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EFFECT OF EXOGENOUS MELATONIN ON ANTIOXIDANT DEFENSE SYSTEM AND OSMO-REGULATORY SOLUTES OF DROUGHT-STRESSED *Morinda citrifolia*

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ABSTRACT

Morinda citrifolia is a small tropical tree that contains active natural metabolites in its leaves, stem, roots, and fruits. Despite these properties, drought stress has always been one of the limiting factors affecting its growth and productivity. This study investigated the role of melatonin in the regeneration of *M. citrifolia in vitro* under simulated drought stress. Nodal cuttings of six-month-old *M. citrifolia* were inoculated into Murashige and Skoog (MS) media supplemented with 2, 4-dichlorophenoxy-acetic acid (0.5 mg/L), indole acetic acid (0.5 mg/L) and varying concentrations of melatonin (0 µM, 50 µM and 100 µM) and polyethylene glycol (PEG) 6000 (0%, 20% and 40%). *M. citrifolia* experienced a significant increase in plant growth, stabilized chlorophyll contents, superoxide dismutase (SOD), and glutathione peroxidase (GPx) activities under drought stress possibly because it is a drought-tolerant plant. However, melatonin was involved in the accumulation of proline and ascorbic acid at 20% PEG. Osmoregulation of solutes stimulated and stabilized the production of catalase, GPx, and SOD activities. Upregulation of glutathione *S*-transferase augmented the biosynthesis of glutathione during drought stress. Also, a high accumulation of carotenoid function as photo-protectants and shields chlorophyll contents from drought-induced reactive oxygen species. Consequently, 40% of hydrogen peroxide was detoxified and plant growth was boosted. Therefore, melatonin acts as a stimulant of carotenoid, compatible solutes, enzymatic and non-enzymatic anti-oxidant defensive system, protects plants against oxidative injury, and boosted the growth of *Morinda. citrifolia in vitro* under drought stress.

Keywords: Antioxidant enzymes and non-enzymes, Osmolytes, *Morinda critifolia*, drought stress, melatonin, plant growth.

INTRODUCTION

Morinda citrifolia (commercially known as Noni) is a small evergreen tree of between 3 to 10 m in height at maturity, belonging to the family Rubiaceae. Its fruit can measure about 5 to 10 cm in length and 3 to 6 cm in width (Nelson, 2005) and might contain up to 260 seeds (Motshakeri and Ghazali, 2015). *M. citrifolia* is native to Southeast Asia and Australia. It is known to be environmentally tolerant and recognized as one of the significant sources of traditional medicines among the population on the Pacific Island (Singh, 2012). It is a multipurpose tree used in the field of phytomedicine and often as a dietary supplement. The root, stem, fruits, and leaves are traditionally used in several East Asian countries for the treatment of numerous diseases such as arthritis, headaches, burns, and even disorders related to tuberculosis, diabetes, and hypertension (Ali *et al.,* 2016). The leaves, roots, and bark have antibacterial, antiviral, antifungal, antitumor, anti-helminthic, and analgesic, hypotensive, anti-inflammatory properties, and immune-enhancing effects (Motshakeri and Ghazali, 2015). The use of *M. citrifolia* is extensive as a food supplement in the treatment of several types of cancer (Torres *et al.*, 2017). It is tagged as one of the functional foods, i.e., those with beneficial effects on one or more target functions in the body. *M. citrifolia* may survive under some adverse conditions, but severe drought stress may limit the growth and yield of the plant. The increase in temperature due to global climate change has made the effect of drought stress particularly prominent, and a major environmental factor limiting the growth and development of plants worldwide including *M. citrifolia* (Qi *et al.*, 2018).

Melatonin (N-acetyl-5-methoxytryptamine) is a naturally auxinic antioxidant multi-regulatory biomolecule that exists in plants as well as animals. It is involved in multiple biological processes

(Calvo *et al.*, 2013; Arnao and Hernández-Ruiz, 2018; Li *et al.*, 2019). It has appreciable ameliorative roles against abiotic stresses like salinity, drought, and extreme temperature (Li *et al.,* 2019). Melatonin has been regarded to be a more potent antioxidant and protective agent compared to even Reduced Glutathione (GSH) (Poeggeler *et al.*, 2002). The GSH enhances plant tolerance by regulating glyoxalase and improving antioxidant enzymes against oxidative damage (Poeggeler *et al.*, 2002). The intrinsic nature of melatonin to function as a nodal antioxidant upon exogenous application to plants has been recently shown in several plant species and under different types of sub-optimal conditions (Su *et al.*, 2019; Qiu *et al.*, 2019). It could induce some specific physiological responses in plants that might serve to enhance photosynthesis, growth, carbon fixation, rooting, seed germination, and defense against several biotic and abiotic stressors. It also works as an important modulator of gene expression related to plant hormones such as in the metabolism of indole-3-acetic acid, cytokinin, ethylene, gibberellin, and auxin carrier proteins (Nawaz *et al.*, 2021). It participates in the activation of pathogenesis-related proteins such as anti-oomycete, chitinase, and antioxidant enzymes like catalase, and superoxide dismutase under stress conditions, in leaf senescence and other morphogenetic features (Arnao and Hernández-Ruiz, 2018).

Drought stress adversely affects the physiological and biochemical processes of plants, leading to a reduction in plant productivity (Qi *et al.*, 2018). Plants try to protect themselves through the activation of their defense system, but severe drought causes dysfunction of this defense system. The imbalance between the generation and scavenging of reactive oxygen species (ROS) leads to oxidative stress (Sharma and Zheng, 2019). Melatonin has the potential to protect plants from the adverse effects of drought stress by enhancing ROS scavenging efficiency. It helps in the protection of photosynthetic apparatus and reduction of drought-induced oxidative stress. Melatonin regulates plant processes at a molecular level, which results in providing better resistance to drought stress (Debnath *et al.*, 2019).

This study sought to establish the activities of melatonin and its underlying biochemical activities in managing drought stress in *M. citrifolia* under *in vitro* conditions.

MATERIALS AND METHODS

Explant Collection and Sterilization

The research was conducted in the Biotechnology laboratory of the Forestry Research Institute of Nigeria (FRIN), Jericho, Ibadan. Nodal cuttings of six (6) month-old *Morinda citrifolia* were collected and sterilized using 70% ethanol for 5 min and subcultured in a Murashige and Skoog (MS) basal medium with vitamins and sucrose.

Media Preparation

The MS media was prepared by dissolving 34.43 g of the MS basal medium with vitamins and sucrose (MM5501, Phytotechnology Laboratories, USA) in 1000 mL distilled water and placed on the magnetic stirrer until a homogenous solution was obtained. The MS Media was supplemented with 0.5 mg/L indole-3-acetic acid (IAA), 0.5 mg/L 2, 4-dichlorophenoxy-acetic acid (2, 4-D), and varying concentrations of polyethylene glycol (PEG 6000) and melatonin (MEL). The pH of the media was adjusted to 5.8, made to solidify with 0.7% agar, dispensed as 20 mL/tube, and sterilized at 121 °C and 15 psi for 15 min (Murashige and Skoog, 1962). The solution was then dispensed into nine (9) beakers and the various treatments were added as follows:

1. MS + 0MEL + 0% PEG
2. MS + 0MEL + 20% PEG
3. MS + 0MEL + 40% PEG
4. MS + 50μM MEL + 0% PEG
5. MS + 50μM MEL + 20% PEG
6. MS + 50μM MEL + 40% PEG
7. MS + 100μM MEL + 0% PEG
8. MS + 100μM MEL + 20% PEG
9. MS + 100μM MEL + 40% PEG

Growth Environment and Measurement

The inoculated tubes were sealed and placed in the growth room under a 16/8 h light/dark photoperiod over 8 weeks at 22 ± 2 °C. Growth parameters measured include plant height, number of leaves, callus weight and shoot weight using a weighing balance (Ohaus PA413 Pioneer analytical balance, Ohaus Corp., USA) after eight weeks of inoculation.

Chlorophyll and Carotenoid Content Determination

The leaf sample (1 g) was homogenized in a 10 mL acetone-hexane (2:3) mixture for two (2) min. Samples were maintained in an ice-water bath to prevent overheating of the sample. Homogenates were centrifuged in 1.5 mL Eppendorf tubes at 5000 rpm for 10 min at 20 ºC. The absorbance spectrum of each supernatant was measured at the absorption maximum of 453, 505, 645, and 663 nm (Yang *et al.*, 1998).

Calculations

Chlorophyll a level (mg/100 mL) = 0.999 x Absorbance $_{663}$ – 0.0989 x Absorbance $_{645}$

Chlorophyll b level (mg/100 mL) = -0.3248 x Absorbance $_{663}$ + 1.77 x Absorbance $_{645}$

Beta-carotene level (mg/100 mL) = 0.261 x Absorbance $_{663}$ – 1.22 x Absorbance $_{645}$ + 0.452 x Absorbance $_{453}$

Antioxidant Assays

Ascorbate Peroxidase (APx) was determined according to Nakano and Asada (1981). Briefly, 0.1 mL plant extract was mixed with solution containing 1.2 mL of the K_3PO_4 buffer (50 mM, pH 7.0), 0.2 mL of 2 mM EDTA, 0.2mL of 0.1mM H_2O_2, and 0.2 mL of 0.5 mM Ascorbate. At 25 ºC, the extract absorbance was taken at 290 nm after 1 min. Ascorbate peroxidase (APx) activity was recorded as the extent of ascorbate oxidation.

APx activity = Δ absorbance/ 2.88 mm/cm^{-1}; where 2.88 is the extinction coefficient of ascorbate

Superoxide dismutase (SOD) activity was assayed based on the inhibition of pyrogallol auto-oxidation as measured at 420 nm (Marklund and Marklund, 1974). Briefly, 20 µL of the plant sample was mixed with 180 µL of 0.05 M Tris-HCl buffer: pH 8.2, and 250 µL of distilled water. Thereafter, 50 µL of 10 mM pyrogallol (dissolved in dilute HCl) was added to the mixture in the cuvette at the point of reading the absorbance. The rate of increase in absorbance was monitored against the blank for 3 min.

SOD Activity (U/mL) = (% inhibition/0.5) * Vt/Vs

% Inhibition = (Δabs/min of Blank – Δ abs/min of sample)/ Δabs/min of blank

Where Vt and Vs are the volume of the whole reaction mixture and the volume of the sample, respectively.

Glutathione peroxidase activity (GPx) measured the rate of oxidation of glutathione (Rotruck, 1973). The remaining GSH in the solution reacts with 2, 4-Dinitrobenzoic acid (DTNB) to form a complex which was absorbed at 412 nm wavelength. Briefly, 25 µL of the sample was mixed with 75 µL of GPx working reagent (containing 0.4 M phosphate buffer; pH 7.0, 2.5mM H_2O_2, 4 mM GSH, and 1mM NaN_3). The mixture was allowed to react for 10 min and was then stopped using 25 µL of 10% trichloroacetic acid (TCA). The precipitated mixture was centrifuged at 4000 rpm for 10 min and 35 µL of the supernatant was taken into another fresh 1.5 mL Eppendorf tube. Then, 350 µL of the GSH working reagent (containing 10 mM DTNB, dissolved in 0.05 M phosphate buffer, pH 7.4) was added to the mixture, and the absorbance was read within 15 min.

Calculation

Activity of GPx (U/mL) = (Absorbance of sample/14150)/10

Catalase activities (CAT) measured the amount of ammonium molybdate that forms a yellow complex with H_2O_2. Plant samples (200 µL) were incubated with 100 µL of buffered H_2O_2 (0.1 M phosphate buffer: pH 7.4 and 65 µmol/mL of H_2O_2) or blank (buffer alone) for 1 min. Thereafter, 500 µL of 32.4 mM of ammonium molybdate was added to the samples on ice. Then, absorbance was read at 405 nm with the blank control (Shangari and O'Brien, 2006).

Calculation

Catalase activity (kU/mL) = ((Absorbance of sample – absorbance of blank)/ (absorbance of blank)) *271; where 271 was obtained from the Goth correctional factor.

Glutathione-S-transferase (GST) activity was determined based on the rate of conjugation of 2, 4-Dinitrochlorobenzene (CDNB) with GSH (Habig *et al.*, 1974). Briefly, 60 µL of the plant sample was mixed with 980 µL of the phosphate buffer (0.1M; pH 6.5). The sample was poured into the cuvette and 10 µL each of 30 mM CDNB, and 30 mM GSH was added to initiate the reactions. The rate of increase in absorbance was taken for 3 min with the blank control at 340 nm.

Non–enzymatic antioxidants

The ascorbic acid concentration was determined according to the method described by Jagota and Dani (1982). In brief, 25 µL of the plant homogenate was added to 100 µL of 10% TCA on ice and centrifuged at 4000 rpm for 10 min. Thereafter, 100 µL of the supernatant was mixed with 400 µL of distilled water. Then, 40 µL of 0.2 M Folin Ciocalteu reagent was added and the absorbance of the sample was measured at 760 nm. The concentration of the ascorbate was calculated from a standard curve prepared by serial dilution of 0.1M ascorbate.

Reduced Glutathione (GSH) is determined by spectrophotometric quantification of a yellow complex formed by DTNB with the thiol group of the sample at 412 nm (Ellman, 1959). Equal volumes of the plant sample and 10% TCA were mixed in an Eppendorf tube and centrifuged for 10 min at 4000 rpm. Then, 350 µL of the GSH working reagent (described above) was added and the absorbance reading was taken at 412 nm. The GSH level was extrapolated from a 0.1 M GSH standard curve.

Calculation

GST activity (µmol/mL/min) = (Δabs/min of sample x Volume of reaction)/9.6 x volume of sample

Where ξ – 9.6 is the molar extinction coefficient of CDNB at 340 nm.

Proline was determined according to the method of Bates *et al.* (1973). Following the addition of 400 µL of absolute ethanol to 20 mg of grounded plant sample, the mixture was heated at 85°C for 20 min on the block heater. The sample was cooled and centrifuged for 5 min at 14,000 g after which 300 µL of the supernatant was measured into a fresh tube. Then, 500 µL of the working reagent (containing 1% ninhydrin in 60% acetic acid and 20% ethanol) was added and heated at 95 °C for 20 min. The mixture was cooled and centrifuged at 2500 rpm for 1 min. The absorbance of the supernatant was taken at 520 nm. The proline level was calculated from a standard curve of 0.04-1 mM L-proline.

Estimation of Hydrogen peroxide content

An equal volume of fresh tissue homogenates and 0.1% Trichloroacetic acid were mixed in an ice bath and separated by centrifugation at 3000 g for 15 min. The supernatant (0.5 mL) was then mixed with 0.5 mL of 10 mM potassium phosphate buffer (pH 7) and 1 mL of 1.0 M potassium iodide, and the absorbance was measured at 390 nm (Junglee *et al.*, 2014).

Statistical Analysis

The plant vegetative growth, proline, ascorbic acid, and H_2O_2 were presented as boxplot graphs (mean values). Plant pigment, GST, and GSH were presented as clustered graphs (mean values) with error bars showing the standard error of the mean (SEM). One-way Analysis of Variance (ANOVA) was used to compare the means of growth and pigment parameters at $P<0.05$. The differences in mean antioxidant enzymes were separated using Duncan's multiple range tests (DMRT). Correlation analysis was used to find the relationship between proline contents and ascorbic acid.

RESULTS

Effect of Melatonin on Plant Growth

An enhanced plant growth (number of leaves and plant height) and callus weight was observed in stressed untreated *M. citrifolia* compared with unstressed untreated plants (Figure 1). In addition, plant height, the number of leaves, and callus weight significantly increased as the concentration of melatonin increased in unstressed (PEG 0%) plants (Figure 1). Also, plant height dramatically increased at 50 µM melatonin in unstressed (PEG 0%) plants (Figure. 1a). Plant height increased progressively with an increase in melatonin concentration under PEG 40% treatment. Also, 100 µM melatonin increased the plant height of *M. citrifolia* by 18%. Moreover, 50 µM melatonin significantly decreased plant height at 20% PEG compared with their control plants (Figure 1a). The number of leaves decreased significantly along with an increase in the concentration of PEG. Under the application of melatonin at different concentrations, the number of leaves increased. Similarly, 0% and 40% PEG showed a similar pattern of increment in the number of leaves as the concentration of melatonin increased (Figure 1b). Compared to the control plants, a sharp increase of 64% in callus weight was observed in plants treated with 50 µM melatonin at 40% PEG (Figure 1c).

Effect of Melatonin on Photosynthetic Pigment Contents

Chlorophyll contents were not affected under untreated *M. citrifolia*. Nevertheless, melatonin increased the concentration of chlorophyll a, b, and carotene with or without drought stress. Melatonin (50 µM) increased chlorophyll contents and carotenoids more than plants treated with 100 µM melatonin (Figure 2; Table 1).

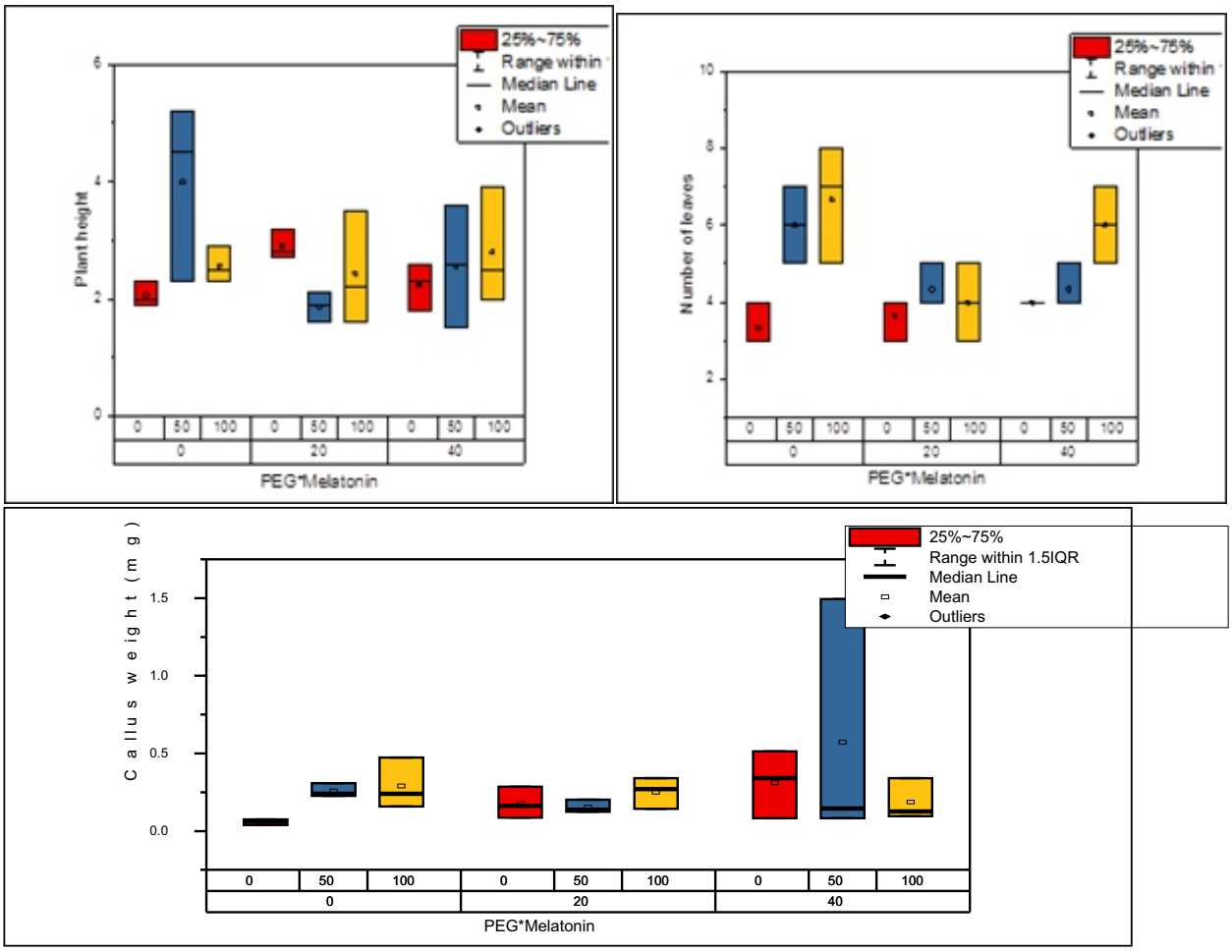

Figure 1: Boxplots showing the influence of Melatonin on growth (plant height (a); number of leaves (b); callus weight (c)) of *M. citrifolia* under osmotic stress. Interaction between PEG (0%, 20% and 40%) and MEL (0 µM, 50 µM and 100 µM).

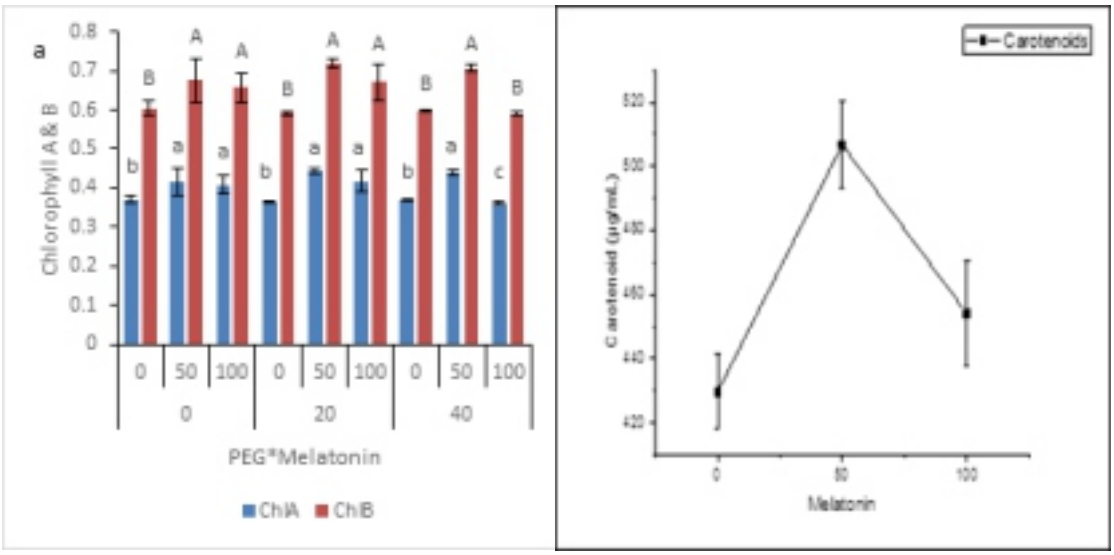

Figure 2: Effect of melatonin on chlorophyll pigments; (A) chlorophyll A and B content (B) Carotenoids of *M. citrifolia* under osmotic stress. Each bar is an average of 3 replicates and means are separated with error bars using mean±standard error. Bar with similar alphabets is not significantly different. Lowercase alphabets are for ChlA while uppercase alphabets are for ChlB. Interaction between PEG (0%, 20% and 40%) and MEL (0 µM, 50 µM and 100 µM).

Table 1: Analysis of variance (ANOVA) table showing mean square values of growth and chlorophyll pigments of *M. citrifolia.*

Source	DF	PH	NL	Carotenoid	CHLA	CHLB
PEG	2	0.547	4.037*	1208	0.000764	0.001954
Melatonin	2	0.3804	8.2593**	13931**	0.009339**	0.023819**
Rep	2	1.1604	0.7037	1331	0.000492	0.001107
PEG*Melatonin	4	1.8437*	2.4259*	1740	0.00123	0.0027
Error	16	0.5725	0.7454	1830	0.000984	0.002552
Total	26					
R-sq		55.77	74.96	57.68	62.38	61.26

Df: degree of freedom, PH- Plant height, NL – Number of leaves, Chl a- Chlorophyll a, Chl b- Chlorophyll b. P-values were significant *, ** and *** at 0.05, 0.01 and 0.001, respectively.

Melatonin effect on Antioxidants Enzymes

Treatments without PEG and melatonin showed no significant changes in the activities of GPx and SOD compared with 20% PEG-treated plants without melatonin. However, there was a variation in the production of CAT. Under drought stress (20 and 40% PEG), there was a remarkable increase in CAT, GPx and SOD activities when treated with 50 µM melatonin (Table 2). However, a significant decrease was observed in CAT, GPx, and SOD at a concentration of 100 µM melatonin compared with control plants. The activity of APx was stabilized in drought-stressed *M. critifolia* when compared with plants without stress (Table 2). Melatonin (100 µM) increased the concentration of GST when *M. citrifolia* was not under drought stress. Under drought stress (20 and 40%), GST was up-regulation in plants treated with 50 µM Melatonin (Figure 3).

Effect of Melatonin on Activities of Glutathione and Glutathione Transferase

Melatonin (100 µM) increased the concentration of GSH and enzymes GST when *M. citrifolia* were

not under drought stress. However, the concentration of GSH produced in an untreated *M. citrifolia* was not significantly different from plants treated with PEG (20 and 40% PEG) without melatonin. The activities of GSH were 15-16% higher than in control plants.

Impact of Melatonin on *M. citrifolia* Osmolytes

Ascorbic acid significantly increased as melatonin concentration increased in the plant without drought stress and with 40% PEG. At 20% PEG stress, ascorbic acid concentration in plants treated with 50 μM melatonin was significantly (22%) higher than in other treatments. Melatonin significantly increased proline contents in plants without drought stress (Figure 4a). During drought stress, there was a wide increase in proline contents at 20% PEG as proline increased along the concentration gradient of melatonin with a 60% increment at 100 μM melatonin. However, at 40% PEG, proline contents gradually reduced their concentration as the melatonin concentration increased (Figure 4b). The correlation between Ascorbic acid and GSH is shown in Figure 5a. Ascorbic acid and GSH relationship decreased along the concentration with *r* below 0.5. However, GSH concentration increased during drought stress than the concentration of ascorbic acid in *M. citrifolia*.

Effect of Melatonin on Hydrogen peroxide production

The production of hydrogen peroxide was significantly higher in control plants treated with melatonin compared with plants without PEG and melatonin. When drought stress (20 and 40% PEG) was induced, melatonin (50 and 100 μM) was highly effective in reducing the production of hydrogen peroxide. Consequently, 50 μM melatonin reduced the production of hydrogen peroxide by 40% more than other treatments under severe drought stress (40% PEG) (Figure 5b).

Principal component analysis

Principal component 1 (PC1) accounted for 77.4% and PC2 accounted for 0.12% of the total variation (Figure 6). Apart from the tolerant ability of the plant, the highest variation in growth of *M. critifolia* was due to CAT at PC 1 (0.75), carotenoid at PC 2 (0.74), APx at PC 3 (0.72), and 4 (0.67), GST at PC 5 (0.97), proline at PC 6 (0.99) and Ascorbic acid (0.33) and chlorophyll B (0.81) at PC 7 (Table 3).

Table 2: Antioxidant enzymes of exogenous treatment of *M. citrifolia* under osmotic stress.

PEG	Melatonin	CAT (kU/L)	GPx (U/mL)	SOD (μmol/g)	APx (U/mL)
	0	198.73c	0.141bc	3.71bc	15.93a
0	50	159.73d	0.125c	3.26d	15.63a
	100	199.55c	0.154b	4.09b	15.61a
	0	95.26e	0.147bc	3.90bc	15.07a
20	50	236.51b	0.176a	4.71a	15.69a
	100	215.57bc	0.141bc	3.71c	15.27a
	0	229.12b	0.139bc	3.65c	15.10a
40	50	261.56a	0.162ab	4.33ab	15.30a
	100	208.18c	0.135c	3.52cd	15.19a
	p-value	***	**	**	Ns

Figures with identical alphabets in each column within each accession are not significantly different at DMRT ($P<0.05$). P-values were significant ** and *** at 0.01 and 0.001, respectively.

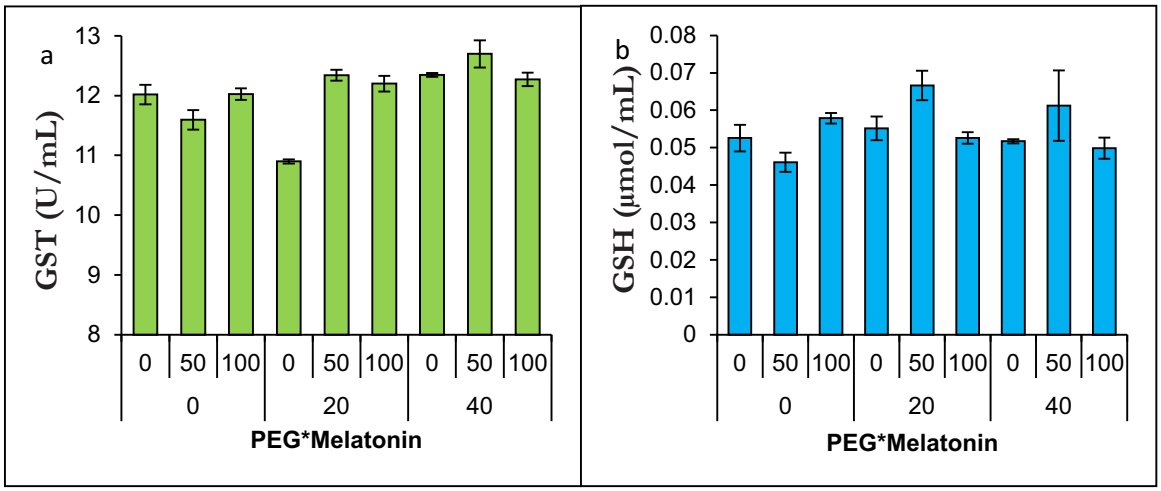

Figure 3: Glutathione Transferase and Glutathione activities of melatonin-treated *M. citrifolia* under osmotic stress. Interaction between PEG (0%, 20% and 40%) and MEL (0 µM, 50 µM and 100 µM). Bars means Error bars with Standard Error.

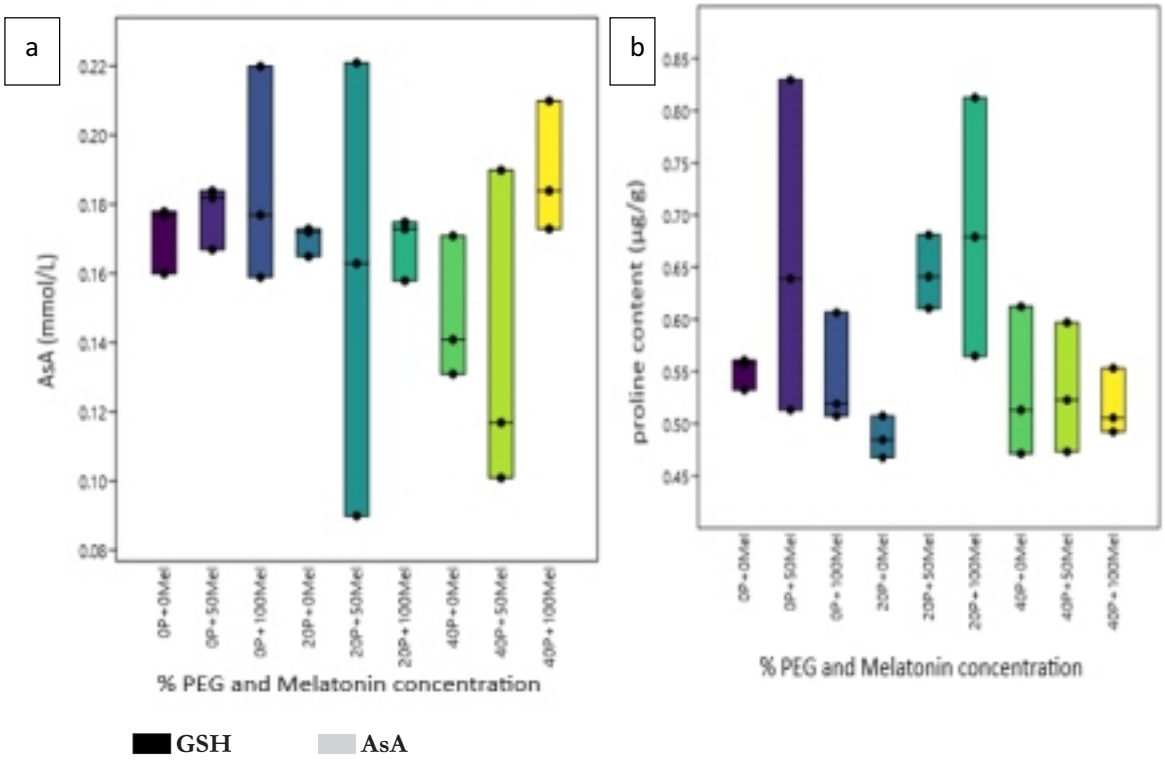

Figure 4: Non-enzymatic activities of drought-stressed *M. citrifolia* under different concentrations of melatonin: a. Ascorbic Acid (AsA) b. Proline Contents. 0P+0MEL: 0% PEG +0 µM, 0P+50MEL: 0% PEG + 500 µM Mel, 0P+100MEL: 0% PEG +100 µM Mel, 20P+0MEL: 20% PEG +0 µM Mel, 20P+50MEL: 20% PEG +50 µM Mel, 20P+100MEL: 20% PEG +100 µM Mel, 40P+0MEL: 40% PEG +0µM Mel, 40P+50MEL: 40% PEG +50 µM Mel, 40P+100MEL: 40% PEG +100 µM Mel , Mel: Melatonin.

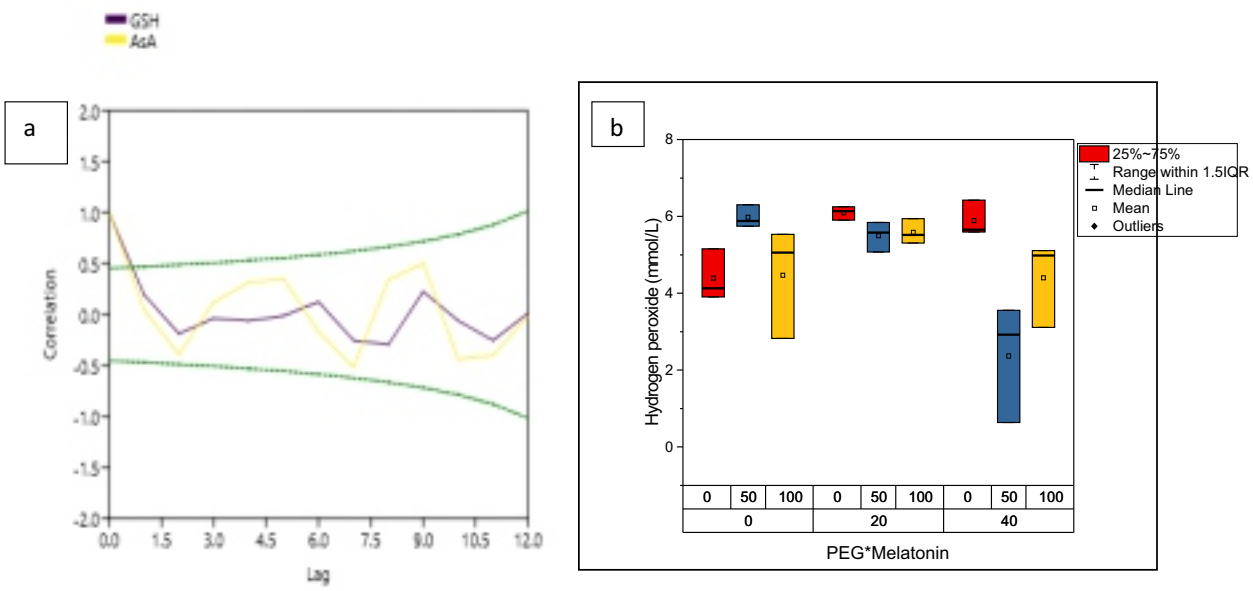

Figure 5: Interaction effect of melatonin on a. Ascorbic and Proline contents b. Production of Hydrogen peroxide in Osmotic stressed *M. citrifolia*. Interaction between PEG (0%, 20% and 40%) and MEL (0 µM, 50 µM and 100 µM). Bars means Error bars with Standard Error.

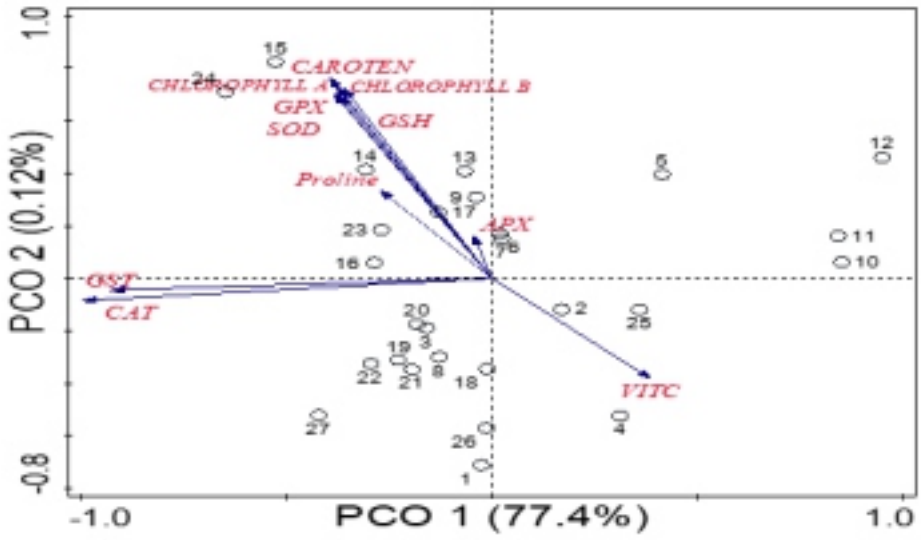

Figure 6: Principal Component (PC) analysis of biochemical markers in Osmotic stressed *M. citrifolia* under different concentrations of melatonin.

Table 3: Principal Component (PC) analysis showing variances in biochemical markers of drought stressed *M. critifolia* under different concentrations of melatonin.

PCA	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6	PC 7
CAT	0.75	-0.67	-0.0028	0.0015	-0.0092	-0.0011	0.00020
GPx	1.6 E-04	1.16E-05	0.024	-0.025	0.0011	0.00094	0.00017
APx	3.7 E-04	0.0012	0.72	0.67	-0.16	-0.065	-0.00098
SOD	0.0048	0.00034	0.69	-0.72	0.033	0.032	0.0057
Ascorbic acid	-0.00024	1.89E-05	- 0.013	0.016	0.041	0.079	0.33
Proline	0.00061	0.00057	0.016	0.053	-0.11	0.99	-0.084
Carotenoid	0.67	0.75	-0.0032	0.0017	0.00027	-0.00092	-0.0012
Chlorophyll b	0.00072	0.00089	0.0031	0.0055	-0.0089	0.054	0.81
Chlorophyll a	0.00047	0.00055	0.0024	0.0022	-0.0088	0.029	0.48
GST	0.0068	-0.0062	0.095	0.14	0.98	0.097	-0.012
GSH	6.9E-05	3.06E-06	0.0097	-0.010	0.00038	2.6E-05	-0.0019
Eigenvalue	3786	1767.96	0.30	0.22	0.042	0.0059	0.00091
% variance	68.16	31.829	0.0054	0.0040	0.00076	0.00011	1.64E-05

DISCUSSION

Melatonin is a dynamic, broad-spectrum antioxidant molecule, which is effective against ROS accumulation and plant physiological functions such as seed morphogenesis, growth, and development, root architecture, and chlorophyll pigment production (Arnao and Hernández-Ruiz, 2018). Melatonin counteracts the effect of drought stress and helps to detoxify excessive ROS, which enhances plant survival under stressful conditions. In this study, drought stress showed little or no effect on the chlorophyll contents and enzymatic antioxidant activities such as APx, GPx, and SOD of *M. citrifolia in vitro,* though increased plant growth could be attributed to the plant tolerance to low water potential and relative water content. Osmo-stability of antioxidant defensive mechanisms is a biochemical marker of drought tolerance (David *et al.*, 2019). *M. citrifolia* had shown a wide variety of habitats ranging from saline soils, and drought to secondary soils (Nelson, 2003). However, elevated production of H_2O_2 under this condition further deduces the prolonged effect of stomata closure on intercellular CO_2 in the Calvin cycle. Thus, the excessive number of electrons generated along the photosynthetic electron chain resulted in the univalent reduction of O_2 forming reactive oxygen species (ROS) (Asada, 2006; Noctor *et al.*, 2014). This study further showed that melatonin (50 and 100 μM) enhanced plant growth which included the number of leaves, plant height, and callus weight *in vitro* under drought stress. However, a high concentration (100 μM) of melatonin is needed to significantly increase plant height under severe drought stress. Exogenous application of a low concentration of melatonin (50 μM) increased chlorophyll a and b with an extremely high accumulation of carotenoid under drought stress. Melatonin enhanced the tolerant ability of *M. citrifolia* to accumulate extremely high carotenoids thus minimizing the degradation of chlorophyll contents and function as photo-protectants. A high concentration of carotenoid suggested its ability to inhibit, stop or reduce the formation of triplet chlorophyll by mopping up excessive electrons that would have been transferred to oxygen molecules and generated ROS during stomatal closure (Mibei *et al.*, 2017). Carotenoids allowed free uptake of energy from agitated ions and degenerated excessively as heat energy. Carotenoids are lipophilic antioxidants that are capable of detoxifying diverse types of radicals such as OH^-, O^{2-}, and H_2O_2 (Young, 1991). This finding supported the report that melatonin treatment increased photosynthesis and chlorophyll content in wheat under abiotic stress (Ni *et al.*, 2018) as well as increased chlorophyll a, chlorophyll b, and total chlorophyll concentrations in *P. frutescens* by melatonin concentration which ranges between 100 - 200 μmol/L (Xiang *et al.*, 2019). Moreover, melatonin effects were attributed to an increase in leaf chlorophyll content and photosynthetic rate observed in an Apricot tree (Medina-Santamarina *et al.*, 2021).

Ascorbic acid and proline are compatible solutes directly involved in the osmoregulation of *M. citrifolia* during drought stress. The study showed that melatonin aided in the accumulation of proline (60%) and ascorbic acid (22%) during drought stress (20% PEG). Proline and ascorbic acid gave an immediate response to drought stress (20% PEG). Osmotic regulation involves an active regulation of cells by enabling the cell to absorb water and accumulate solutes. The reduced osmotic potential aided the cells in maintaining the turgidity required for cell growth under low water potential (Osakabe *et al.*, 2014). Osmotic regulation substances such as proline, soluble sugar, and glycine betaine (GB) gave a protective measure to resist drought stress in plants (Ashraf and Foolad, 2004). Exposure of plants to drought resulted in a high concentration of proline, thereby, increasing the total concentration of responsive amino acids that are responsible to fight oxidative stress in plants (Lum *et al.,* 2014). Only soluble sugars can take the place of water molecules and form hydrogen bonds with proteins to maintain the specific structure and function of proteins while proline stimulates the activity of enzymes APx, CAT, and SOD (Yang *et al.*, 2021). *M. citrifolia* plants treated with 50 μM of melatonin stimulated activities of CAT, GPx, and SOD under drought stress (20% and 40%). Melatonin extensively stimulates cellular redox homeostasis by increasing the activity of enzymatic antioxidants (Nawaz *et al.*, 2018). This is an indication that melatonin subdued drought-induced superoxide anions (O^{2-}) in *M. citrifolia* by enhancing SOD activity. Excessive synthesis of SOD improved plant tolerance to survive oxidative stress (Stevens *et al.*, 2008). In addition, catalase showed a greater possibility to decompose H_2O_2 into H_2O and O_2 instantly which shows plant stability under drought stress. Melatonin-pretreated seedlings are a relative transcript abundance of enzymatic SOD, CAT, and POD (Jahan *et al.*, 2019). These findings were consistent with prior findings that Melatonin enhanced SOD, peroxidase (Xiang *et al.*, 2019), and GPx activities (Bela *et al.*, 2015; Cui *et al.*, 2017) resulting in an efficient ROS scavenging. The GPx is efficient in eliminating H_2O_2, hydroperoxides, and lipid peroxide (Bela *et al.*, 2015). A high concentration of melatonin (100 μM) elicits adverse effects on plant antioxidant defense mechanisms. Therefore, it should be applied at the required dosage.

Additionally, melatonin is accompanied by upward regulation of glutathione transferase (GST) which led to more accumulation (16%) of glutathione (GSH) in drought-stressed *M. citrifolia*. Glutathione is a major reduced thiol non-enzymatic antioxidant that acts as an electron donor (Grozeff *et al.*, 2017). Melatonin (50 μM) stimulated glutathione which protects *M. citrifolia* against oxidative damages. The higher level of GSH enabled the melatonin seedlings to maintain higher GPx enzyme activity. Consequently, 40% hydrogen peroxide radical was scavenged. This suggests that melatonin was actively involved in osmoregulation, modulation of antioxidant enzymes, and accumulation of GSH which controlled the overproduction of hydrogen peroxide. The high GSH/GSSG ratio helped the Mel-treated seedlings to maintain cellular osmoticum and low MG content even under stress (Banerjee and Roychoudhury, 2019). GST activity and its gene expression are upregulated by melatonin which contributed to the reduction of the lethal effect induced by drought stress in wheat plants (Cui *et al.*, 2017). Also, melatonin treatment not only increases the concentration of ascorbic acid and GSH contents in drought-stressed plants but was accompanied by a reduced H_2O_2 content (Liu *et al.*, 2015). Melatonin is known to be directly involved in the scavenging of H_2O_2 in guard cells (Reiter *et al.*, 2007; Galano *et al.*, 2013; Li *et al.*, 2015). This counteracts the imbalance in redox homeostasis caused during drought stress.

CONCLUSION

The findings from this study showed that melatonin increased growth (plant height and the number of leaves), callus weight, chlorophyll contents, and substantial-high concentration of total carotenoid in drought-stressed *M. citrifolia*. Also, melatonin contributed to the accumulation of compatible solutes, proline, and ascorbic acid, and corroborates the solute osmoregulatory functions to stimulate antioxidant enzymes (CAT, GPx, and SOD). Melatonin was involved in the upregulation of GST which resulted in the accumulation of GSH and eventually detoxified 40% H_2O_2 under drought conditions.

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