stage bud-grafted plants in the open and glasshouse condition showed a clonal variation in closing of stomata which results in a reduction of photosynthesis rate under water stress conditions (Sumesh et al., 2011). Other evaluations that had been performed on polybag stage bud-grafted plants in the nursery included variation in rooting characteristics, water use efficiency and osmoregulation, and leaf stomata regulation in relation to soil moisture (Ahmad, 1999). Thus, an assessment of the currently used or available rootstocks is required to compare their physiological performance under water stress condition.

Physiological traits such as chlorophyll content, leaf wax content and chlorophyll fluorescence have been employed to assess the potential tolerance of *Hevea* genotypes or clones to water stress. In addition, leaf epicuticular wax and chlorophyll content have also been implicated as significant physiological parameters in the evaluation of drought-tolerant and susceptible genotype (Sumesh et al., 2011; Thomas et al., 2015). Similar genotypic differences were observed in wheat, whereby drought-tolerant genotypes accumulated higher epicuticular wax content compared to their drought-sensitive counterparts (Guo et al., 2016). Furthermore, water stress condition was shown to promote the development and accumulation of leaf wax content , while humid condition or rainfall resulted in loss of epicuticular wax (Skoss, 1955). Thus, it is plausible that epicuticular wax developed under water stress is essential to reduce loss of humidity via transpiration and maintenance of chlorophyll pigment that is required for photosynthesis.

In addition, plants can alter water relations to maintain cellular metabolic function, such as osmotic adjustment through accumulation of proline and sucrose solute (Molinari et al., 2007). Both proline and sucrose facilitate in the defence or recovery mechanism of relief from water stress. Concerning osmotic adjustment, the damaging of plant cells caused by disintegration of plasma membrane can be estimated by measuring membrane stability index (Tardieu, 2012). However, in rubber seedlings, the status of membrane and chlorophyll stability caused by water stress have yet to be investigated. Thus, the present study was aimed at evaluating four different *Hevea* clonal seedlings using selected physiological traits under water stress conditions. The underlying adaptation to water stress is discussed.

MATERIALS AND METHODS

The experiment was conducted using a completely randomized design (CRD) in the glasshouse at Rubber Research Institute of Malaysia, Sungai Buloh, Selangor. The temperature during the study period in the glasshouse ranged from 24°C to 39°C, and relative humidity was between 41% and 97%. The experiment comprised 72 pots in total that consisted of four *Hevea* clonal seedlings *viz*. RRIM 2002, RRIM 2020, RRIM 2023 and RRIM 2024 which were applied with two water treatments, which were well-watered and water stress. Three replications were allocated for each clonal seedlings and water treatment.

The seeds were collected from the middle of monoclonal block plantings at Field 101 in Pelepah, Kota Tinggi, Johor. The seeds collected were raised in polyethylene polybags size 18 cm (diameter) x 38 cm (height), until two whorls harden leaves stage (approximately three months) and acclimatised in the greenhouse for 2 weeks before application of the water treatments (Figure 1). Before treatment application, the seedlings were watered daily and fertilised with Kokei (slow-release fertiliser). The water stress was imposed when they attained two whorls harden of leaves/fully expanded leaves by withholding irrigation for 14 days, and the well-irrigated plants were watered at field capacity as a control.

Figure 1. *Hevea* seedlings raised in 18cm x 38cm pots and arranged in the greenhouse.

Field capacity

Field capacity (FC) of the soil was determined using the gravimetric method. The soil was air-dried for 14 days before 7 kg of soil was filled up in the pots (18 cm diameter and 38 cm height). The pots were then saturated with water and left to drain for 48 h without disturbance. After 48 h, the pots were weighed and recorded as an initial weight for field capacity. To re-establish the right field capacity, each pot was weighed using electronic digital balance every day and the appropriate amount of water was added slowly to the soil surfaces until it reached an initial weight of FC.

Soil moisture

The soil moisture status of the plants was recorded using a soil moisture meter (Spectrum Technologies, Inc. USA). The probe was inserted to 30 cm soil depth at three different points per polybag. Before the soil moisture reading, the soil moisture meter was calibrated using the calibration trimmer adjustment slot for a meter reading of “10”. Then, the determination of soil moisture content was done following five scales that have been established, that are 10 (wet), 6 to 8 (average wet), 4 to 6 (average), 2 to 4 (average dry) and 0 (dry).

Epicuticular wax determination

Epicuticular wax content was estimated gravimetrically using chloroform (Ebercon et al., 1977). The middle leaflets of the leaf in uniform sizes were cut and the leaf area was recorded (cm^2). The leaf was rinsed repeatedly for 15 to 20 seconds in a 20 mL chloroform in pre-weighed (W_1) beaker. The rinsed leaf was then kept in an oven, overnight at 50°C, and then the weight of the beaker (W_2) was measured using a balance (Shimadzu, TX323L). The epicuticular wax content on a leaf (g/cm^2) was calculated as follows:

$$(W_2\text{-}W_1)/A$$

Where, W_1=Initial weight of empty beaker (g), W_2= Final weight of (with wax) beaker (g), A= Leaf area (cm^2)

Chlorophyll stability

Chlorophyll a, chlorophyll b and total chlorophyll content were estimated following Witham et al. (1986). Fresh leaves (0.2 g) were selected from mature or immature leaf whorls. The leaf samples were immediately immersed in 20 mL of 80% acetone in an aluminium foil covered glass bottle for approximately 72 h and kept in the dark to induce bleaching of the green pigment. The absorbance value of the leaf leachate was recorded at 645 nm and 663 nm using Spectrophotometer (Shimadzu UV-1800).

Membrane stability

The membrane stability index (MSI) was measured using a conductivity meter (Eutech CON 450). Leaf samples (200 mg) were thoroughly washed in double distilled water and placed in two separate 10 mL tubes containing distilled water. One tube was heated for 30 min at 40°C in a water bath and electrical conductivity was measured (C1). The duplicate tube was boiled for 10 min at 100°C in a water bath and electrical conductivity was measured (C2) (Khanna-Chopra and Selote, 2007).

The MSI was estimated using the equation given below:

$$MSI = \left\{1 - \frac{(C1)}{(C2)}\right\} \times 100$$

Membrane injury (MI) was estimated as the ratio of MSI of drought-stressed plants and MSI of control plants as given by Dhanda et al. (2004).

$$MI\ (\%) = \left\{1 - \frac{(MSId)}{(MSIc)}\right\} \times 100$$

Relative water content

Relative water content (RWC) is an indication of the water status of the plant responses to the different environment. The measurement of leaf RWC was performed on newly expanded leaves collected from the potted clonal seedlings. A puncture (diameter = 0.6 cm) was used to cut 20 leaf discs, weighing a minimum of 0.5 g fresh weight (FW). Then, the leaves were floated in distilled water in a covered petri dish for 24 h at room temperature to get the turgid weight (TW). After incubation, the leaf discs were placed in a pre-heated oven at 80°C for 48 h to obtain the weight (DW). RWC of leaves was calculated according to the formula by Barrs and Weatherly (1962):

$$RWC\ (\%) = [(FW-DW)/(TW-DW)] * 100$$

Sucrose and proline

Sucrose content was determined using the Anthrone method, as described by Dische (1962). A standard curve was established with a range of sucrose concentrations (0.01 mM, 0.025 mM, 0.05 mM, 0.1 mM, 0.2 mM and 0.5 mM). For determination of sucrose in leaf samples, 0.5 g leaf tissue was extracted in 10 mL of 80% ethanol in a water bath pre-set at 85°C, for 30 min. Then, the extraction was centrifuged (Thermo Scientific, Heraeus Multifuge X IR) at 11,180 g until a clear separation of supernatant and pellet was obtained. The supernatant (15 µL) was added to 135 µL of distilled water, 350 µL of 2.5% trichloroacetic acid and 3 mL Anthrone. The mixture was incubated in a water bath at 100°C for 15 min, prior to measurement of absorbance at 627 nm using a spectrophotometer (Shimadzu UV-1800).

Proline content was measured as described by Bates et al. (1973). A standard curve was established with a range of proline concentration in 1 mL (20, 40, 60, 80 and 100 nmoL). For determination of proline in leaf samples, 1 mL of the total extracts in 3% sulphosalicylic acid was mixed with 1 mL of glacial ninhydrin and 1 mL glacial acetic acid. Both the standard and the samples were boiled for 1 h, with the test tubes covered with aluminium foil. After heating for 1 h at 100°C, the test tubes were placed on ice and 2 mL of toluene was added and the mixture was mixed vigorously for 15 to 20 s. Finally, the proline content was measured from the upper phase at 520 nm using a spectrophotometer (Shimadzu UV-1800).

Statistical analysis

All data were subjected to a two-way analysis of variance (ANOVA) to determine differences among *Hevea* clonal seedlings and water treatments for each variable at 7 and 14 days of the water stress period. The significant differences between means were determined using the LSD test at $p < 0.05$ level. Statistical tests were performed with SAS 9.4 for windows (SAS Institute Inc, NC, USA).

RESULTS

Under water stress, soil moisture content showed a decreasing trend throughout experiment in all clonal seedlings. A significant difference in soil moisture status was found between the seedlings from day two until day 10. However, there was no significant difference between clonals from day 11 to 14. The greatest decrease was observed in RRIM 2023, and the least decrease in RRIM 2002 (Figure 2A). Relative water content began to reduce from day seven until the end of the experiment. However, no significant difference between seedlings was observed on days 0, 7 and 14 (Figure 2B).

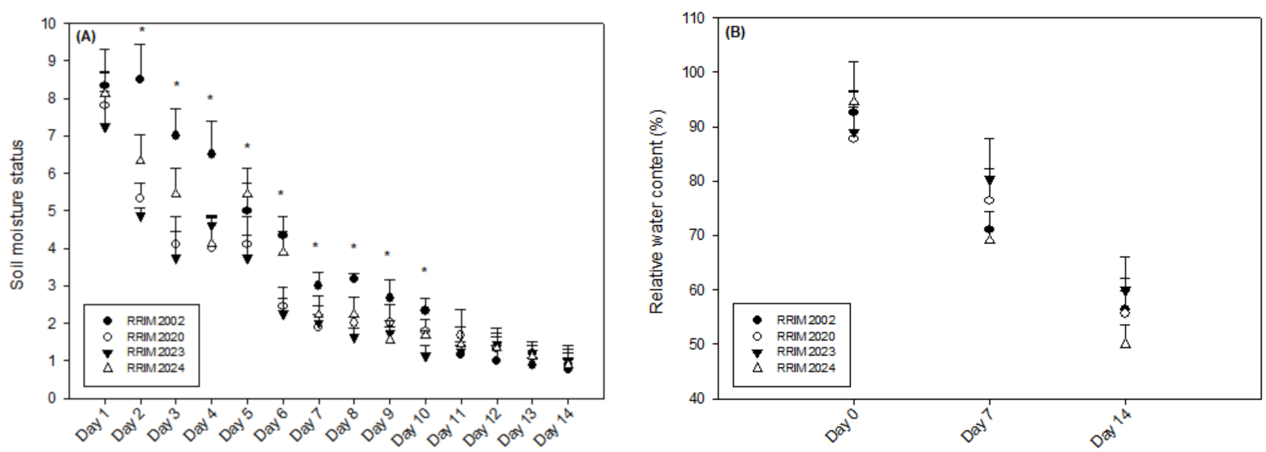

Figure 2. Effect of water stress on soil moisture content (A) and relative water content (B) in seedlings of RRIM 2002, RRIM 2020, RRIM 2023 and RRIM 2024 throughout the 14 days duration of the experiment. Values were means of four plants with error bars which indicated standard error. The asterisk (*) above the means on the day of measurement were significantly different according to LSD test, ($p < 0.05$).

Water stress caused a decrease in membrane stability index (MSI) (Figures 3A, B) and an increase in membrane injury (MI) (Fig. 4A, B) from day 7 onwards (to day 14). The magnitude of the decline in MSI from day 7 onwards (to day 14) was the greatest in RRIM 2020, followed by RRIM 2024, RRIM 2002 and RRIM 2023 by 40%, 26%, 23%, 8%, respectively (Figures 2A, B). RRIM 2023 maintained significantly higher MSI and exhibited lower MI than RRIM 2024, RRIM 2002 and RRIM 2020 (Figures 4A, B). Seven days after water stress, chlorophyll a, b and total chlorophyll (a+b) contents were significantly higher in RRIM 2024 and RRIM 2023 relative to RRIM 2020, but no difference was observed on 14 days of stress (Table 1). Despite the variability in chlorophyll content, epicuticular wax content (ECW) also presented variability on both day 7 and day 14 of water stress, whereby the values were significantly varied, ranging from 0.78 mg to 15.33 mg on day 7, and ranging from 6.96 mg to 51.29 mg on day 14. The variation was more pronounced on day 14 of water stress. The highest ECW content was detected in RRIM 2023, followed by RRIM 2024, RRIM 2002 and RRIM 2020 (Table 1).

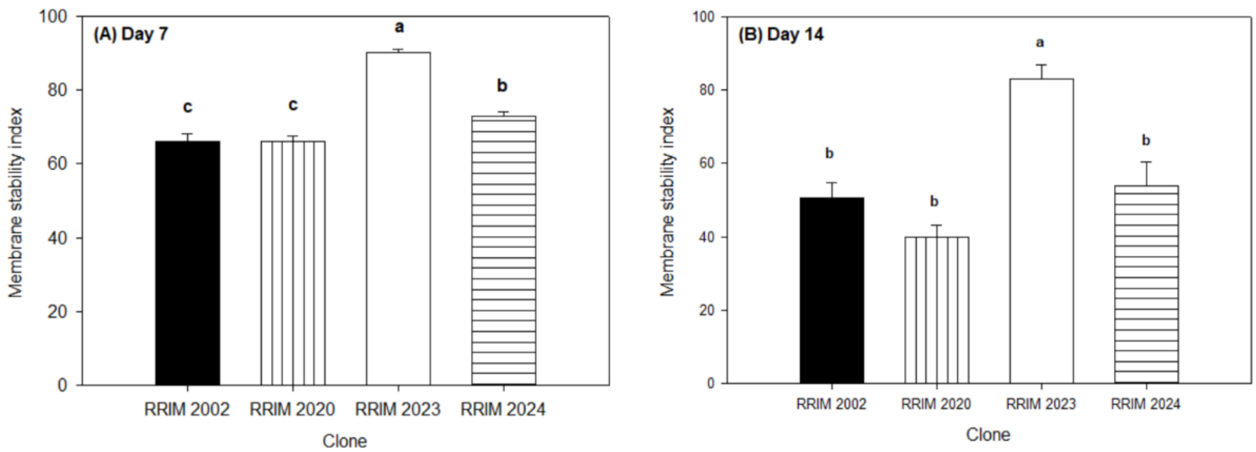

Figure 3. Effect of water stress on membrane stability index at (A) day 7 and (B) day 14 of the four test seedlings (black column, RRIM 2002; vertical line column, RRIM 2020; white column, RRIM 2023; horizontal line column, RRIM 2024). Values were means of four plants with error bars which indicated standard error. Different letters indicated a $p < 0.05$ obtained by LSD test.

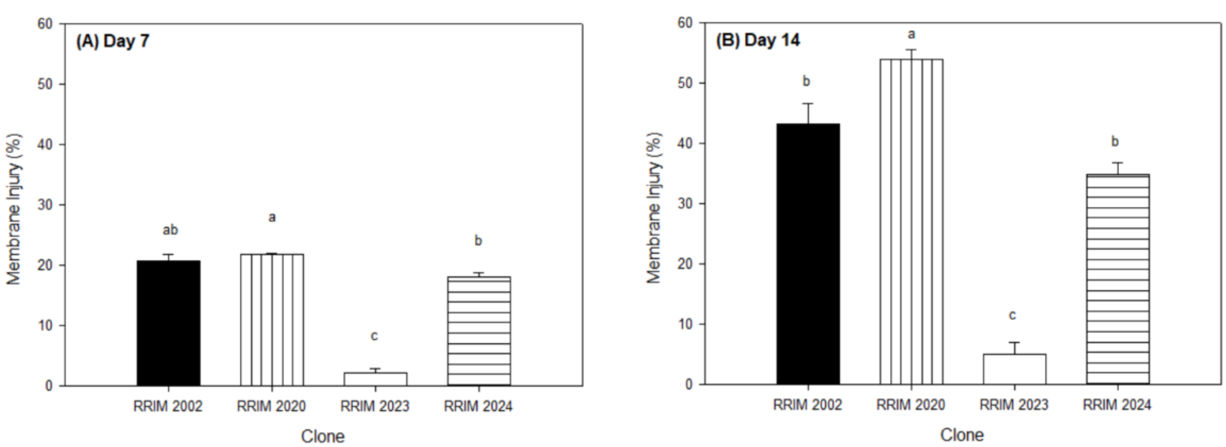

Figure 4. Effect of water stress on membrane injury at (A) day 7 and (B) day 14 on the four test seedlings (black column, RRIM 2002; vertical line column, RRIM 2020; white column, RRIM 2023; horizontal line column, RRIM 2024). Values were means of four plants with error bars which indicated standard error. Different letters indicated a $p < 0.05$ obtained by LSD test.

Table 1. Effect of water stress on chlorophyll a, b and (a+b), and epicuticular wax contents on the four test seedlings (RRIM 2002, RRIM 2020, RRIM 2023 and RRIM 2024) at day 7 and day 14. Means values in each column followed by different letters differed significantly at $p < 0.05$ obtained by LSD test.

	Clone	Chlorophyll *a* (mg/g)	Chlorophyll *b* (mg/g)	Chlorophyll *a+b* (mg/g)	Epicuticular wax content (mg)
Day 7	RRIM 2002	20.0^{ab}	14.8^{ab}	14.8^{ab}	0.78^{b}
	RRIM 2020	15.8^{b}	10.0^{b}	10.3^{b}	6.66^{ab}
	RRIM 2023	22.7^{a}	18.6^{a}	18.4^{a}	15.33^{a}
	RRIM 2024	25.6^{a}	19.4^{a}	19.5^{a}	14.16^{a}
Day 14	RRIM 2002	17.1^{a}	13.1^{a}	13.8^{a}	9.39^{a}
	RRIM 2020	16.8^{a}	9.8^{a}	9.6^{a}	6.96^{b}
	RRIM 2023	17.8^{a}	14.2^{a}	14.0^{a}	51.29^{a}
	RRIM 2024	16.2^{a}	14.8^{a}	14.1^{a}	37.84^{ab}

Among the four seedlings, sucrose accumulation showed no difference after 7 days of water stress (Figure 5A). Further exposure to water stress until 14 days exhibited a variable response with significant higher sucrose in RRIM 2023 relative to RRIM 2002, RRIM 2020 and RRIM 2024 (Figure 5B). It could be inferred that despite the 14 days of water stress treatment, sucrose content was relatively stable except a significant increase in RRIM 2023. Proline content in the four clonal seedlings increased as water stress intensified, as seen on days 7 and 14 (Figures 6A, B). RRIM 2020 showed a pronounced accumulation of proline compared to the other seedlings, as observed on days 7 and 14 of water stress treatment. The magnitude of increase from 7 to 14 days of water stress were 13%, 17%, 37% and 19% in RRIM 2002, RRIM 2020, RRIM 2023 and RRIM 2024, respectively.

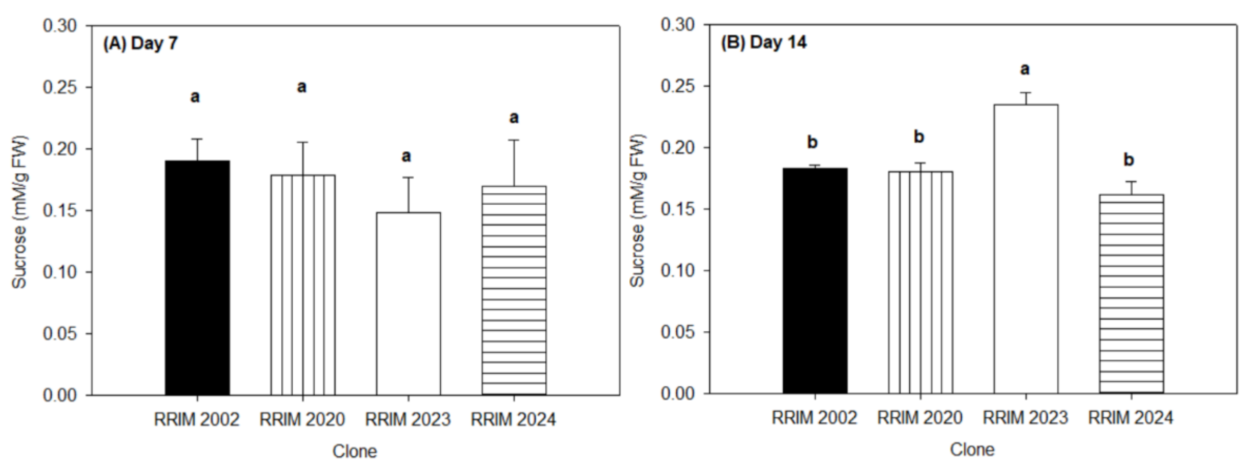

Figure 5. Effect of water stress on sucrose at (A) day 7 and (B) day 14 of the four test seedlings (black column, RRIM 2002; vertical line column, RRIM 2020; white column, RRIM 2023; horizontal line column, RRIM 2024). Values were means of four plants with error bars which indicated standard error. Different letters indicated a $p < 0.05$ obtained by LSD test.

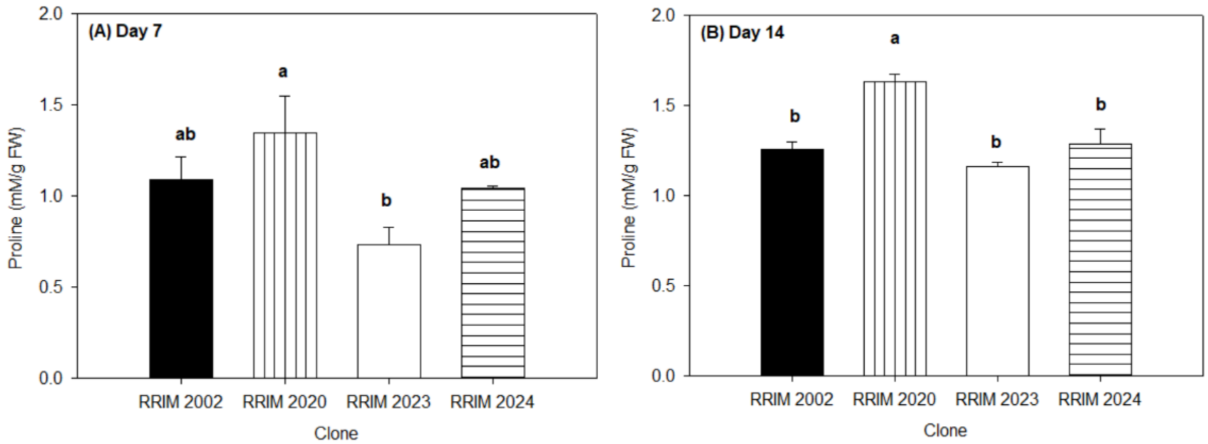

Figure 6. Effect of water stress on proline at (A) day 7 and (B) day 14 of four seedlings (black column, RRIM 2002; vertical line column, RRIM 2020; white column, RRIM 2023; horizontal line column, RRIM 2024). Values were means of four plants with error bars which indicated standard error. Different letters indicated a $p < 0.05$ obtained by LSD test.

DISCUSSIONS

Environmental stress, such as drought or water stress are the factors that restrict growth and rubber productivity (Chandrashekar et al., 1990; Mohana Krishna et al., 1991). Thus, under water stress, soil water loss occurs mainly through transpiration, consequently, reduced water availability for plant uptake leads to turgor loss (Anjum et al., 2011). In the present study, soil moisture content showed a decreasing trend in all clonal seedlings tested. A significant difference was found among the test seedlings from day 2 until day 10 and then no difference until day 14. The greatest decrease was observed in RRIM 2023, and the least decrease was recorded in RRIM 2002 (Figure 2A). The greatest decrease in RRIM 2023 might be due to the rooting characteristics of the RRIM 2023 efficiency in absorbing water relative to other clonal seedlings. However, in this study there is no growth and morphological analysis, and further investigations are needed to evaluate the ability of the roots in water uptake under drought stress conditions. Reduction in soil moisture content tallied with a decrease in relative water content (RWC) in plants under water stress (Figure 2B). RWC is a plant-based water stress indicator for plant water status that indicates the equilibrium in cell volume between water absorbed by the plant and released through transpiration process (Parkash and Singh, 2020). In this study, the lack of significant differences of RWC between seedlings might indicate that a similar mechanism is involved in the regulation of plant water status beyond a certain point (day 10) as the soil dries.

At 14 days after water stress treatment, epicuticular wax content was recorded higher in RRIM 2023 followed by RRIM 2024, whilst lowest in those of RRIM 2002 and RRIM 2020 (Table 1). Leaf wax is an impermeable layer on the leaf that reduces transpirational water loss through stomata (Bueno et al., 2019). Studies in *Hevea* have indicated that leaf epicuticular wax is related to genotypes and wild accessions that are tolerant to water stress or drought condition are distinguished by high wax content (Thomas et al., 2015). It has also been reported in cotton and wheat (Bondada et al., 1996; Zhang et al., 2015) that epicuticular wax could assist in equilibrating water in plants as an adaptation under drought condition by reducing stomatal conductance and transpiration rate (Rao et al., 1988; Zhang et al., 2005). We further observed that RRIM 2023 exhibited the highest membrane stability index (MSI) and the least membrane injury (MI) (Figures 3 and 4). These results indicated that RRIM 2023 maintained water stability through the epicuticular wax barrier on the leaf surface during the water stress period and consequently reduced membrane injury. Thus, diurnal relationship between epicuticular wax content and membrane stability index in a longer period under water stress (more than 14 days) is worth investigating further. This is to better understand the mechanisms that are at play in *Hevea* seedlings in regulating water reserves.

Under water stress conditions, accumulation of proline has been implicated in controlling the osmotic adjustment and is associated with water stress conditions in many plant species (Liu et al., 2011; Sanier et al., 2013). Proline accumulation was reported to be significantly higher in drought-tolerant than drought-sensitive cultivars of mulberry (Reddy et al., 2004), and olive (Ahmed et al., 2009). Increase in proline level in the leaves of seedlings at day 14 compared to day 7 of water stress suggested that the plants could counterbalance the change in osmotic condition, notably in RRIM 2020 seedlings (Figures 6A and B). Furthermore, water stress has been shown to result in the accumulation of sucrose in black poplar (Regier et al., 2009) and mango cultivar (Elsheery and Cao, 2008), which reflects on tolerance to water stress. Elsewhere, sucrose content has been reported to fluctuate in concomitant with the increase in water stress (Wang, 2014). In contrast, a relatively similar sucrose content recorded under water stress in this study except for a significant increase in RRIM 2023 (Figures 5A and B), perhaps a more pronounced trend of proline and sucrose levels could be observed in a prolonged period of water stress.

Water stress has been shown to negatively affect leaf pigment content and accelerate chlorophyll breakdown (Demmig-Adams and Adams, 1996). As expected, a decrease of pigment content (chlorophyll a, b and total chlorophyll) was observed in all the seedlings at day 14 compared to day 7 (Table 1). The significantly higher pigment content recorded in seedlings of RRIM 2023 and RRIM 2024 compared to RRIM 2020 at day 7 indicated on their adaptation to water stress condition where such response had been correlated to lower decomposition of the chlorophyll pigments (Ying et al., 2015). However, pigment content was lower across the board with less variation among the test seedlings at day 14 of water stress. This might indicate that chlorophyll decomposition was involved in the initial phase of water stress before decreasing and reaching an equilibrium state as the water stress intensity increased.

CONCLUSIONS

In conclusion, although RRIM 2023 seedling seems much affected by water stress, the seedling demonstrated better adaptation, possibly due to mechanism involving a complex progression in physiological stress sensing (epicuticular wax content), cellular osmoregulation (membrane stability) and pigments regulation. Thus, these significant traits could be the preliminary information on the performance of clonal seedlings in the selection of drought tolerant rootstocks.

AUTHORS CONTRIBUTION

NMZ conceived and designed the work, and performed the analysis. NMZ wrote the paper, and checked and approved the submission.

CONFLICT OF INTEREST

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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