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Water Stress Response in Different *Jatropha curcas* Accessions from Different Geographical Zones of Botswana: Biochemical & Physiological Perceptive

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Abstract

Jatropha curcas L. is one climate smart drought-resistant multipurpose plant with a variety of properties that have conjured interest all over the world due to its potential to produce biofuel. In this study, *Jatropha curcas* accessions were collected from three different climate zones of Botswana; Northern region (Maun), Central region (Mmadinare) and Southern region (Thamaga). These accessions were subjected to water stress to study their biochemical and physiological responses. Results showed that water stress increased malondialdehyde (MDA) content, electrolyte leakage as well as proline content in all the accessions. It is worth-noting that Maun accession exhibited highest proline content, when subjected to water stress. Maun accession also displayed less MAD and electrolyte leakage than the other two accessions, an indication of less perturbation to membranes under water stress. This could be attributed in part, to its higher catalase and superoxide dismutase contents, which presumably prevented lipid peroxidation by mopping up reactive oxygen species. The slightly higher dry weights exhibited by Mmadinare and Maun accessions could be ascribed to their ability to maintain membrane integrity under water stress conditions. It can therefore be concluded that Maun and Mmadinare accessions can be grown under drought conditions commonly experienced in Botswana.

Keywords

Jatropha curcas, Malondialdehyde, Proline, Soluble Protein, Superoxide Dismutase, Catalase

1. Introduction

Water deficit is one of the major types of abiotic stress that diminishes plant growth and development. Plants exposed to drought undergo several mechanisms (physiological, biochemical and molecular) to survive water scarcity in the soil [1]. Water deficit results in elevated production of Reactive Oxygen Species (ROS), which causes oxidative damage and impairs cellular functions [2]. Oxidative stress is a state of imbalance between the production of ROS and the neutralization of the free radicals by antioxidants, leading to the damage of cellular components such as lipids, nucleic acids, metabolites and proteins, which ultimately causes cell death [3].

Water deficiency in plants stimulates the generation of ROS, which brings about deleterious effects and causes lipid peroxidation, consequently leading to membrane injuries, protein degradation and enzyme inactivation as well as the formation of protease-resistant cross-linked aggregates [4]. Lipid peroxidation, which is a decomposition of polyunsaturated lipids in the plasma membrane, takes place in a stressed plant and it is used as an indicator to determine the extent of lipid damage under severe drought conditions by quantifying Malondealdeyde, a product of lipid peroxidation [4].

In response to oxidative damage, plant species develop multiple photo-protective and antioxidant defense mechanisms to counteract oxidative damage. ROS can act as secondary messengers in the signaling of the activation of defense responses, and this significantly promotes the acclimatization of plants to water stress conditions [5].

Furthermore, among the various mechanisms used by the plant to tolerate water stress, many plant species undergo accumulation of soluble organic compounds such as osmoregulators. This is to allow for osmotic adjustment, which promotes the maintenance of cellular turgor and encourages water absorption (movement of water molecules into the cell) [6]. Consequently, there is biosynthesis of non-toxic molecules of low molecular weight in the vacuole and cytosol, such as inorganic ions, soluble sugars, amino acids, proline and glycine-betaine. These assist in the maintenance of cell membrane and protein integrity, which are essential for metabolic activities. The accumulation of these molecules has been used as an indicator in the evaluation of drought tolerance [7]. It is for this reason that responses of plants to various stresses are determined using specific biochemical parameters such as protein content and stress-related antioxidants enzymes such as superoxide dismutases, peroxidases and catalases [7].

Jatropha curcas L. is a drought-resistant multipurpose plant with a variety of properties that have conjured interest all over the world due to its potential to produce biofuel [8]. *Jatropha curcas* seeds play a significant role in the production of biodiesel. They contain about 35% to 40% of oil [9]. Also, the seeds cannot be used for human consumption because they contain toxic chemical elements such as cursin, toxalbumine called curine, cyanic acid related to ricimic acid and toxic phorbol esters [8].

The present study was aimed at evaluating water stress tolerance of *Jatropha curcas* by employing partial biochemical characterization involving soluble protein, proline content and activities of potential antioxidant enzymes.

2. Materials and Methods

2.1. Experimental Material and Planting

Three *Jatropha curcas* accession seeds collected from the North, South and Central regions of Botswana (Maun [19˚58'S, 23˚25'E]; Thamaga [24˚40'S, 25˚32'E] and Mmadinare [21˚56'S, 27˚37'E] respectively) were germinated in Petri dishes at 25˚C. After emergence, the seedlings were transferred to pots filled with potting soil (Sanitas, Gaborone, Botswana). The pots with seedlings were set up in a completely randomized designed with three replications. Each pot contained one plant. The plants were raised in a greenhouse at 27˚C and relative humidity of 65% - 70%. When the plants were one month old, they were separated into the control and water-stressed sets. Water stress was imposed by withholding water [10].

2.2. Treatments

Potted plants of the three *Jatropha curcas* accessions (35 days old) were subjected to water stress by withholding water, while for the control, plants were given 250 mL of half-strength Hoagland's solution in alternate days, for 49 days.

2.3. Determination of Lipid Peroxidation (Malondialdehyde Content)

The level of lipid peroxidation was measured by estimating malondialdehyde (MDA) content according to the method developed by [11] at the end of water stress exposure. Leaf tissue (0.2 g) from fully expanded leaves was homogenized in 1.0 mL 5% trichloroacetic acid (TCA) solution in cold pestle and mortar. Homogenate was centrifuged at 14,000 rpm for 10 min at room temperature. The supernatant was collected for the estimation of MDA. The reaction mixture contained 1.0 mL of an aliquot of the supernatant and 4 mL of 0.5% thiobarbituric acid (TBA) in 20% TCA solution (freshly prepared), heated at 90˚C for 30 min. After 30 min, the reaction was stopped by quickly placing tubes in ice-chilled water following centrifugation at 10,000 rpm for 10 min. Absorbance was recorded with a spectrophotometer UV mini-1240 UV-VIS (Shimadzu, Tokyo, Japan) at 532 nm, and non-specific absorbance was taken at 600 nm. Lipid peroxidation was calculated by subtracting the absorbance value at 600 nm from the values measurements at 532 nm. MDA content was calculated by the [12] formula using its absorption coefficient of 155 $mM^{-1} \cdot cm^{-1}$ expressed as nmol $MDA \cdot g^{-1}$ fresh weight;

$$MDA = \frac{\left[\left(A_{532} - A_{600}\right)V \times 1000/\varepsilon\right]}{W} \quad (1)$$

where ε is the specific extinction coefficient (155/cm Mcm), V is the volume of

crushing medium in mL, and W is the fresh weight of leaf sample in grams.

2.4. Electrolyte Leakage

Electrolyte leakage was measured based on the method by [13] at the end of water exposure. Fully expanded leaves were cut into 1 cm segments and washed three times with deionized water to remove surface adhered electrolytes. After washing, they were placed in individual stoppered vials containing 10 mL deionized water and incubated at room temperature (25˚C) on a shaker (100 rpm) for 24 h. The electrical conductivity of a bathing solution (EC1) was measured after incubation with a digital electrical conductivity meter (Ex stick EC 400 conductivity/TDS/Salinity Meter, Extech instruments). Samples were then placed in a thermostatic water bath at 95˚C for 15 min, and the last conductivity (EC2) was measured after cooling to room temperature. The electrolyte leakage percentage was determined as follows.

$$\text{Electrolyte leakage}(\%) = (\text{EC1}/\text{EC2}) \times 100 \quad (2)$$

2.5. Superoxide Dismutase Assay

Superoxide dismutase (SOD) activity was determined according to [14] method. 0.2 g leaf tissue collected from fully expanded leaves was homogenized thoroughly using a chilled mortar and pestle in a medium consisting of 50 Mm phosphate buffer (pH 7.8) and 100 mg of polyvinyl polypyrolidone as a phenolic binder. The homogenate was then centrifuged at 16,000 ×g for 15 min in a refrigerated centrifuge at 4˚C. The supernatant was then collected to be used for the assay. The sets of assay systems were prepared separately; assay system, dark control and light control. The reaction mixture contained 0.1 mL 1.5 M sodium carbonate, 0.3 mL of 0.13 M methionine, 0.3 mL of 10 μM EDTA, 0.3 mL of 13 μM riboflavin and 0.3 mL of 0.63 mM nitroblue tetrazolium. The nitroblue tetrazolium was not added to the light control system. To the reaction system, 0.1 mL enzyme extract (sample) was added to the blank and the light control system. The reaction mixture was then made up to 3.0 mL with 50 Mm phosphate buffer (pH 7.8). The tubes with dark control samples(blank; all reagents and sample added) were kept in the dark chamber, and the tubes for the light control samples (all reagents except nitroblue tetrazolium, with sample added just before absorbance reading) and the assay system(all reagents and sample) were kept under a fluorescent lamp. After 30 min of incubation, the absorbance readings were taken at 560 nm using a spectrophotometer (UV Mini-1240 UV-VIS, Shimadzu, Tokyo, Japan). One unit of SOD was defined as the enzyme activity that inhibited the photoreduction of nitroblue tetrazolium to blue formazan by 50% or the amount of enzyme which reduced the absorbance reading to 50% in comparison with the tubes lacking the enzyme. SOD was expressed in Units per milligram (Unit/mg).

$$\text{SOD activity} = \frac{A_0 - A_1}{A_0} \div 50\% \times \frac{\text{System Volume}}{\text{Sample Volume}} \quad (3)$$

A_0 is the absorbance in the absence of enzyme extract, A_1 is the absorbance in the presence of enzyme extract, and the enzymatic activity that causes 50% inhibition in the system is defined as one unit [15].

2.6. Catalase Assay

Catalase was measured according to the [16] method after water stress exposure. The enzyme extract was prepared by grinding 0.2 g leaf tissue from fully expanded leaves in a chilled mortar and pestle by adding 4 mL 50 Mm phosphate buffer, pH 7.0, mixed with 100 mg polyvinyl polypyrolidone as a phenolic binder and centrifuged for 15 min at 4000 rpm (4˚C). The assay consisted of 1.0 mL of 50 mM phosphate buffer pH 7.0, 2.0 mL of enzyme extract and 1.0 mL of 3% hydrogen peroxide. The phosphate buffer and the enzyme extract were pipetted out and mixed well in a test tube. To this, hydrogen peroxide was added to initiate the enzyme activity. Immediately after the addition of hydrogen peroxide, enzyme activity was measure at 240 nm for 180 seconds at 15 seconds interval using a Spectrophotometer, with 1 mL phosphate buffer used as a blank. Formula according to [17] was used as follows;

$$\text{Catalase activity} = \Delta Abs \times V_t / \varepsilon_{240} \times d \times V_s \times C_t \times 0.001 \quad (4)$$

where, ΔAbs is the difference between the initial and final absorbance, V_t is total volume of reaction, ε_{240} is the molar extinction coefficient for H_2O_2 at OD_{240} (34.9 mol^{-1} cm^{-1}), d is optical path length of cuvette (1 cm), V_s is volume of sample, C_t is the total protein concentration in the sample and 0.001 is absorbance change caused by 1 U of enzyme per min at 240 nm OD.

2.7. Proline Content

Samples of fresh leaf tissue from fully expanded leaves and root tissue were obtained per plant after the drought exposure period. Proline analysis was carried out on the samples following the method by [18]. First, Acid ninhydrin was prepared by warming 1.25 g of ninhydrin in 30 mL glacial acetic acid and 20 mL of 6 M phosphoric acid until dissolved. The reagent was kept cool at 4˚C. 0.5 g samples of fresh tissue were homogenized in 10 mL 3% sulfosalicylic acid. The homogenate was then filtered through Whatman filter paper. 2 mL of the filtrate was reacted with 2 mL acid ninhydrin and 2 mL glacial acetic acid in a test tube for 1 hour at 100˚C in a water bath to develop the colors. Immediately after removal from the water bath, the test tubes were cooled in an ice bath and proline extracted by adding 4 mL toluene followed by shaking with a Vortex mixer for 15 - 20 seconds. The chromophore containing toluene was aspirated from the aqueous phase, warmed to room temperature, and the absorbance was taken using a spectrophotometer (UV Mini-1240 UV-VIS, Shimadzu, Tokyo, Japan) at 520 nm, using toluene as a blank. Proline content in fresh tissue was determined by comparing the sample absorbance with the standard proline curve and calculated on a fresh weight basis as follows:

$$\left[\left(\mu g \text{ proline}/mL \times \text{toluene } mL\right)/115.5\mu g/\text{mole}\right]/\left[\left(g \text{ sample}\right)5\right]$$
$$= \mu\text{mol proline}/g \text{ of fresh weight tissue}$$

2.8. Soluble Protein Content

The amount of protein in the enzyme extract was determined according to the procedure of [19] at the end of water stress using the Bradford method. 0.2 g plant tissue from the fourth fully expanded leaves was ground using pestle and mortar in 4.0 mL of phosphate buffer (pH 7). These samples were kept overnight for complete extraction of protein and followed by centrifugation at 16,300 × g for 20 min. The supernatant was used for protein analysis, and the pellet discarded. 0.5 L of supernatant was adjusted to 1.0 mL volume with phosphate buffer followed by mixing with 5 mL of Bradford reagent and absorbance recorded at 595 nm against reagent blank using UV mini-1240 UV-VIS spectrophotometer (Shimadzu, Tokyo, Japan). The concentration of protein was determined from the standard curve. Protein content was expressed in milligrams per milliliter solution (mg/mL).

2.9. Total Dry Weight

Dry weight (g) was determined after drying plant material (roots, stem and shoot) at 70˚C (until a constant weight was achieved), and the mass measured.

2.10. Data Analysis

One-way ANOVA was used to determine the effects of drought treatments at $P < 0.05$. All statistical analyses were performed using Sigma plot 11.

3. Results

Malondialdehyde (MDA) content was significantly ($P < 0.05$) higher in water-stressed plants than the control ones in all the accessions (**Figure 1**). It is worth-noting that Thamaga accession displayed a considerably high amount of MDA than the other two accessions, when exposed to water stress (**Figure 1**). Thamaga and Mmadinare accession exhibited higher electrolyte leakage percentage than Maun accession (**Figure 2**).

When plants were subjected to water stress, they all appeared to increase their Superoxide dismutase (SOD) activity more or less the same, with Thamaga accession displaying a slightly higher SOD activity than the other two accessions (**Figure 3**). Maun accession appeared to exhibit a higher catalase activity when exposed to water stress than Mmadinare and Thamaga accession (**Figure 4**). Maun accession exhibited a significant ($P < 0.05$) increase in proline content compared to the other accessions (**Figure 5**) and displayed the highest proline content in both the control and water-stressed plants. Maun accession exhibited the highest soluble protein content followed by Mmadinare accession, and Thamaga accession showed the least protein content (**Table 1**). The three accessions exhibited significant ($P > 0.05$), more or less the same dry weight (**Figure 6**). Mmadinare

Lipid peroxidation

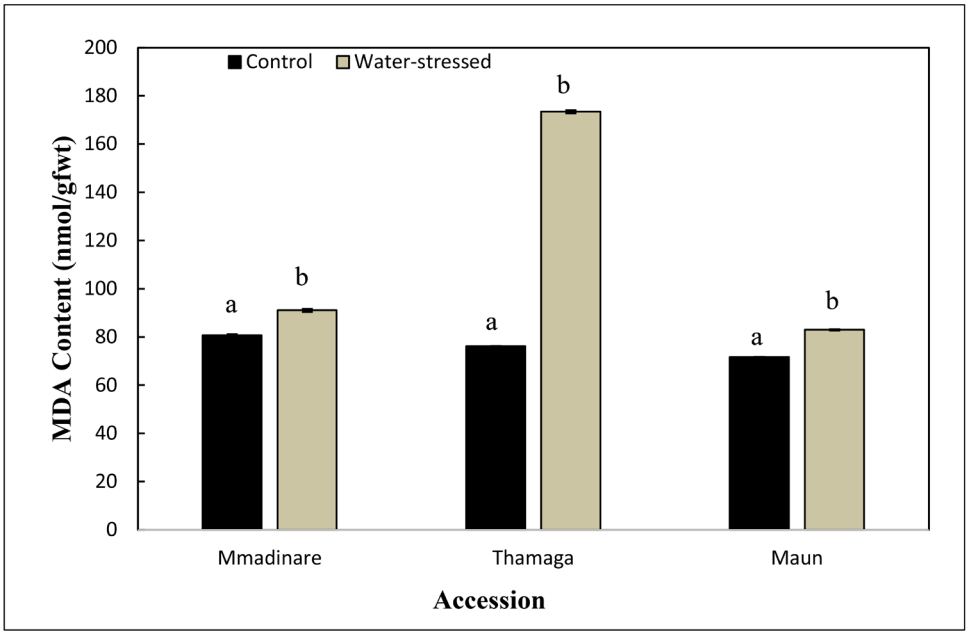

Figure 1. Effect of water stress on malondialdehyde (MDA) content in *Jatropha curcas* leaves of Mmadinare, Maun and Thamaga accession. Bars represent standard error of means (n = 3). Lowercase letters indicate significant difference between different water treatments (control and water-stressed) within accession at the 0.05 level.

Electrolyte leakage

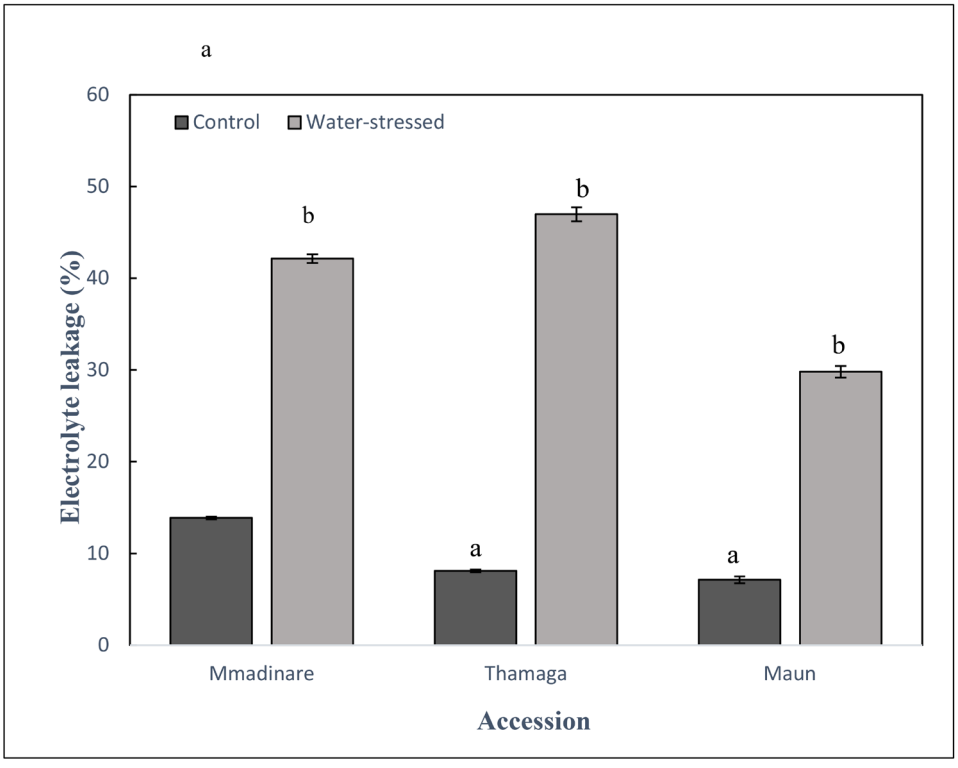

Figure 2. Effect of water stress on electrolyte leakage in three *Jatropha curcas* accessions. Bars represent standard error of means (n = 3). Lowercase letters indicate significant difference between different water treatments (control and water-stressed) within accession at the 0.05 level.

Superoxide dismutase activity (SOD)

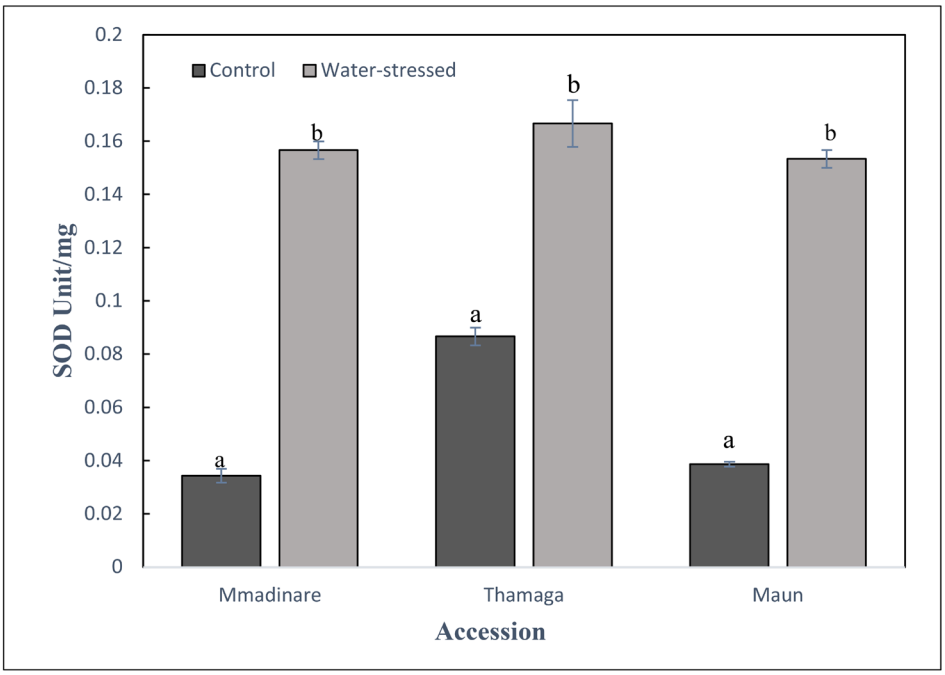

Figure 3. Effect of water stress on the superoxide dismutase activity in *Jatropha curcas* in Mmadinare, Maun and Thamaga accessions at the end of water stress exposure (49 Days after exposure). Bars represent standard error of means (n = 3). Lowercase letters indicate significant difference between different water treatments (control and water-stressed) within accession at the 0.05 level.

Catalase activity

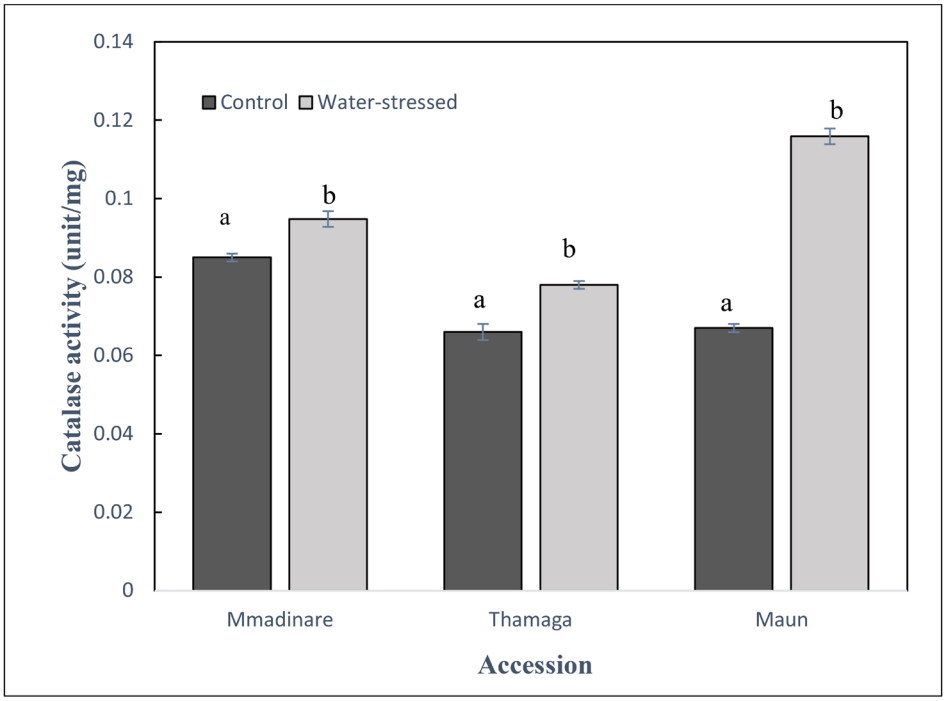

Figure 4. Effect of water-stress on the catalase activity in *Jatropha curcas* leaves in Mmadinare, Maun and Thamaga accessions at the end of water stress exposure (49 DAE). Bars represent standard error of means (n = 3). Lowercase letters indicate significant difference between different water treatments (control and water-stressed) within accession at the 0.05.

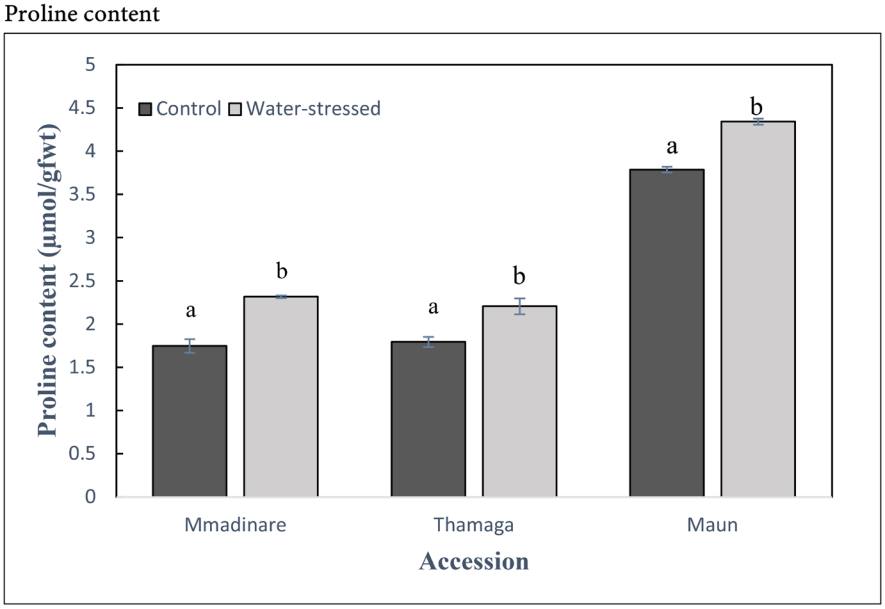

Figure 5. Effect of water stress on the proline content in three *Jatropha curcas* accessions. Bars represent standard error of means (n = 3). Lowercase letters indicate significant difference between different water treatments (control and water-stressed) within accession at the 0.05 level.

Table 1. Effect of water stress on soluble protein content of *Jatropha curcas* accessions.

Accession	Absorbance at 595 nm	Soluble Protein Content (mg/mL)
Mmadinare		
Control	1.34 ± 0.02	1.30 ± 0.02[a]
Water-stressed	1.43 ± 0.03	1.55 ± 0.03[b]
Thamaga		
Control	1.31 ± 0.04	1.22 ± 0.01[a]
Water-stressed	1.35 ± 0.04	1.33 ± 0.02[b]
Maun		
Control	1.21 ± 0.02	0.94 ± 0.01[a]
Water-stressed	1.57 ± 0.04	1.94 ± 0.06[b]

Different lowercase letters in the same column represent significant difference at 0.05 level among different water treatments.

accession displayed a slightly higher mass of 3.0g followed by Maun accession (2.8g) and lastly Thamaga accession (2.74g).

4. Discussion

Higher malondialdehyde (MDA) levels were exhibited by water-stressed plants than control plants (**Figure 1**). These higher MDA levels indicated cell membrane damage, as MDA one of the final products of polyunsaturated fatty acids peroxidation in the cells. These results were consistent with those obtained by [20] in wheat seedlings. By comparison of the MDA content of all three water-

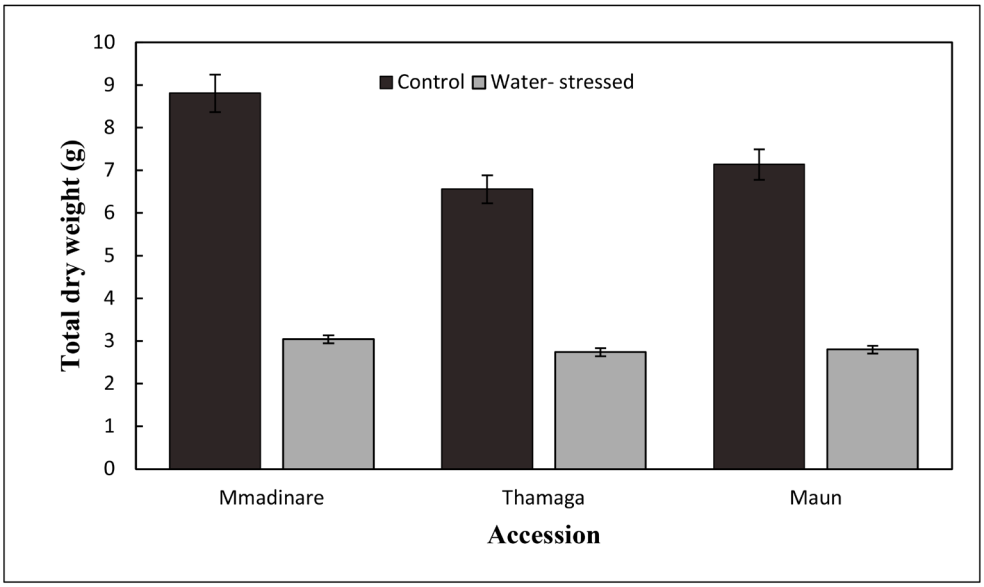

Figure 6. Effect of water stress on the dry weight of the three *Jatropha curcas* accessions (Mmadinare, Thamaga and Maun). Bars represent standard error of means (n = 5).

stressed accessions, Maun and Mmadinare accessions appeared to display the lowest lipid peroxidation, indicating maintenance of membrane integrity under water stress conditions compared to Thamaga accession. Lipid peroxidation (estimated from MDA content) due to water stress can be ascribed to oxidative damage through generation of reactive oxygen species (ROS) [8]. Lipid peroxidation results in an increase in the permeability of the cell membrane, as attested to by electrolyte leakage percentage, which was lower in Maun accession compared to the other two accessions (**Figure 2**).

The lower electrolyte leakage exhibited by Maun and Mmadinare accessions under water stress conditions (**Figure 2**), further demonstrated that these accessions exhibited less damage to the cell membrane as attested to by lower MDA content than Thamaga accession. It should be noted that Thamaga accession which displayed the highest MDA content, also exhibited a higher electrolyte leakage (**Figure 2**). The highest electrolyte leakage percentage could be ascribed to physiological structure of biological membranes and their chemical composition being modified by dehydration, which increased cell membrane permeability [21].

The maintenance of membrane integrity under water stress conditions in Maun and Mmadinare accessions could further be attributed to their ability to more effectively scavenge reactive oxygen species (ROS) (as attested to by lower electrolyte leakage and lower MDA content) than Thamaga accession. The ability of the plant to maintain membrane integrity depends, in part, on its ability to generate oxygen metabolizing enzymes (such as superoxide dismutase and catalase) to mop up ROS, to counteract lipid peroxidation. **Figure 3** showed that all the *Jatropha curcas* accessions exhibited more or less the same increase in superoxide dismutase (SOD) activity when exposed to water stress. It was worth noting that

when plants experience oxidative stress, superoxide radical is produced at high levels, and therefore superoxide dismutase is needed to scavenge this radical. If this radical is not scavenged by SOD, it disturbs vital biomolecules [22]. With respect to catalase (CAT), another oxygen metabolizing enzyme, all the three accessions displayed the activity of catalase under water stress (**Figure 4**), but Maun accession exhibited the highest catalase activity. The higher catalase activity exhibited by Maun accession (**Figure 4**) than the other accessions can be ascribed to its ability to scavenge reactive oxygen species as attested to by its low MDA content (**Figure 1**) and lower electrolyte leakage (**Figure 2**). Catalase converts hydrogen peroxide to water in the peroxisomes [23]. In agreement with these results, [24] reported that catalase activity was up-regulated in the drought-tolerant CE704 genotype (maize), while catalase levels were decreased in the drought-sensitive genotype 2023.

It should be pointed out that apart from the mopping up of radicals to reduce lipid peroxidation to keep the cell membrane intact, there was also production of proline (**Figure 5**) and accumulation of soluble proteins in water-stressed plants (**Table 1**). Proline is an important variable amino acid in that plays a role in the protection of the cell membrane from oxidative damage and also scavenge reactive oxygen species (ROS) underwater stress [25]. Soluble proteins have been proposed to play an important role in membrane protein stability and osmotic adjustment [26] [27] [28]. Maun and Mmadinare accessions displayed a higher proline (**Figure 5**) and soluble protein content (**Table 1**) under water stress. The higher proline content exhibited by Maun and Mmadinare accessions when subjected to water stress, appear to confer water stress tolerance in these accessions, consistent with previous findings where proline pretreatment also minimized the toxicity of Hg^{2+} in rice (*Oryza sativa*) by scavenging ROS, such as H_2O_2 [29], therefore resulting in better maintenance of cell membrane integrity (as shown by its lower electrolyte leakage percentage and lower MDA content).

Maun and Mmadinare accessions also exhibited the highest amount of soluble protein (**Table 1**). These results are in agreement with [30], where they found out that soluble protein content in the leaves of cotton under drought treatment was much higher than that of conventional soil water treatment. In contrast, [31] found out that Banana cultivars exhibited a slight decrease in soluble protein content despite being considered tolerant to water stress.

Jatropha curcas plants that were subjected to water stress showed a generally low dry weight compared to the control (**Figure 6**). There was no significant difference ($P > 0.005$) between dry weights of the three accessions. The slightly higher dry weights exhibited by Mmadinare and Maun accessions, than Thamaga accession, could ascribed to their ability to effectively scavenge for ROS when exposed to water stress. The small differences in dry weight between the accessions could be attributed to the fact that during water stress, the plants prioritized on developing survival traits to promote synthesis of protective compounds, instead of the actual growth of the plant. This is consistent with [32], who reported that

water stress can limit plant growth and biomass production, and alter the allocation pattern of biomass. Furthermore, it has been reported that water stress influences cell enlargement more than cell division [33]. Therefore, the slightly higher dry weight exhibited by Maun and Mmadinare accessions, than Thamaga accession could attest to the fact that they channeled more resources to protective mechanisms than growth.

5. Conclusion

It could be concluded that among the three *Jatropha curcas* accessions, Maun (Northern region) and Mmadinare (Central region) accessions appeared to maintain membrane integrity compared to Thamaga accession (Southern region) under water stress conditions. Maintenance of membrane integrity under water stress conditions can be ascribed, in part, to their ability to mop up reactive oxygen species through the production of oxygen metabolizing enzymes (superoxide dismutase and catalase), as well as the accumulation of proline and soluble proteins as protective mechanisms.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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