MANNITOL-INDUCED WATER DEFICIT STRESS IN OIL PALM (*Elaeis guineensis* Jacq.) SEEDLINGS

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ABSTRACT

The aim of this study was to investigate the biochemical, physiological and morphological responses of oil palm seedlings to mannitol-induced water deficit. Proline content and the percentage of relative membrane leakage in seedlings increased when subjected to water deficit, depending on the degree of water deficit. Relative membrane leakage was positively associated with chlorophyll degradation. Chlorophyll a (Chl$_a$), chlorophyll b (Chl$_b$), total carotenoids (C$_{x+c}$), maximum quantum yield of PSII (F$_v$/F$_m$) and photon yield of PSII (Φ$_{PSII}$) in the seedlings under water deficit conditions dropped significantly in comparison to the control group, leading to a reduction in net photosynthetic rate (P$_n$) and growth. In addition, the physiological changes and growth parameters of the plants responding to severe water deficit underwent a greater reduction than those of the plants responding to mild water deficit. These data may be applied to establish criteria for water deficit tolerance screening in breeding programmes.

INTRODUCTION

The oil palm is one of the most important oil-producing crops in the world and is widely cultivated in the tropical zone, especially in Southeast Asia (Malaysia, Indonesia and Thailand) (Wilcove and Koh, 2010). Yield, including oil yield and productivity, is the focus of oil palm genetic improvement in the form of breeding programmes (Jalani *et al.*, 1997; Cochard *et al.*, 2005). Oil yield is dependent not only on genetic background, but also on environmental factors, *i.e.* relative humidity, water availability, soil structure, fertiliser application,

agricultural management and light conditions (Kallarackal *et al.*, 2004; Henson and Harun, 2005). Hybrid seed production has been used to exploit heterosis or hybrid vigour for the improvement of productivity (Wahid *et al.*, 2005). As the next step, tolerance to abiotic stresses, such as water deficit, extreme temperature, mineral deficiency, heavy metal toxicity and ultraviolet irradiation, will be the target in oil palm improvement. The water content of the soil in oil palm plantations may play a key role in plant growth (Henson and Harun, 2005), and may also function as a signal for female sex representation (Jones, 1997). In non-irrigated areas, there is a higher proportion of male flowers and growth retardation, leading to low productivity. Basic knowledge relating to water shortage responses in oil palm is a fruitful topic which should be investigated further for application in water deficit tolerance screening.

Water deficit is a major problem worldwide, limiting the growth and productivity of many crop species, especially in rainfed agricultural areas (totalling >1.2 billion hectares) (Chaves and Oliveira, 2004; Passioura, 2007). Plants show physio-biochemical changes, such as decreased Rubisco activity, reduced photochemical efficiency,

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enhanced accumulation of stress metabolites, increased antioxidant enzymes, loss of membrane stability, reduced leaf water potential, pigment degradation, decreased stomatal conductance, reduced internal CO_2 concentration, reduced net photosynthetic rate (P_n) and inhibited growth prior to plant death in response to water deficit (Chaves and Oliveira, 2004; Cattivelli et al., 2008). Mannitol-induced water deficit has been widely explored in many plant species, e.g. tomato (Weng, 2000), sugar-cane (Cha-um and Kirdmanee, 2008), rice and sorghum (Cha-um et al., 2009). In the case of the palm family, ecophysiology in coconut palms, including membrane lipid composition (Repellin et al., 1997), photosynthetic ability and growth performance have been investigated (Gomes and Prado, 2007; Gomes et al., 2008). Polyethylene glycol (PEG) is well-known as a candidate osmoticum for water deficit induction being an agent of membrane injury (Ahmad et al., 2007). Moreover, PEG not only plays a role as an osmoticum but also reduces oxygen-dissolution in the culture medium. The toxic symptoms of plants grown under PEG treatment are more evident than those induced by mannitol in terms of iso-osmotic potential (Pandey et al., 2004; Slama et al., 2007).

The aim of this study was to investigate the responses of oil palm seedlings, in terms of proline accumulation, relative membrane leakage, photosynthetic ability and growth characters, to mannitol-induced water deficit.

MATERIALS AND METHODS

Plant Materials

Oil palm fruits were obtained from Suksomboon Palm Co. Ltd, located in Chonburi province, an eastern region of Thailand. The kernel of each fruit was removed. The seeds, with seed coat intact, were dried in a hot-air oven at 45°C for 12 hr, then the seed coat was broken. The embryos, along with the endosperm, were surface-disinfected once in 15% Clorox® for 20 min and once in 5% Clorox® for 30 min. The embryos were then excised and germinated on MS media (Murashige and Skoog, 1962). The media were adjusted to pH 5.7 before autoclaving. Oil palm seedlings were cultured in vitro under conditions of 25±2°C ambient temperature, 60±5% relative humidity (RH) and 60±5 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) provided by fluorescent lamps with a 16 hr per day photoperiod. After two months, the seedlings were transferred aseptically to MS-liquid sugar-free media. The uncovered vessels containing photoautotrophic seedlings were transferred aseptically to culture box chambers (Carry Box Model P-850, size 26×36×19 cm, Japan) where RH

was controlled at 65±5% by 1.5 litres saturated NaCl solution. The number of air exchanges in the culture box chambers was increased to 5.1±0.3 µmol CO_2 hr^{-1} by punching 32 holes in the sides of the plastic chambers and placing gas-permeable microporous polypropylene film (0.22 µm pore size) over the holes (Cha-um et al., 2003). The seedlings were acclimatised for 14 days by placing the chambers in a plant growth incubator under a temperature shift of 28±2°C/25±2°C (light/dark), CO_2 concentration of 500±100 µmol mol^{-1}, RH of 60±5%, and PPFD of 120±5 µmol m^{-2} s^{-1} provided by fluorescent lamps with a 16 hr per day photoperiod. Mannitol in the culture media was adjusted to -0.23 (control), -0.392, -0.674, -0.939 or -1.205 MPa water deficit maintained for a period of one month. Relative membrane leakage, proline content, contents of photosynthetic pigments, chlorophyll a fluorescence, net photosynthetic rate (P_n) and growth characters were measured.

Experiment Design and Statistical Analysis

The experiment was arranged as a completely randomised design (CRD) with eight replicates (n=8). The means obtained were compared by the Duncan's New Multiple Range Test (DMRT), with analyses performed using the SPSS software. The correlations between physiological, biochemical and growth parameters were evaluated using Pearson's correlation coefficients.

Data Collection

Relative membrane leakage (%) was determined according to the Dionisio-Sese and Tobita (1998) method. Leaves were cut into pieces of 5.0±0.2 mm length, and placed in glass vessels (Opticlear®; KIMBLE, Vineland, New Jersey, USA) containing 10 ml deionised water. The glass vessels were capped and maintained at room temperature (25°C) for 15 min. Initial electrical conductivity (EC_0) was measured using an electrical conductivity meter. The leaf tissue was then incubated at 100°C in a water bath for 15 min, cooled down to 25°C before electrical conductivity (EC_1) was measured again.

Proline in the root and leaf tissues was extracted and analysed according to the method of Bates et al. (1973). Fifty milligrams of fresh material were ground with liquid nitrogen in a mortar. The homogenate powder was mixed with 1 ml aqueous sulfosalicylic acid (3% w/v) and filtered through filter paper (Whatman #1, England). The extracted solution was reacted with an equal volume of glacial acetic acid and ninhydrin reagent (1.25 mg ninhydrin in 30 ml glacial acetic acid and 20 ml 6M H_3PO_4), then incubated at 95°C for 1 hr. The reaction was terminated by placing the container in an ice bath. The reaction mixture

was mixed vigorously with 2 ml toluene. After cooling to 25°C, the chromophore was measured by a spectrophotometer DR/4000 at 520 nm using L-proline as a standard.

Contents of chlorophyll *a* (Chl_a), chlorophyll *b* (Chl_b) and total chlorophyll (TC), were analysed following the methods of Shabala *et al.* (1998), and total carotenoid (C_{x+c}) concentration was assayed according to Lichtenthaler (1987). One hundred milligrams of leaf materials were collected and placed in a 25-ml glass vial, along with 10 ml 95.5% acetone, and blended using a homogeniser. The glass vials were sealed with parafilm to prevent evaporation, and then stored at 4°C for 48 hr. The Chl_a and Chl_b concentrations were measured using a UV-visible spectrophotometer at 662 nm and 644 nm wavelengths, respectively. The C_{x+c} concentration was also measured by the spectrophotometer at 470 nm. A solution of 95.5% acetone was used as a blank.

Chlorophyll *a* fluorescence emission from the adaxial surface on the leaf was measured using a fluorescence monitoring system in the pulse amplitude modulation mode, as previously described by Loggini *et al.* (1999). A leaf, adapted to dark conditions for 30 min using leaf-clips, was initially exposed to the modulated measuring beam of a far-red light (LED source with typical peak at a wavelength of 735 nm). Original (F_0) and maximum (F_m) fluorescence yields were measured under weak modulated red light (<0.5 µmol m^{-2} s^{-1}) with 1.6 s pulses of saturating light (>6.8 µmol m^{-2} s^{-1} PAR), and calculated using Fluorescence Monitoring System (FMS) software for Windows®. Variable fluorescence yield (F_v) was calculated by the equation of F_m–F_0. The ratio of variable to maximum fluorescence (F_v/F_m) was calculated as the maximum quantum yield of PSII photochemistry. Photon yield of PSII (Φ_{PSII}) in light was calculated by $\Phi_{PSII} = (F_m'-F)/F_m'$ after 45 s of illumination, when steady state was achieved. In addition, non-photochemical quenching (NPQ) was calculated as described by

Maxwell and Johnson (2000).

Net photosynthetic rate (P_n) was calculated by comparing the different concentrations of CO_2 inside (C_{in}) and outside (C_{out}) the glass vessel containing the oil palm seedlings. The CO_2 concentrations at steady state were measured by gas chromatography (GC; Model GC-17A, Shimadzu Co. Ltd., Japan). The P_n of the *in vitro* cultivated seedlings was calculated according to the method of Fujiwara *et al.* (1987).

Shoot height (SH), root length (RL), leaf area (LA), fresh weight (FW) and dry weight (DW) of the oil palm seedlings were measured. The seedlings were dried at 80°C in a hot-air oven for two days, and then incubated in desiccators before measuring dry weight. Leaf area of the seedlings was measured using a leaf area meter DT-scan.

RESULTS AND DISCUSSION

Proline content and relative membrane leakage in the leaf tissues of oil palm seedlings increased in relation to the reduction in water potential caused by mannitol in the culture media (*Figure 1*). Compared to the control conditions ($\Psi w = -0.238$ MPa), proline content and relative membrane leakage in leaf tissues under extreme water deficit ($\Psi_w = -1.205$ MPa) increased by 3.68 and 3.05 times, respectively. The increase in relative membrane leakage was positively related to chlorophyll *a* (Chl_a) degradation (*Figure 2A*). The Chl_a, Chlorophyll *b* (Chl_b), total chlorophyll (TC) and total carotenoids (C_{x+c}) levels in the leaf tissues dropped significantly when the plants were exposed to water deficit stress (Ψ_w values ranged from -0.674 to -1.205 MPa) (*Table 1*). Under extreme water deficit conditions ($\Psi_w = -1.205$ MPa), the degradation percentages of Chl_a, Chl_b, TC and C_{x+c} in the leaf tissues of the oil palm seedlings were 59.08%, 63.28%, 60.32% and 34.09%, respectively. The Chl_a content in the water-deficit stressed leaves correlated with maximum quantum
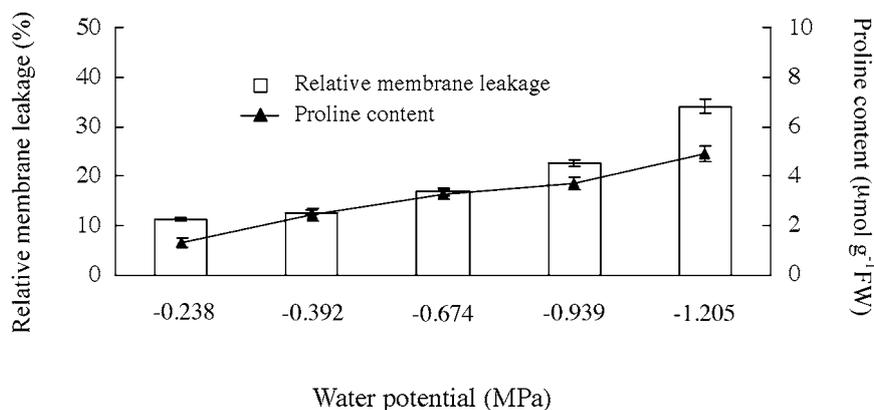

Figure 1. *Relative membrane leakage and proline content of oil palm seedlings grown under mannitol-induced water deficit for one month. Error bars represent ±SE.*
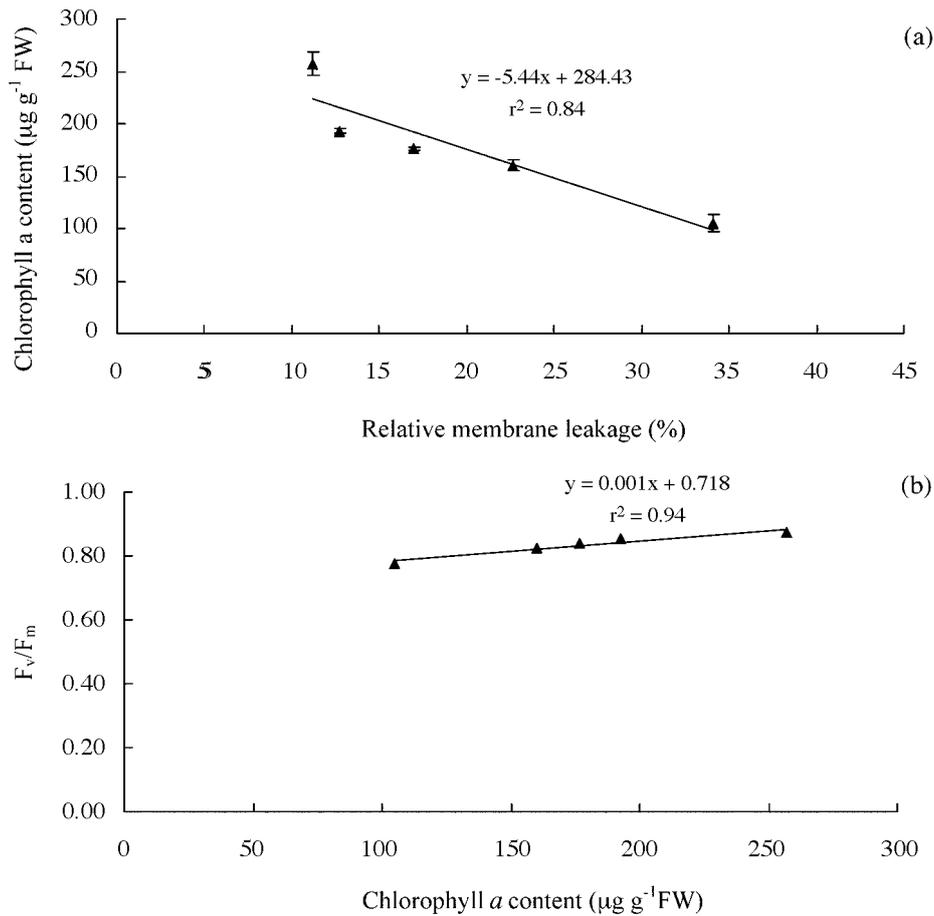
Figure 2. Relationships between relative membrane leakage and chlorophyll a *content (A), chlorophyll* a *content and maximum quantum yield of PSII (F_v/F_m) (B) of oil palm seedlings grown under mannitol-induced water deficit for one month. Error bars represent ±SE.*

TABLE 1. CHLOROPHYLL *a* (Chl_a), CHLOROPHYLL *b* (Chl_b), TOTAL CHLOROPHYLL (TC) AND TOTAL CAROTENOID (C_{x+c}) CONTENTS OF OIL PALM GROWN SEEDLINGS UNDER MANNITOL-INDUCED WATER DEFICIT FOR ONE MONTH

Water potential (MPa)	Chl_a ($\mu g\ g^{-1}$ FW)	Chl_b ($\mu g\ g^{-1}$ FW)	TC ($\mu g\ g^{-1}$ FW)	C_{x+c} ($\mu g\ g^{-1}$ FW)
-0.238 (control)	257.1a	108.1a	365.2a	65.7a
-0.392	192.8ab	79.2b	272.0b	59.1b
-0.674	176.5b	70.7b	247.2b	55.1bc
-0.939	160.2bc	62.6b	222.8bc	51.4c
-1.205	105.2c	39.7c	144.9c	43.3d
ANOVA	**	**	**	**

Note: values bearing different letters in each column show significant differences at $p \le 0.01$ (**) according to Duncan's New Multiple Range Test (DMRT).
FW – fresh weight.

yield of PSII (F_v/F_m) (*Figure 2B*) and with photon yield of PSII (Φ_{PSII}) (*Figure 3A*). The F_v/F_m and Φ_{PSII} in the leaf tissues of plants undergoing water deficit treatments decreased significantly, while non-photochemical quenching (NPQ) increased, especially under extreme conditions of water deficit (Ψ_w = -1.205 MPa) (*Table 2*). A positive correlation between Φ_{PSII} and net photosynthetic rate (P_n) was demonstrated in *Figure 3B*. It was found that P_n was significantly reduced in plants exposed to water deficit treatments (*Table 2*). Bio-chemical and physiological parameters including Chl_a, Chl_b, TC C_{x+c}, F_v/F_m, Φ_{PSII} and P_n were found to have positive correlations using Pearson's correlation coefficients, except in the case of proline, which displayed a negative relationship (*Table 3*). LA, FW and DW of water deficit stressed seedlings were drastically retarded in comparison to those under control conditions (*Table 4*). In contrast, SH and RL in the plants subjected to mild water deficit
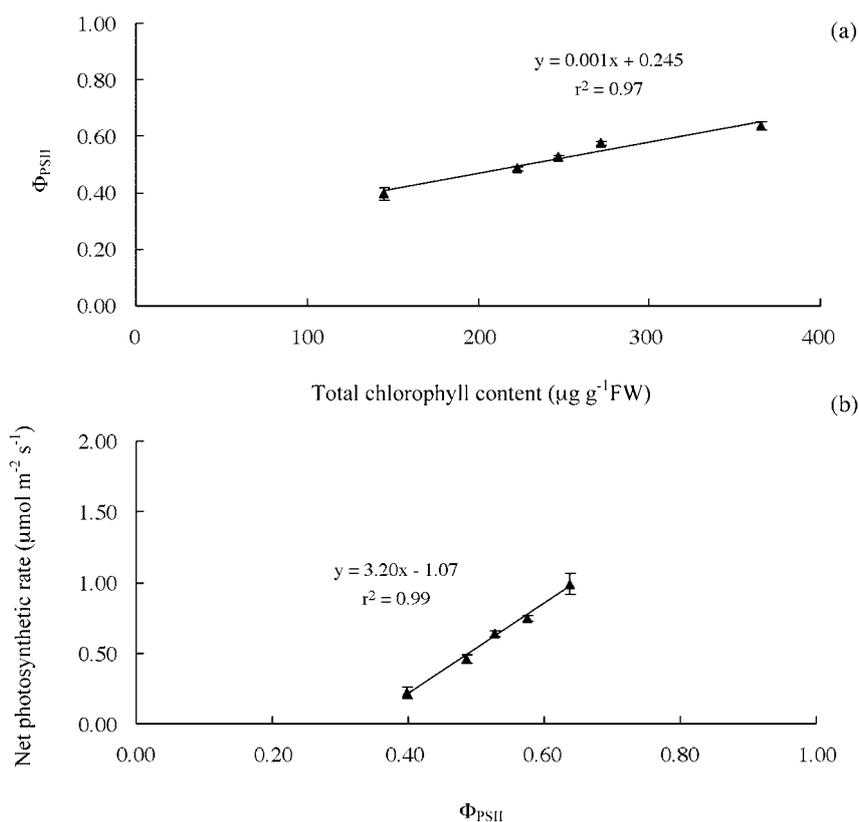
Figure 3. Relationships between total chlorophyll content and quantum efficiency of PSII (Φ_{PSII}), and between Φ_{PSII} and net photosynthetic rate of oil palm seedlings grown under mannitol-induced water deficit for one month. Error bars represent ±SE.

TABLE 2. MAXIMUM QUANTUM YIELD OF PSII (F_v/F_m), PHOTON YIELD OF PSII (Φ_{PSII}), NON-PHOTOCHEMICAL QUENCHING (NPQ) AND NET PHOTOSYNTHETIC RATE (P_n) OF OIL PALM SEEDLINGS GROWN UNDER MANNITOL-INDUCED WATER DEFICIT FOR ONE MONTH

Water potential (MPa)	F_v/F_m	Φ_{PSII}	NPQ	P_n ($\mu mol\ m^{-2}\ s^{-1}$)
-0.238 (control)	0.875a	0.637a	0.012c	0.99a
-0.392	0.854b	0.575b	0.024bc	0.75b
-0.674	0.839bc	0.527bc	0.033bc	0.64b
-0.939	0.824c	0.486c	0.041b	0.46c
-1.205	0.777d	0.397d	0.099a	0.22d
ANOVA	**	**	**	**

Note: values bearing different letters in each column show significant differences at $p \leq 0.01$ (**) according to Duncan's New Multiple Range Test (DMRT).

TABLE 3. CORRELATION COEFFICIENTS BETWEEN PHYSIOLOGICAL AND BIO-CHEMICAL PARAMETERS OF OIL PALM SEEDLINGS GROWN UNDER MANNITOL-INDUCED WATER DEFICIT FOR ONE MONTH

Parameter	Chl_a	Chl_b	TC	C_{x+c}	PRO	F_v/F_m	Φ_{PSII}	P_n
Chl_a	-	-	-	-	-	-	-	-
Chl_b	0.984**	-	-	-	-	-	-	-
TC	0.999**	0.992**	-	-	-	-	-	-
C_{x+c}	0.885**	0.937**	0.903**	-	-	-	-	-
PRO	-0.765**	-0.824**	-0.785**	-0.897**	-	-	-	-
F_v/F_m	0.838**	0.895**	0.859**	0.957**	-0.929**	-	-	-
Φ_{PSII}	0.891**	0.933**	0.907**	0.976**	-0.932**	0.977**	-	-
P_n	0.924**	0.856**	0.936**	0.971**	-0.896**	0.953**	0.981**	-

Note: highly significant level at $p \leq 0.01$ is represented by ** using Pearson's correlation coefficients.
TC – total chlorophyll.
P_n – net photosynthetic rate.

TABLE 4. SHOOT HEIGHT (SH), ROOT LENGTH (RL), LEAF AREA (LA), FRESH WEIGHT (FW) AND DRY WEIGHT (DW) OF OIL PALM SEEDLINGS GROWN UNDER MANNITOL-INDUCED WATER DEFICIT FOR ONE MONTH

Water potential (MPa)	SH (cm)	RL (cm)	LA (cm²)	FW (mg)	DW (mg)
-0.238 (control)	20.64a	10.58a	41.02a	1 179a	200a
-0.392	19.56ab	9.04ab	26.46b	728b	145b
-0.674	16.16bc	7.16ab	19.06c	477c	115bc
-0.939	15.52bc	5.10b	14.41cd	437c	90c
-1.205	14.80c	4.50b	11.07d	335c	72c
ANOVA	*	**	**	**	**

Note: values bearing different letters in each column show significant differences at $p \leq 0.01$ (**) and $p \leq 0.05$ (*) according to Duncan's New Multiple Range Test (DMRT).

($\Psi_w = -0.392$ MPa) were maintained, but dropped significantly under high levels of water deficit at -0.674 MPa and -0.939 MPa (*Table 4*). Under extreme water deficit ($\Psi_w = -1.205$ MPa), SH, RL, LA, FW and DW were reduced by 28.30%, 57.47%, 73.01%, 71.59% and 64.00% when compared to the control. In addition, LA was decreased by 35.5%, 53.5%, 64.9% and 73.0% when subjected to -0392, -0.674, -0.939 and -1.205 MPa mannitol-induced water deficit, respectively. Similarly, DW was reduced by 27.5%, 42.5%, 55.0% and 64.0%, respectively, when subjected to -0392, -0.674, -0.939 and -1.205 MPa water deficit. A similar trend of reduction in all growth characters was demonstrated in relation to the strength of the mannitol-induced water deficit in the culture media.

Data presented above demonstrated that the proline content in the leaf tissues of oil palm seedlings increased depending on the degree and duration of the stress treatments. Proline accumulation in higher plants has been well established as a good indicator of stress response because proline alleviates water deficit stress by means of antioxidation, osmoregulation and energy preservation. In olive trees, proline levels in both young and old leaves of plants exposed to water deficit stress were higher than in plants grown under well-irrigated conditions (Ahmed *et al.*, 2009). Proline content in mature leaves of drought-stressed sugar beet was 1.5 times higher than in well-irrigated plants (Chołuj *et al.*, 2008). In addition, ornithine-δ-aminotransferase (δ-OAT), glutamate dehydrogenase (GDH) and proline-5-carboxylase reductase (P5CR) activities of the proline biosynthesis pathway in drought-stressed mulberry (Chaitanya *et al.*, 2009) and *Sesuvium portulacastrum* (Slama *et al.*, 2006) increased in relation to the contents of proline. The levels of proline accumulation changed depending on the degree of water deficit (Chaitanya *et al.*, 2009), exposure period (Slama *et al.*, 2006) and specific plant organs (Chołuj *et al.*, 2008; Ahmed *et al.*, 2009).

In this study, we observed that relative membrane leakage in oil palm seedlings grown under mannitol-induced water deficit increased in a similar trend as that of proline accumulation. Relative membrane leakage of different plant species in response to water deficit stress has been shown to increase depending on the degree of stress (Beltrano and Ronco, 2008), genotype (Sayar *et al.*, 2008; Hu *et al.*, 2010) and exposure period (Hu *et al.*, 2010). In the case of bermudagrass grown under water deficit conditions, relative membrane leakage in drought-sensitive cultivars, C299 and Tifeagle, was greater than in drought-tolerant cultivars (Tifway, Kan 1, Sportbermuda, H10 and H19) (Hu *et al.*, 2010).

The photosynthetic pigments, Chl_a, Chl_b, TC and C_{x+c}, in the leaf tissues of oil palm seedlings were degraded, leading to a decrease in F_v/F_m, Φ_{PSII} and P_n in response to water deficit stress, especially under severe water deficit ($\Psi_w = -1.205$ MPa). Although the decrease in Chl content in response to water deficit is well-known, different effects on C_{x+c} have been reported for coconut (Gomes *et al.*, 2008) and olive (Ahmed *et al.*, 2009; Guerfel *et al.*, 2009). The C_{x+c} levels in water deficit stressed olive plants decreased significantly (Ahmed *et al.*, 2009), while they were maintained in coconut (Gomes *et al.*, 2008). The C_{x+c} is believed to play an important role in protection against photo-oxidative damage, as represented by low NPQ (Müller *et al.*, 2001; Omasa and Takayama, 2003). In the case of oil palm, C_{x+c} in the severe water deficit conditions was significantly degraded which may have caused the NPQ enrichment. In oil palm, the degree of degradation of the photosynthetic pigments and the CO_2 assimilation rate in response to severe water deficit were closely correlated to F_v/F_m, Φ_{PSII} and P_n reduction. Similar results have been reported in coconut (Gomes *et al.*, 2008), perennial grass (Xu *et al.*, 2009), maize (Li *et al.*, 2009) and olive (Boussadia *et al.*, 2008). In addition, P_n in coconut palms was reduced significantly in both the Una and Jiqui cultivars by 37.28% and 43.09%, when

exposed to water deficit stress (Gomes *et al.*, 2008), in order to limit the CO_2 assimilation rate through the stomatal apertures (Cornic, 2000). Limited CO_2 assimilation through stomatal pores of water-deficit stressed plants leads to low P_n (Chaves, 1991; van Heerden *et al.*, 2007; Dias and Brüggemann, 2010). Biomass production in higher plants is achieved by the plant's photosynthetic ability, which is inhibited by water deficit. The growth characters, *i.e.* SH, RL, LA, FW and DW, of oil palm seedlings were retarded in response to water shortage. Inhibition of plant growth when subjected to water deficit conditions has also been demonstrated in perennial grass (Xu *et al.*, 2009), olive (Guerfel *et al.*, 2009), *Sesuvium portulacastrum* (Slama *et al.*, 2006) and *Dianthus* (Álvarez *et al.*, 2009).

In conclusion, relative membrane leakage in oil palm seedlings increased in response to water deficit, leading to damage to the photosynthetic pigments. This in turn diminished the photosynthetic ability and reduced the growth performance of the seedlings grown under short-term water shortage conditions. The physiological and growth characters of oil palm seedlings also decreased significantly, depending on the degree of water deficit and the exposure period. These data provide the basis for the establishment for multivariate criteria for water deficit tolerance screening in oil palm breeding programmes.

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