Physio-Biochemical Responses of Oil Palm (*Elaeis guineensis* Jacq.) Seedlings to Mannitol- and Polyethylene Glycol-Induced Iso-Osmotic Stresses

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Abstract: The aim of this investigation was to comparatively examine the physio-biochemical responses of oil palm seedlings to mannitol- and PEG-induced iso-osmotic stresses. The water content of osmotically stressed oil palm seedlings decreased, but the proline content and the electrolyte leakage of the seedlings increased with decreasing water potential ($\Psi_w$). However, the responses varied with the strength of osmotic stress and type of osmotic agent. Relative electrolyte leakage (REL) was negatively correlated to chlorophyll content in the osmotically stressed leaves. Chlorophyll a (Chla), chlorophyll b (Chlb), total carotenoids ($C_{x+c}$) and total chlorophyll (TC) in the seedlings were significantly reduced by osmotic stress, subsequently reducing maximum quantum yield of PSII ($F_{v}/F_{m}$) and photon yield of PSII ($\Phi_{psii}$), thereby lowering net-photosynthetic rate ($P_n$) and inhibiting growth. Physio-biochemical parameters, including REL, $F_{v}/F_{m}$, and $\Phi_{psii}$ in oil palm seedlings were reduced more greatly by PEG-induced osmotic stress than by mannitol-induced stress. A deterioration in morphological characters, including leaf chlorosis, leaf burn, and green leaf area reduction were demonstrated in oil palm seedlings under osmotic stress induced by either mannitol or PEG. However, the toxic symptoms in oil palm seedlings under PEG-induced stress were severer than in those under mannitol-induced iso-osmotic stress, especially under severe osmotic stress.

Key words: Chlorophyll fluorescence, Net-photosynthetic rate, Osmotic stress, Photosynthetic pigment, Proline, Relative electrolyte leakage.

Southeast Asia, including Malaysia, Indonesia and Thailand, is a tropical zone, and is the main area of cultivation of the oil palm, one of the most important oil production plants in the world (Yusof and Chan, 2003; Wahid et al., 2005). Oil yield and productivity are major concerns of oil palm breeders (Jalani et al., 1997; Cochard et al., 2005). The oil yield trait is not only dependent on genetic factors, but is also affected by environmental conditions, such as relative humidity, water availability, soil structure, fertilizer application, agricultural management and light conditions (Henson and Dolmat, 2003; Kallarackal et al., 2004; Henson and Harun, 2005).

Another issue is tolerance to abiotic stress, such as water-deficit, extreme temperature, mineral deficiency, heavy metal toxicity and ultraviolet irradiation. This is an alternative target for oil palm breeding. The water content of soil plays a key role in oil palm growth and development (Henson and Harun, 2005) and may also trigger female sex representation. In arid zones, the proportion of male flowers is high, which retards growth, leading to low productivity (Jones, 1997).

Water deficit stress is a major problem worldwide, limiting plant growth and leading to low productivity in many crop species, especially in arid zones (>1.2 billion hectares) (Chaves and Oliveira, 2004; Kijne, 2006; Passioua, 2007). The response of plants to water stress, such as decreased Rubisco (ribulose-1,5-bisphosphatase carboxyase/oxygenase) activity, reduced photochemical efficiency, enhanced accumulation of stress metabolites (proline, glycinebetaine, polyamine, glutathione, polyanime, sugars, sugar alcohols and $\alpha$-tocopherol), and increased antioxidant enzymes (superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase) has been well discovered (Chaves et al., 2002; Parry et al.,...
2002; Flexas et al., 2006). Proline accumulation in plants exposed to water deficit conditions has been generally regarded as an osmotic adjustment or osmoregulation defense mechanism (Gomes et al., 2010), which has been investigated as a biochemical marker of water deficit tolerance screening in many plant species (Ashraf and Foolad, 2007). The physiological changes, including loss of membrane stability, reduced leaf water potential, pigment degradation, decreased stomatal conductance, reduced internal CO₂ concentration, net photosynthetic rate (Pₚ) reduction and growth inhibition have been studied intensively (Chaves and Oliveira, 2004; Reddy et al., 2004; Cattivelli et al., 2008). Generally, biochemical and physiological responses of plants to water deficit stress have been studied in field trials and hydroponic systems. In the present study, an environmental control system for photoautotrophic in vitro culture was developed (Cha-um et al., 2003) and applied as a prototype for salt tolerance screening in rice (Cha-um et al., 2010a).

Mannitol-induced water stress has been widely applied to many plant species i.e. tomatoes (Weng, 2000), sugarcane (Cha-um and Kirdmanee, 2008), rice and sorghum (Cha-um et al., 2009). Polyethylene glycol (PEG) is an osmoticum for inducing water deficit (Ionenko and Anisimov 2001), and 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 (Hiller and Dexter, 1997; Verslues et al., 1998). The physio-biochemical responses of oil palm seedlings under either mannitol or PEG-induced iso-osmotic stress have not been reported previously. In the Arecaceae family, ecophysiological expression in coconut palms, including membrane lipid composition (Repellin et al., 1997), photosynthetic ability and growth performance under water deficit stress, using PEG solution, has been investigated (Gomes and Prado, 2007; Gomes et al., 2008).

Also, we have investigated the physio-biochemical responses of oil palm seedlings grown under PEG-induced water deficit (Cha-um et al., 2010b). In the present study, water content, relative electrolyte leakage (REL), proline accumulation, photosynthetic pigment content, chlorophyll fluorescence, net photosynthetic rate (Pₚ) and growth performance of oil palm seedlings under mannitol- and PEG-induced iso-osmotic stresses were evaluated.

Materials and Methods

1. Plant materials

Oil palm fruits were obtained from Suksomboon Palm Oil Co Ltd., Chonburi province, in the eastern region of Thailand. The kernel of the fruit was removed. The seeds, with the seed coat, were dried in a hot air oven at 45°C for 12 h, and the seed coat scarified. 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 allowed to germinate in MS media (Murashige and Skoog, 1962) containing 3% (w/v) sucrose without plant growth regulators. 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 from fluorescent lamps with a 16 h d⁻¹ photoperiod. After two months, the seedlings were transferred aseptically to MS-liquid sugar-free media (photoautotrophic growth). The uncovered vessels containing the seedlings were transferred aseptically to culture box chambers (Carry Box Model P-850, size 26×36×19 cm, Eastern Premium Co. Ltd., Bangkok, Thailand) with RH controlled at 65±5% with 1.5 L saturated NaCl solution. The number of air exchanges in the culture box chambers was increased to 5.1±0.3 μmol CO₂ hr⁻¹ by punching the side of the plastic chambers with 32 holes and placing gas-permeable microporous polypropylene film (0.22 μm pore size) over the holes (Cha-um et al., 2003). Oil palm seedlings were acclimated for 14 days by placing the chambers in a Plant Growth Incubator controlled at 28±2°C/25±2°C (light/dark), 500±100 μmol CO₂ mol⁻¹ concentration, 60±5% RH, 120±5 μmol m⁻² s⁻¹ PPFD provided from fluorescent lamps with a 16 hr d⁻¹ photoperiod. Osmotic potential in the culture media was adjusted to −0.238 (control), −0.674 MPa (mannitol), −0.674 MPa (PEG6000), −0.939 MPa (mannitol), or −0.939 MPa (PEG6000) and the seedlings grown on for 30 days. Water content (WC), relative electrolyte leakage (REL), proline content, photosynthetic pigments, chlorophyll fluorescence, net-photosynthetic rate (Pₚ) and growth characters were measured.

2. Data collection

REL (%) was determined according to the Dionisio-Sese and Tobita (1998) method. Leaf tissue was cut into pieces 5.0±0.2 mm in length, and placed in glass vessels (OptiClear; KIMBLE, Vineland, New Jersey, USA) containing 10 mL deionized water. The glass vessels were capped and maintained at room temperature (25°C) for 15 min. Initial electrical conductivity (ECᵢ) 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 and electrical conductivity (ECᵢ) was measured.

Proline in the leaf tissues was extracted and analyzed according to the method of Bates et al. (1973). The fresh material (50 mg) was 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
Table 1. Water content (WC), and contents of chlorophyll a (Chl\textsubscript{a}), chlorophyll b (Chl\textsubscript{b}), total chlorophyll (TC) and total carotenoids (C\textsubscript{x+c}) in oil palm grown under mannitol- and PEG-induced iso-osmotic stresses.

| Water potential (MPa) | WC (%) | Chl\textsubscript{a} (µg g\textsubscript{FW}) | Chl\textsubscript{b} (µg g\textsubscript{FW}) | TC (µg g\textsubscript{FW}) | C\textsubscript{x+c} (µg g\textsubscript{FW}) |
|-----------------------|--------|---------------------------------|-----------------|-----------------|-----------------|
| -0.238 (Control)      | 79.6 a | 242.1 a                         | 149.2 a         | 391.3 a         | 73.1 a          |
| -0.674 mannitol       | 78.1 a | 220.9 a                         | 108.5 b         | 329.4 b         | 66.1 a          |
| -0.674 PEG            | 75.4 b | 110.2 b                         | 42.4 c          | 152.6 c         | 36.0 b          |
| -0.939 mannitol       | 72.9 c | 51.7 c                          | 20.6 cd         | 72.3 d          | 19.2 c          |
| -0.939 PEG            | 70.3 d | 37.0 c                          | 15.0 d          | 52.0 d          | 13.4 c          |

ANOVA ** ** ** ** **

Different letters in each column show significant difference at \( p \leq 0.01 \) (***) by Duncan’s New Multiple Range Test (DMRT).

Chlorophyll fluorescence emission from the adaxial surface of the leaf was measured using a fluorescence monitoring system (model FMS 2; Hansatech Instruments Ltd., Norfolk, UK) 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 662 nm and 644 nm wavelengths. The C\textsubscript{x+c} concentration was also measured by spectrophotometer at 470 nm. A solution of 95.5% acetone was used as a blank.

Chlorophyll fluorescence emission from the adaxial surface of the leaf was measured using a fluorescence monitoring system (model FMS 2; Hansatech Instruments Ltd., Norfolk, UK) 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 far-red light (LED source with a peak at wavelength 735 nm). Original (F\textsubscript{0}) and maximum (F\textsubscript{m}) fluorescence yields were measured under weak modulated red light (<0.5 µmol m\textsuperscript{-2} s\textsuperscript{-1}) with 1.6 s pulses of saturating light (>6.8 µmol m\textsuperscript{-2} s\textsuperscript{-1} PAR) and calculated using FMS software for Windows. The variable fluorescence yield (F\textsubscript{v}) was calculated by the equation of F\textsubscript{m}-F\textsubscript{0}. The ratio of variable to maximum fluorescence (F\textsubscript{v}/F\textsubscript{m}) was calculated as maximum quantum yield of PSII photochemistry. The photon yield of PSII (Φ\textsubscript{PSII}) in the light was calculated by Φ\textsubscript{PSII}=(F\textsubscript{m}'-F)/F\textsubscript{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\textsubscript{n}) was calculated by comparing the concentrations of CO\textsubscript{2} inside (C\textsubscript{i}) with those outside (C\textsubscript{e}) the glass vessel containing the oil palm seedlings. The CO\textsubscript{2} concentrations at steady state were measured by gas chromatography (GC; Model GC-17A, Shimadzu Co. Ltd., Tokyo, Japan). The P\textsubscript{n} of in vitro cultivated seedlings was calculated according to the method of Fujisawa et al. (1987).

Shoot height (SH), root length (RL), leaf area (LA), fresh weight (FW) and dry weight (DW) of oil palm seedlings was calculated according to the method of Fujiwara et al. (1987).

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3. Experimental design and statistical analysis

The experiment was arranged in a completely randomized design (CRD) with eight replicates (\( n=8 \)). The mean values obtained were compared by Duncan’s New Multiple Range Test (DMRT) and analyzed using SPSS software.

Results

1. Water content, proline content and relative electrolyte leakage

The water content in the leaf tissues of oil palm seedlings decreased, relating to water potential in the culture media and the type of osmoticum (Table 1). Water content dropped significantly in response to mild PEG-induced osmotic stress (−0.674 MPa) for 75.4%, while it decreased significantly in severe mannitol-induced osmotic stress conditions (−0.939 MPa) for 72.9%. Proline content and relative electrolyte leakage in the leaf tissues of oil palm seedlings showed a similar trend and increased following water potential reduction under either PEG- or mannitol-induced stress. The proline level and relative electrolyte leakage in extreme PEG-induced osmotic stress (\( Ψ\textsubscript{w} = -0.939 \) MPa) were 5.25 µmol g\textsuperscript{-1} FW and 40.57%,...
respectively which were 10.94 and 2.90 times larger than the values of the control (Fig. 1). Electrolyte leakage showed a correlation with Chla damage (Fig. 2A).

2. Photosynthetic pigment, chlorophyll fluorescence and net photosynthetic rate

Chl contents of the leaf tissues under mild and severe PEG-induced stresses were 54.48 and 84.72% lower than that of the control seedlings, respectively. In contrast, Chl content under mild mannitol-induced stress was only 8.75% lower than in the control. A similar pattern of Cx+c decrease was demonstrated, as shown in Table 1. Chl, and TC contents of the leaf tissues were also greatly decreased by osmotic stress (Table 1). The degradation of Chl<sub>a</sub>, Chl<sub>b</sub>, TC and Cx+c in the leaf tissues by osmotic stress was significant, especially in PEG solution. For example, the Chl<sub>a</sub> content under mild mannitol-induced stresses was 27.28 and 86.19% lower, respectively, and that in PEG solution

![Graph](https://via.placeholder.com/150)

Fig. 1. Relative electrolyte leakage and proline content of oil palm grown under mannitol- and PEG-induced iso-osmotic stresses. Error bars indicate ±SE.

![Graph](https://via.placeholder.com/150)

Fig. 2. Relationship between relative electrolyte leakage and chlorophyll a content (A) and between chlorophyll a content and maximum quantum yield of PSII ($Fv/Fm$) (B) in oil palm grown under mannitol- and PEG-induced iso-osmotic stress. Error bars indicated by ±SE. ** represents a highly significant difference.

![Graph](https://via.placeholder.com/150)

Fig. 3. Relationship between total chlorophyll content and quantum efficiency of PSII ($F_{PSII}$) (A) and between quantum efficiency of PSII ($F_{PSII}$) and net photosynthetic rate ($P_n$) (B) in oil palm grown under mannitol- and PEG-induced iso-osmotic stresses. Error bars indicate ±SE. ** represents a highly significant difference.
height (SH) and root length (RL) were maintained under mild osmotic stress ($\Psi_w = -0.674$ MPa), but decreased significantly under severe osmotic stress, $-0.939$ MPa (Table 3). The reduction percentage of plant dry weight under $-0.674$ MPa mannitol- and PEG-induced iso-osmotic stresses was 32.37 and 67.89%, respectively, and that under $-0.939$ MPa mannitol- and PEG-induced iso-osmotic stresses was 67.11 and 75.26%, respectively. There was a similar trend in leaf area and plant fresh weight. Leaf chlorosis, leaf burn and reduction of leaf expansion were evident in the severe osmotic stress induced either by PEG or mannitol (Fig. 1).

### Table 3. Growth performance, shoot height (SH), root length (RL), leaf area (LA), fresh weight (FW) and dry weight (DW) of oil palm grown under mannitol- and PEG-induced iso-osmotic stresses.

| Water potential (MPa) | SH (cm) | RL (cm) | LA (cm²) | FW (mg) | DW (mg) |
|-----------------------|---------|---------|-----------|---------|---------|
| Control (-0.238)      | 21.88 a | 21.21 a | 76.38 a   | 1864 a  | 380 a   |
| Mannitol (-0.674)     | 21.03 a | 18.30 ab| 40.59 b   | 1172 b  | 257 b   |
| PEG (-0.674)          | 15.00 b | 17.20 ab| 13.83 c   | 495 c   | 122 c   |
| Mannitol (-0.939)     | 13.90 bc| 14.23 b | 11.80 c   | 461 c   | 125 c   |
| PEG (-0.939)          | 11.23 c | 14.10 b | 2.40 c    | 316 c   | 94 c    |

ANOVA ** * ** ** **

Different letters in each column show significant difference at $p \leq 0.01$ (**) by Duncan’s New Multiple Range Test (DMRT).

3. Growth performance

The growth characters, leaf area (LA), fresh weight (FW) and dry weight (DW) of the seedlings were drastically inhibited by osmotic stress (Table 3). In contrast, shoot height (SH) and root length (RL) were maintained under mild osmotic stress ($\Psi_w = -0.674$ MPa), but decreased significantly under severe osmotic stress, $-0.939$ MPa (Table 3). The reduction percentage of plant dry weight under $-0.674$ MPa mannitol- and PEG-induced iso-osmotic stresses was 32.37 and 67.89%, respectively, and that under $-0.939$ MPa mannitol- and PEG-induced iso-osmotic stresses was 67.11 and 75.26%, respectively. There was a similar trend in leaf area and plant fresh weight. Leaf chlorosis, leaf burn and reduction of leaf expansion were evident in the severe osmotic stress induced either by PEG or mannitol (Fig. 4).

### Discussion

In general, the water available for the plant is reduced by adding an osmoticum such as PEG or sugar alcohol (sorbitol and mannitol), leading to a slower rate of water absorption, uptake and translocation, as identified by low water content and low water potential (Gomes et al., 2010). Proline accumulation in higher plants is an indicator of stress as it alleviates water stress by means of antioxidant and osmoregulation defense mechanisms. The proline level was higher in plants exposed to water stress than in well-watered plants (Chohuj et al., 2008; Ahmed et al., 2009; Cha-um et al., 2010b). In rice (cv. Kranti), the proline content of the plants was higher under PEG-induced stress.
than under mannitol-induced iso-osmotic stress (Pandey et al., 2004). In contrast, proline accumulation in the leaf tissues of *Sesuvium portulacastrum* under PEG-induced stress was lower than plants grown under mannitol-induced iso-osmotic stress (Slama et al., 2007). Proline accumulation in stress conditions was not only dependent on the type of stressor but also the plant species. In addition, the ornithine-δ-aminotransferase (δ-OAT), glutamate dehydrogenase (GDH), and proline-5-carboxylase reductase (P5CR) in the proline biosynthesis pathway in drought-stressed mulberry (Chaitanya et al., 2009) and *Sesuvium portulacastrum* (Slama et al., 2006) have been investigated. In this study, REL in oil palm seedlings was increased under PEG- and mannitol-induced osmotic stress, as was proline accumulation. REL in different plant species in response to water stress, has been shown to increase, depending on the degree of stress and the type of stressor (Bajji et al., 2001; Beltrano and Ronco, 2008; Cha-um et al., 2010b; Xu and Huang, 2010).

Levels of the photosynthetic pigments, Chl<sub>a</sub>, Chl<sub>b</sub>, TC and C<sub>x+c</sub> in the leaf tissues of oil palm seedlings decreased, leading to diminution of F<sub>r</sub>/<F<sub>m</sub>> and Φ<sub>PSII</sub> and P<sub>n</sub> in response to iso-osmotic stress, especially severe PEG-induced stress (Ψ<sub>w</sub>=−0.939 MPa). However, in olive plants the C<sub>x+c</sub> level has been reported to decrease by osmotic stress (Ahmed et al., 2009; Guerfel et al., 2009), while it was maintained in coconut (Gomes et al., 2008). C<sub>x+c</sub> plays an important role in protection against photo-oxidative damage, as represented by low NPQ (Müller et al., 2001; Omasa and Takayama, 2003). In oil palm, C<sub>x+c</sub> was reduced significantly under severe water deficit conditions, which might cause NPQ enrichment. In oil palm, the photosynthetic ability dropped significantly under PEG-induced osmotic stress (Cha-um et al., 2010b) and the degree of the degradation of photosynthetic pigments and the CO<sub>2</sub> assimilation rate under severe osmotic stress were closely correlated with diminution of F<sub>r</sub>/<F<sub>m</sub>> and reduction of Φ<sub>PSII</sub> and P<sub>n</sub>. In addition, P<sub>n</sub> in coconut cultivars, Una and Jiqui were reduced by 37.28% and 43.09%, respectively, under water deficit stress (Gomes et al., 2008), to limit the

![Fig. 4. Morphological characters of oil palm seedlings grown under mannitol- and PEG-induced iso-osmotic stresses at −0.238 MPa (control) (A), −0.674 MPa-mannitol (B), −0.674 MPa-PEG (C), −0.939 MPa-mannitol (D) and −0.939 MPa-PEG (E).](image-url)
CO₂ assimilation through the stomatal apertures (Cornic, 2000). In the present study, the toxic symptoms in oil palm seedlings grown under PEG-induced osmotic stress were greater than those under mannitol-induced iso-osmotic stress. Similar results have been observed in Sesuvium portulacastrum (Slama et al., 2007) and rice (Pandey et al., 2004).

Biomass production in higher plants is achieved by the photosynthetic ability, which is inhibited by osmotic stress. Growth characters, i.e. SH, RL, LA, FW and DW of oil palm seedlings were retarded by water shortage, especially in plants under PEG-induced osmotic stress. Similar results in the overall growth performance have been recorded in maize coleoptiles (Hohl and Schopfer, 1991). Osmotic stress induced by PEG, especially high-molecular-weight PEG (MW>1000) has been reported to limit water uptake and O₂-dissolution (Lawlor, 1970; Janes, 1974), whereas mannitol has been used only as an osmoticum with low absorption rate by the root tissues of plants (<5% radioactive labeling) (Smith and Smith, 1973; Flora and Madore, 1993; Vitéva et al., 2002).

In conclusion, water content of the leaf tissues in palm seedlings decreased under osmotic stress, inducing relative electrolyte leakage and damage to photosynthetic pigments. The degradation of pigments and diminution of photosynthetic ability in oil palm seedlings grown under osmotic stress were positively related to the reduction of net photosynthetic rate and overall growth performance. In oil palm seedlings, the toxic effect of PEG-induced osmotic stress was greater than that of mannitol-induced stress.

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