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APPLICATION OF SEAWEED EXTRACTS IN OIL PALM (*Elaeis guineensis*) SEEDLINGS SUBJECTED TO WATER DEFICIT: PHYSIOLOGICAL, BIOCHEMICAL AND ENZYMATIC RESPONSES

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ABSTRACT

Water deficit can negatively affect physiological and biochemical responses in plants. The stress-mitigation potential of seaweed extracts (SWEs) Caulerpa lentillifera *(green seaweed) and* Gracilaria edulis *(red seaweed) on oil palm seedlings subjected to water deficit was investigated. Plant height, relative water content, chlorophyll content, soluble protein concentration and the expression of genes coding for thiamine (vitamin B1) biosynthesis enzymes were evaluated. SWE treatments were applied to four-month-old oil palm seedlings under full irrigation (control) and water deficit conditions. Overall, SWE did not affect the height of the seedlings after 60 days of treatment, but the seedlings managed to maintain relative water content of up to 90.00% and chlorophyll and leaf protein content increased up to 50.00% in seedlings treated with red seaweed extract compared to the control. The application of* G. edulis *and* C. lentillifera *SWE induced downregulation of THIC (up to 15.00%) and THI4 (up to 15.46%) expression respectively on day 2 post-treatment as compared to the control. This finding is in line with the postulated role of thiamine in protection against stress in plants and suggests the role of SWE as a biostimulant.*

Keywords: biostimulant, seaweed extracts, thiamine, vitamin B1, water deficit.

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INTRODUCTION

Elaeis guineensis (Jacq.) is native to Africa and is the commercial species of oil palm planted in Malaysia since 1870 (Basiron, 2007). It started as an ornamental plant and has become a huge industry due to increasing global demands for palm oil in many applications such as food products, cosmetics, and bio-energy (Alam *et al*., 2015). Currently, Malaysia is reported to be the second-largest world producer and exporter of palm oil (Kushairi *et al*., 2018). However, challenges still remain and largely affect the oil palm growth and productivity, and among them is the water deficit (Noor *et al*., 2011). The means to control this situation unfortunately is still very limited. According to Henson and Harun (2005), oil yields depend not only on genetic resources but also on environmental factors such as availability of water, relative humidity, use of fertilisers, soil texture, and cultural practices. Thus, a need to search for alternative stress mitigation techniques to maintain and improve crop production would be very beneficial.

Under water deficit conditions, plants often respond by reducing their growth and metabolic activities (Bonjoch & Tamayo, 2001) as ways to improve plant adaptability towards stress. Apart from that, thiamine has also been reported to have

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a role in plant protection against stress, where the level of thiamine content in maize leaves increased when subjected to water deficit (Rapala-Kozik *et al*., 2009). This may be due to thiamine's role in plant defense mechanism through systemic acquired resistance (SAR) by induction of SAR-related genes (Subki *et al*., 2018; 2020). THIC and THI4 gene transcripts in oil palm, key genes in the thiamine biosynthesis pathway, showed an increase in gene expression in conjunction with increasing concentrations of osmotic, salinity, and oxidative stresses (Abidin *et al*., 2016; Idris *et al*., 2018; Rahman *et al*., 2017; Wong *et al*., 2016). Plus, biotic stress and the colonisation of harmless endophytes have also been shown to increase the expression of thiamine biosynthesis genes as well as the accumulation of the vitamin and its intermediates (Kamarudin *et al.,* 2017a; 2017b; Yusof *et al*., 2015).

Seaweeds have been widely utilised in agriculture and horticulture as a complement to fertilisers because of their various positive effects on crop production, stress tolerance, nutrient uptake, and the quality of products after harvest (Fan *et al*., 2013). The same effects of the physiological responses in crops treated with plant growth-regulatory substances were reported in seaweed extracts (SWEs)-treated plants (Ghaderiardakani *et al*., 2019). SWEs exhibited effects at low concentrations and the effects may involve the synergy between plant-growth promoters (Ghaderiardakani *et al*., 2019). Battacharyya *et al*. (2015) reported that the application of different types of SWEs could improve plants' tolerance towards a wider range of abiotic stresses.

In this study, *Caulerpa lentillifera* and *Gracilaria edulis* were extracted and applied to oil palm seedlings through soil application. *Caulerpa lentillifera* is also known as sea grapes (Guo *et al*., 2015) and belongs to the Chlorophyta phylum. It has high polyunsaturated fatty acids (PUFAs), multiple essential amino acids, dietary fibres, minerals, vitamin C and vitamin A (Chen *et al.,* 2019). *Gracilaria edulis*, a member of the Rhodophyta phylum, produces phycocolloid agar for the biotechnology, medicine, cosmetics, pharmaceutical and food industries (Lee *et al*., 2016). It contains high dietary fibre, ash, essential amino acids, minerals and vitamins (Sakthivel & Devi, 2015). Even though the usefulness of SWEs in attenuating the negative effects of water deficit has been proven in many plants, studies on oil palm have not been addressed to date. It is crucial to find alternatives to stress control in oil palm plantations and the dependency on synthetic and chemical control must be reduced. Hence, the objective of this study is to investigate the physiological and biochemical effects of SWEs on oil palm seedlings subjected to water deficit.

MATERIALS AND METHODS

Collection of Seaweeds

Caulevpa lentillifera and *G. edulis* were collected from Teluk Kemang, Port Dickson, Negeri Sembilan, Malaysia (2°27'14.039"N, 101°51'18.719"E) as shown in *Figure 1. Table 1* shows the dominant compounds found in the extract using gas chromatography-mass spectrometry (GC-MS). The seaweeds were handled as described by Aziz *et al.* (2019a) and washed with seawater to remove sand particles, epiphytes and foreign particles followed by water to remove the remaining salt. The seaweeds were freeze-dried to a constant weight before grinding into fine powder and kept at -20°C until further analysis.

Preparation of Seaweed Crude Extracts

The crude SWEs (*C. lentillifera* and *G. edulis* extract) were prepared by using the Soxhlet extraction method as described previously by Aziz *et al.* (2019c). Ten gram of seaweed powder was packed into a Soxhlet thimble and placed inside a Soxhlet extractor with dichloromethane as solvent. The concentrated crude extract was then kept at 4ºC until further use.

Plant Materials and Experimental Design

Four-month-old oil palm seedlings (*Dura* x *Pisifera*) were procured from Felda Global Ventures (FGV) Holdings Berhad, Malaysia. The seedlings were grown and maintained in polyethylene bags under greenhouse conditions at the Transgenic Greenhouse, Institute of Plantation Studies (IKP), UPM, Malaysia. The seedlings were acclimatised under normal nursery practices. *i.e.*, watered with 200 mL twice daily (morning and evening) by using a drip irrigation system. This experiment consists of two watering regimes: Full irrigation (200 mL of water) and water deficit condition (100 mL of water) (Najihah *et al.,* 2019) and four soil application treatments; control (distilled water), positive control (Green Amino-G liquid fertiliser) and liquid SWE (*C. lentillifera* and *G. edulis*). Green Amino-G liquid fertiliser, an organic biostimulant derived from marine products fortified with a range of vitamins and trace minerals was prepared by adding 3-4 mL of fertiliser to 1 L of water. The SWEs with a concentration of 1 mg/mL with a final volume of 20 mL of distilled water were prepared (Salim, 2016). The same amount of 20 mL of each soil application treatment was soil drenched onto the oil palm seedlings every 15 days and sampled at day 60 for physiological analyses (plant height, relative water content, chlorophyll content, and protein content). While

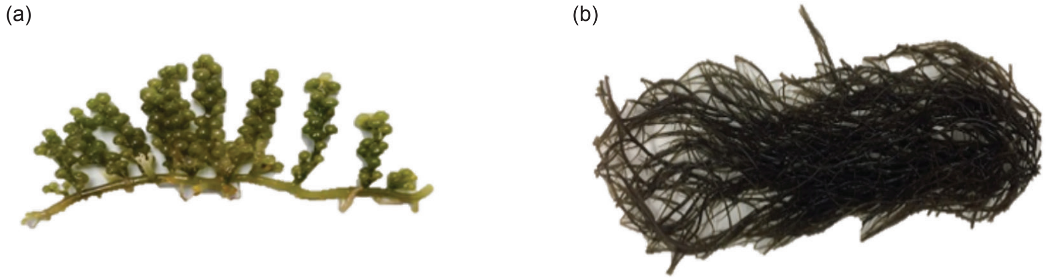

Figure 1. Seaweeds collected in this study are found in abundance in Port Dickson (a) Caulerpa lentillifera, *and (b)* Gracilaria edulis.

TABLE 1. IDENTIFIED COMPOUNDS OF SEAWEED EXTRACTS WITH BIOSTIMULANT POTENTIAL

Compounds
Phytol (Diterpene alcohol)
9,12,15-Octadecatrienoic acid, (Z,Z,Z) (Fatty acid)
n-Hexadecanoic acid (Fatty acid)
Tetradecanoic acid (Fatty acid)

Source: Aziz *et al.* (2019b).

for gene expression analysis, the spear leaves of the seedlings were sampled at day 0, 1 and 2 post-SWE treatment (control, *C. lentillifera* and *G. edulis*). Both experiments were arranged in a completely randomised design (CRD) with three replicates (n=3).

Physiological Analyses

Plant height. Plant height was measured as performed by Sapak *et al.* (2008). The plant height was measured by measuring the seedlings 1 cm above the soil level to the tip of the leaves.

Relative water content (RWC). RWC of the leaves was analysed as done by Sun *et al.* (2011). Six leaf samples of the same size were weighed and recorded as fresh weight (FW). The turgid weight (TW) of the leaf samples was recorded by immersing them in a petri dish with water for 24 hr. The dry weight (DW) of the leaf samples was then recorded after oven-drying at 70ºC for 24 hr. RWC (%) was calculated using Equation (1):

$$[(FW-DW)/(TW-DW)]\times100 \qquad (1)$$

Chlorophyll concentration. The chlorophyll concentration of the leaf samples was determined as performed by Godlewska *et al.* (2016) with a slight modification. Leaf samples at 0.2 g were immersed in 200 mL of methanol for 30 min. The sample was filtered using Whatman filter paper before the absorbance readings were recorded at wavelengths of 645 and 663 nm. The concentration of total chlorophyll (Total Chl) was determined from Equation (2):

$$Total\ Chl = 8.02 \cdot A\ (663) + 20.2 \cdot A\ (645) \qquad (2)$$

Total soluble proteins. The proteins were extracted using TCA/acetone extraction method as performed by Hassan *et al.* (2014) with a slight modification. The quantification of the protein content was determined using Bradford's (1976) method. The protein concentrations of the samples were calculated using the Equation (1) described previously.

Gene Expression Analysis

Total RNA was extracted as described by Kamarudin *et al.* (2017a) and stored at -80ºC until further use. Reverse transcription was performed using Tetro cDNA Synthesis Kit (Bioline) and stored at -20°C until further use. PCR was performed using MyTaq^{TM} Red Mix (Bioline). Three pairs of primers were used in this study namely actin (housekeeping gene), THIC, and THI4. Actin was used to normalise mRNA levels between different samples. THIC encodes the first enzyme of the pyrimidine branch located in chloroplast, chloroplast stroma and plastid, while, THI4 encodes the enzyme responsible for the biosynthesis of thiamine precursor thiazole. Primers used in this study are shown in *Table 2*.

The PCR cycling conditions include initial denaturation at 95°C for 1 min for 1 cycle, followed by 30 repetitive cycles of denaturation step at 95°C for 15 s, annealing step at 60°C for all primers for 15 s, and extension step at 72°C for 10 s. The final extension step was set at 72°C for 3 min for 1 cycle and then held at 4°C. The PCR product was then subjected to agarose gel electrophoresis and the intensities of the bands obtained were analysed using ImageJ software.

TABLE 2. NUCLEOTIDE SEQUENCES OF THE PRIMERS USED IN THIS STUDY

Gene	Primer sequence (5′-3′)	Amplicon size (bp)
Actin	F : 5′ – CTT GCT CCA AGC AGC ATG A – 3′ R : 5′ – AGA AGC ACT TCC GGT GCA CG – 3′	168
THIC	F : 5′ – AAT GAA GGT CCA GGG CAT – 3′ R : 5′ – GCT GAG GTG ATG TGA TCA – 3′	188
THI4	F : 5′ – ATC GTG GAG CAG TCC GTC T– 3′ R : 5′ – AGC TCG TCG AGG AAG AGG T– 3′	104

Statistical Analysis

Results are expressed as mean ± standard deviation (SD). Statistical analysis was performed using two-way ANOVA and Dunnet post-test. The significant level was set at *P*<0.05 using GraphPad Prism 8.

RESULTS AND DISCUSSION

Physiological Effects of Seaweeds Extract on Oil Palm Seedlings

To observe the effects of the application of SWE on various physiological parameters, data and samples were taken for plant height, relative water content, chlorophyll content, and protein content. In this study, the plant height of seedlings under both conditions showed no significant difference in all treatments as compared to the controls when measured after 60 days (*Figure 2a*). Theoretically, plants respond to any biotic or abiotic stress by decreasing their normal metabolic activities leading to growth reduction (Bonjoch & Tamayo, 2001). SWE has been reported to have a significant effect on the vegetative growth of many crops including soybean (Noli *et. al.*, 2021), coriander (Tursun, 2022), sugarcane (Chen *et al.*, 2021), and tomato (Kumari *et al.*, 2011). However, this could not be seen in this current study as the time point taken may be a bit too short to be able to see any difference in plant height.

On the other hand, oil palm seedlings not treated with SWEs and subjected to a 50% water deficit showed a reduction in RWC as compared to the controls (*Figure 2b*). The result obtained is in accordance with the findings reported by Sun *et al.* (2011) which can vary from one species to the other. However, it could be assumed that under water deficit conditions, the RWC of the seedlings applied with both *C. lentillifera* and *G. edulis* extracts can retain the water content as well as the seedlings applied with the Greenamino-G liquid fertiliser. In this study, the findings agree with what was reported by Santaniello *et al.* (2017), in which SWEs treated plants were able to maintain their RWC of up to 90% throughout the dehydration period. Increased RWC in the stressed plants after the application of seaweed indicated that plants encountered less stress due to high water status. According to Trivedi *et al.* (2017), the compound presents in SWE contributes to greater cytoplasmic water volume and cell expansion for the higher RWC. It is known that the closure of stomata acts as the primary plant defense when subjected to drought stress to save water and maintain turgor (Santaniello *et al.*, 2017; Skirycz & Inzé, 2010). Therefore, an increase in RWC was the likely consequence of stomata closure in preventing excessive water losses.

Under water deficit, the damaged photosynthetic pigments and the thylakoid membrane affect the chlorophyll content. The total chlorophyll content of seedlings under normal and water deficit conditions increased significantly by all treatments as compared to negative control under normal conditions (*Figure 2c*). The results obtained coincide with what has been reported by Latique *et al.* (2013), where bean plants treated with SWEs showed higher chlorophyll content than untreated controls. The same finding by Ghaffar *et al.* (2019) also showed that the water deficit decreased the chlorophyll content in the control treatment. It is well understood that the photosynthetic capability of plant tissues is indicated by the chlorophyll content in the leaves (Hassanzadeh *et al.*, 2009). In addition, the compounds of SWE such as betaines and cytokinin have a role in inhibiting leaf chlorophyll content degradation (Ali *et al.*, 2019).

Protein content in plants is also a good indicator of the status of plant health. The leaf protein content of the seedlings under both conditions increased significantly when applied with *G. edulis* extracts as shown in *Figure 2d*. Previously, Hidangmayum and Sharma (2017) reported that SWEs play an important role in increasing the leaf protein content of onions. The increase in leaf protein may be due to the increased availability and absorption of necessary elements (Ca, Na, K, Mg, N and Zn) present in the SWEs (Erulan *et al.* 2009; Ramya *et al.*, 2011). Low protein concentration in plants is an indicator of damage caused by stress (Bonjoch & Tamayo, 2001). Most plants under stress will activate their stress coping mechanisms which include *de novo* synthesis of stress proteins such as heat shock protein to tolerate abiotic stresses (Akhzari & Pessarakli, 2016). The accumulation of leaf-soluble proteins acts as a nitrogen reserve

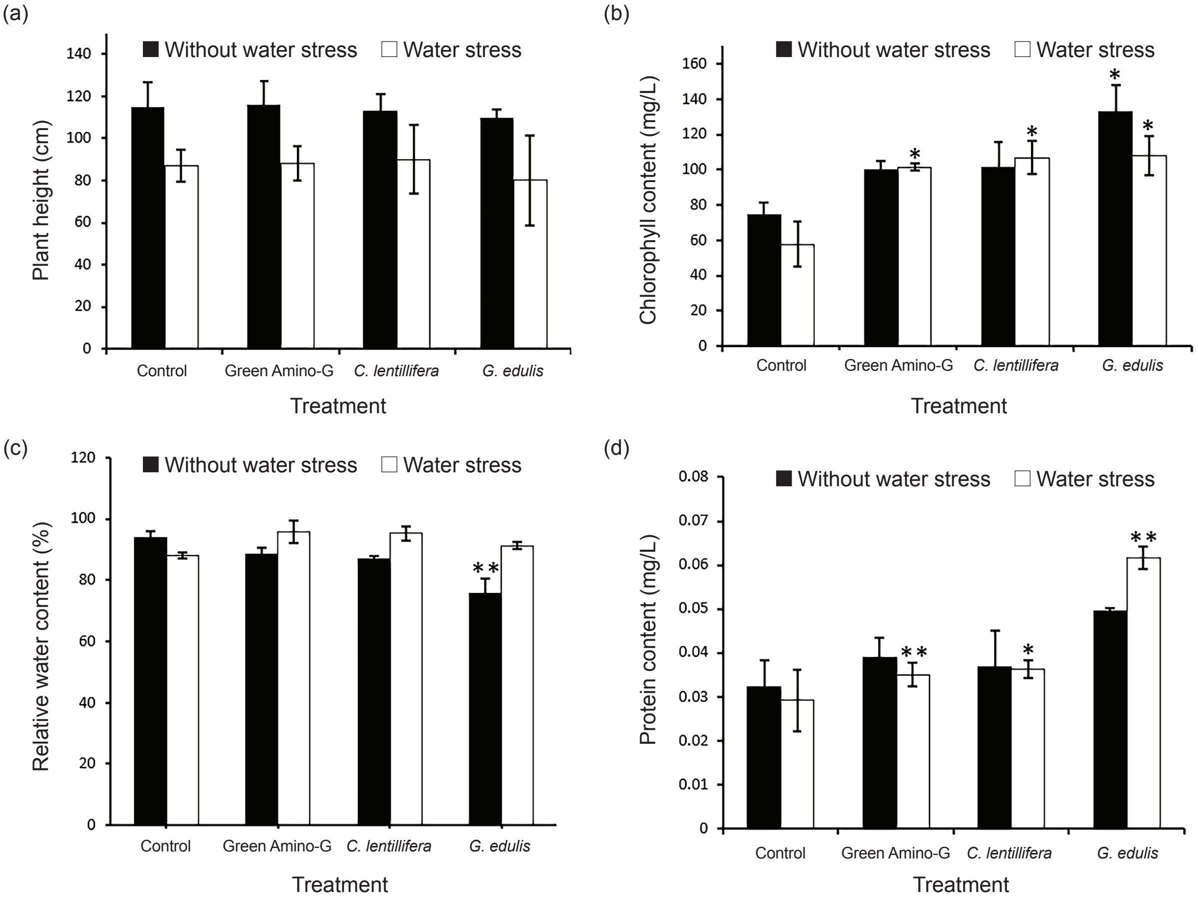

Figure 2. Effects of seaweed extract application towards (a) the plant height, (b) chlorophyll content, (c) relative water content and (d) protein content in oil palm seedlings with and without water deficit and with treatments after 60 days. Data presented are the mean ± standard deviation of three replicates with significant differences of *P<0.05, **P<0.01, *and* ***P<0.001 vs. *control, using one-way ANOVA (Dunnett post-test) on the raw data.*

which will then be used during recovery (Bonjoch & Tamayo, 2001).

Expression of THIC and THI4 Gene Transcripts

Thiamine has been postulated to have a role in protection against stress where studies have shown the upregulation of thiamine biosynthesis genes transcripts in plants treated with various biotic and abiotic stresses (Abidin *et al.*, 2016; Kamarudin *et al.*, 2017b; Wong *et al.*, 2016; Yusof *et al.*, 2015). In this study, the expression of THIC and THI4 gene transcripts were analysed from oil palm spear leaves samples with water deficit following the treatment with 0.1 g/100 mL SWE to determine if SWE may contribute to the mitigation of stress in the plants. The actin, THIC, and THI4 gene fragments were successfully amplified from samples collected on days 0, 1, and 2 post-treatment application. The expression of actin remained constant throughout the experiment (*Figure 3*). However, the expression of both THIC and THI4 gene fragments was postulated to be downregulated in oil palm seedlings under water deficit treated with SWE as the band intensity observed was lower than the control (*Figure 4a* and *4b*).

To further verify the data obtained from the gel electrophoresis, the intensity of the bands amplified was analysed using ImageJ software. The actin band amplified from all samples showed stable intensities throughout the treatment period (*Figure 5a*). Actin is a highly conserved, key cytoskeletal protein involved in numerous structural and functional roles, therefore, regardless of any condition, the actin gene will still be expressed (Ladt *et al.*, 2016). The ImageJ software analysed the gene expression by measuring the peak area under the curve which accounts for the band intensity. Each intensity of the band has different values according to their pixel value read by the ImageJ software. The expression of THIC gene transcript in oil palm seedlings under water deficit without SWE treatment increased from day 0 to day 1 indicating the effects of the stress on the seedlings (*Figure 5b*). However, on day 2, the expression for both genes started to remain constant or decreased slightly indicating the adaptation of the oil palm seedlings towards the water deficiency condition. However, the expression of THIC gene transcript in oil palm seedlings under water deficit treated with SWE showed a decrease in expression as compared to the control. There was a decrease of up to 15.00% in THIC gene expression on day 2 in oil

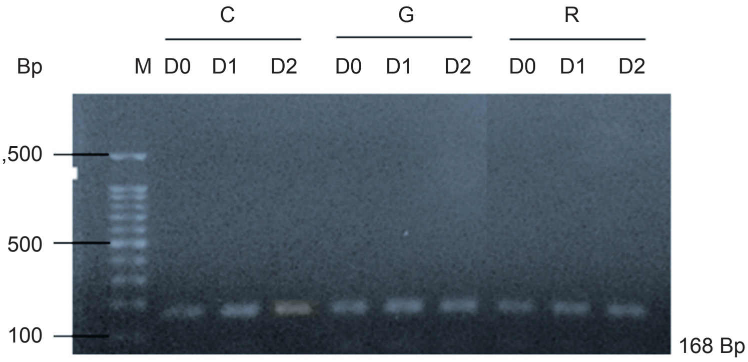

Figure 3. The expression of actin gene transcript from oil palm seedlings with and without SWE treatment from day 0 until day 2 (D0, D1, and D2). M represents 100 bp DNA ladder, C represents oil palm seedlings under water deficit without SWE treatment (control), G represents oil palm seedlings treated with C. lentillifera *(green seaweed) and R represents oil palm seedlings under water deficit treated with* G. edulis *(red seaweed).*

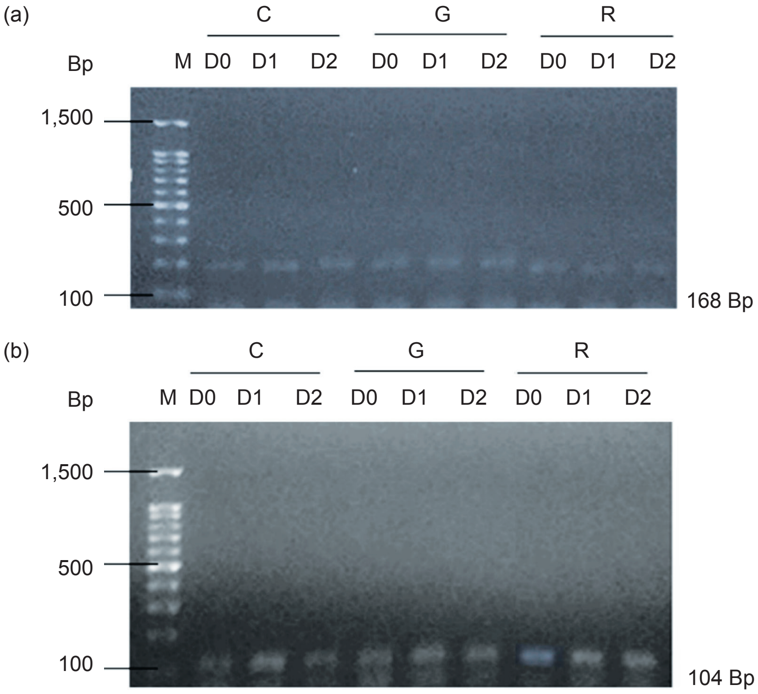

Figure 4. The expression of (a) THIC and (b) THI4 gene transcripts in oil palm seedlings under water deficit with and without SWE treatment from day 0 until day 2 (D0, D1, and D2). M represents 100 bp DNA ladder, C represents oil palm seedlings under water deficit without SWE treatment (control), G represents oil palm seedlings treated with C. lentillifera *(green seaweed) and R represents oil palm seedlings under water deficit treated with* G. edulis *(red seaweed).*

palm seedlings treated with *G. edulis*. The THI4 gene transcript also showed a somewhat similar trend where the expression showed a decrease throughout day 0 to day 2 post-treatment, with up to 15.46% decrement on day 2 in oil palm seedlings treated with *C. lentillifera* (*Figure 5c*).

A study by Abidin *et al.* (2016) and Yee *et al.* (2016) and reported that THIC and THI4 gene transcripts in oil palms increased in conjunction with increasing concentrations of osmotic, salinity and oxidative stresses. These suggest that thiamine biosynthesis is tightly modulated during stress sensing and adaptation. The increase in both genes' expression was also due to the non-cofactor role and cofactor role of thiamine in the metabolic pathways in combating abiotic stress (Goyer, 2010; Jabeen *et al.*, 2020). Thiamine is required as a cofactor in carbohydrate metabolism, NADPH, and ATP synthesis. A huge amount of energy is needed by the oil palm to combat drought stress. Thus, the expression of these two genes must be high enough to synthesise thiamine. Besides that, thiamine also induced the pathway of salicylic acid in the plant. Salicylic acid is known to provide a defense mechanism to the plant. A decrease in gene expression in stress oil palm seedlings treated with SWEs further proved that SWEs could act as a biostimulant

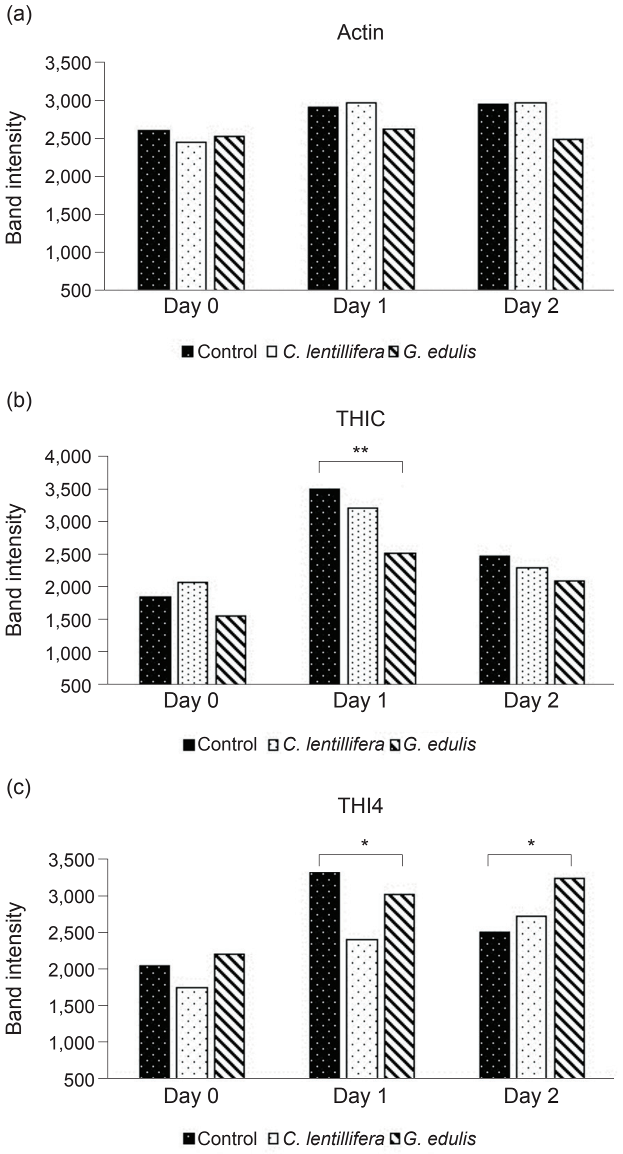

*Figure 5. Gene expression analysis of (a) actin, (b) THIC, and (c) THI4 in oil palm seedlings under water deficit with and without SWE treatment from day 0 until day 2 was analysed using ImageJ. Data presented are the mean ± standard deviation of three replicates with significant differences *P<0.05, **P<0.01, and ***P<0.001 vs. control, using one-way ANOVA (Dunnett post-test) on the raw data.*

as it helps the oil palm seedlings combat the drought stress. Thiamine possesses an antioxidant capacity as it has O^{2-}/OH^{-} scavenger properties (Ahn, 2007).

Thiamine is believed to mitigate the effect of water deficit by up-regulating thiamine biosynthesis genes in plants (Tunc-Ozdemir *et al.,* 2009). Under stress conditions, thiamine concentration is known to be high in seeds, roots, and leaves to protect the plant (Ghaffar *et al.*, 2019; Goyer, 2010; Kaya *et al.*, 2015). Particularly, THIC genes are immediately triggered by stress and become the early stress response in *Arabidopsis* plants (Rapala-Kozik *et al.*, 2012). Moreover, thiamine compounds have been proposed to act as antioxidants (Rosado-Souza *et al.,* 2020). The studies reported by Ghaffar *et al.* (2019) and Sayed and Gadallah (2002) also proved that the application of thiamine lessens the negative effects of water deficit on several plants. The upregulation of THIC and THI4 gene transcripts in oil palm seedlings under water deficit without SWE treatment was probably due to the suggested role of thiamine as a signalling molecule in plant response to biotic and abiotic stresses (Goyer, 2010). This finding is consistent with that of Fitzpatrick and Chapman (2020) who reported that the up-regulation of thiamine biosynthesis occurred under stress conditions.

On the other hand, the downregulation of THIC and THI4 gene transcripts in oil palm seedlings treated with SWE could be the stimulatory effects of the SWEs in aiding the plant to combat the water deficiency condition which caused the less requirement of thiamine. Besides, the SWEs application downregulated the expression of THIC and THI4 genes due to the ability of SWEs to reduce the effect of water deficit by stimulating the expression of stress-responsive genes, and suppressing reactive oxygen species (ROS) (Shukla *et al.,* 2019) rather than focusing on thiamine biosynthesis. Furthermore, SWE also showed a significant increase in abscisic acid (ABA) accumulation (Irani *et al.,* 2021) which play a role in inducing various gene expression that encodes important proteins for both biochemical and physiological responses.

It was suggested by Mansori *et al.* (2016) that SWEs treatment influences the ability of the water deficit plants to maintain a balance between the formation and detoxification of oxygen species, leading to amelioration of oxidative stress. A study by Abidin *et al.* (2016) and Wong *et al.* (2016) reported that the expressions of THIC and THI4 gene transcripts in oil palm increased in conjunction with increasing concentrations of osmotic, salinity, and oxidative stresses. These studies suggest that thiamine biosynthesis is tightly modulated during stress sensing and adaptation. The increase in both genes' expression was also due to the non-cofactor role and cofactor role of thiamine in the metabolic pathways (Goyer, 2010). Thiamine is required as a cofactor in carbohydrate metabolism, NADPH, and ATP synthesis. A huge amount of energy is needed by the oil palm to combat drought stress. Thus, the expression of these two genes must be high enough to synthesise thiamine. Besides that, thiamine also induced the pathway of salicylic acid in the plant. Salicylic acid is known to provide a defence mechanism to the plant. A decrease in thiamine biosynthesis gene expression in oil palm seedlings under water deficit treated with SWE further proved that SWE could act as a biostimulant as it helps the oil palm seedlings to combat the stress.

CONCLUSION

In conclusion, *C. lentillifera* and *G. edulis* described in this study have the potential in increasing the chlorophyll content, protein content, and RWC as well as downregulating the expression of the first two enzymes in the thiamine biosynthesis pathway, THIC and THI4 in oil palm seedlings under water deficit. The increment of important biomolecules, as well as water retention capabilities, may be favorable in maintaining stress tolerance in oil palm seedlings. The decrease in the expression of thiamine biosynthesis gene fragments further proved that SWEs could act as biostimulant as it helps the oil palm seedlings combat the stress. This study hoped to open up further possibilities in the exploration of the use of seaweeds as one of the strategies for maintaining the sustainability of the palm oil industry in Malaysia.

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