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Physiological responses and *P5CS* gene expression of transgenic oil palm plantlet induced by drought stress

Respons fisiologis dan ekspresi gen P5CS pada planlet kelapa sawit transgenik terhadap induksi cekaman kekeringan

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Abstrak

Kekeringan merupakan salah satu faktor pembatas dalam budidaya tanaman, seperti halnya pada kelapa sawit (Elaeis guineensis Jacq.)*. Pendekatan transgenik diharapkan mampu meningkatkan toleransi tanaman terhadap cekaman kekeringan dan meminimalisir rendahnya produktivitas saat terjadinya kekeringan. Prolin sebagai salah satu senyawa osmoprotektan pada tanaman yang biosintesisnya melibatkan gen* P5CS *dijadikan target rekayasa dalam penelitian ini. Penelitian ini bertujuan mengevaluasi tingkat ketahanan planlet kelapa sawit transgenik* P5CS *terhadap cekaman kekeringan menggunakan senyawa polietilena glikol 6000 (PEG-6000). Pada penelitian ini planlet kelapa sawit transgenik yang disisipi gen* P5CS *dan non-transgenik diperlakukan dengan PEG-6000 0, 2, dan 4% secara* in vitro*. Rancangan acak lengkap faktorial dengan tiga ulangan digunakan dalam penelitian ini. Skor tingkat kekeringan, kandungan klorofil total, kandungan karotenoid, kandungan prolin, dan ekspresi gen* P5CS *pada jaringan daun diamati pada 7 dan 14 hari setelah perlakuan cekaman. Hasil penelitian menunjukkan bahwa tanaman transgenik mempunyai skor tingkat kekeringan yang lebih rendah dibandingkan non-transgenik. Cekaman PEG-6000 pada konsentrasi 4% menurunkan kandungan klorofil total dan karotenoid yang lebih besar dibandingkan dengan konsentrasi 2% pada tanaman non-transgenik pada 7 dan 14 hari setelah perlakuan (HSP). Selain itu, tanaman transgenik mengalami peningkatan akumulasi prolin dan ekspresi gen* P5CS *selama perlakuan cekaman. Hasil ini menunjukkan bahwa transgen* P5CS *mampu meningkatkan toleransi tanaman kelapa sawit terhadap cekaman kekeringan.*

[Kata kunci: karotenoid, klorofil, kekeringan, toleransi kekeringan, prolin]

Abstract

Drought is one of the limiting factors in crop cultivation, such as in oil palm (*Elaeis guineensis* Jacq.). The transgenic approaches are expected to increase plant tolerance to drought stress and minimize low productivity when drought occurs. Proline is an osmoprotectant compound in plants which its biosynthesis involved the *P5CS* gene. The objective of this study was to evaluate the tolerance level of *P5CS*-transgenic oil palm to drought stress induced by polyethylene glycol 6000 (PEG-6000). In this present study, the transgenic and non-transgenic oil palms were treated by 0, 2, and 4% PEG-6000 under *in vitro* conditions. The experiment was arranged as a factorial completely randomized design with three replications. The drought level score, total chlorophyll content, carotenoids, and proline content, as well as *P5CS* gene expression in leaf tissues were observed at 7 and 14 days after stress treatments. The result showed that transgenic plantlets had a lower drought level score than those of non-transgenic lines. A concentration of 4% PEG-6000 treatment reduced the total chlorophyll and carotenoids contents than that of 2% concentration in non-transgenic plantlets at 7 and 14 day after treatments (DAT). In addition, proline content and *P5CS* gene expression level in transgenic had been significantly increased during stress treatment. Based on these results, it can be concluded that the *P5CS* transgene increased the drought stress tolerance of oil palm.

[Keywords: carotenoids, chlorophyll, drought, drought tolerance, proline]

Introduction

Oil palm (*Elaeis guineensis* Jacq.) is one of the important economic oil crops in the world. Oil palm has the highest yield per hectare of all oil crops, such as soy bean, rapeseed, and sunflower.

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Moreover, palm oil is the largest source of vegetable oil (Corley & Tinker, 2016). Indonesian Statistics (BPS, 2019) reported that during the year 2000-2018, the production of Indonesian oil palm increased 80.87%, while export activity of crude palm oil (CPO) increased 85.27%.

Since the drought stress has become a main problem in various crop cultivation, the breeding strategy to develop drought tolerance varieties has long been attempted, include in oil palm. Drought has negative effect on crop growth and development, and its effect varies among the severity of stress, growth stage, and genetic background (Din *et al*., 2011; Farooq *et al*., 2012; Lum *et al*., 2014; Yehouessi *et al*., 2019). Previous studies also reported that drought stress reduce the photosynthesis activity and increase the free proline level in tall fescue (*Festuca arundinacea*) (Man *et al*., 2011), sugarcane (Abbas *et al*., 2014), rice (Todaka *et al*., 2017), teak (*Tectona grandis* L.f.) (Galeano *et al*., 2019), and maize (Voronin *et al*., 2019). Effects of drought stress on morpho-physiological, biochemical, molecular level have been reported in oil palm (Cha-um *et al*., 2010; Jazayeri *et al*., 2015; Rivera-Mendes *et al*., 2016; Kaur & Asthir, 2017; Yehouessi *et al*., 2019; Fauzi & Putra, 2019; Amanah *et al*., 2019). Darlan *et al.* (2016) found that oil palm production due to drought caused by *El Nino* phenomenon in Indonesia could reach 30-60% reduction.

Drought stress tolerance in plants is one of the complex traits and controlled by many genes (Ashraf, 2010). Maintaining excess water loss during stress through osmoprotectants or compatible solutes known as a tolerance strategy to drought. Proline accumulation is an adaptive response and also known as an osmoregulatory solute in plants under hyperosmotic stresses, such as drought (Iskandar *et al*., 2014). The Δ^1-pyrroline-5-carboxylate synthetase (P5CS) is a rate limiting enzyme that has a role in proline biosynthesis which is encoded by *P5CS* gene (Kishor *et al*., 2005). Proline also reported as a biochemical marker under drought stress in plants (Toruan-Mathius *et al*., 2004; Ashraf, 2010; Fichman *et al*., 2015; Zarattini & Forlani, 2017). In addition, drought stress has also been reported to interfere the photosynthetic system of plants as indicated by decreasing of chlorophyll content (Din *et al*., 2011; Jazayeri *et al*., 2015). Besides reducing chlorophyll, drought stress also reduces carotenoids due to the increase of reactive oxygen species production (Mibei *et al*., 2017). As common non-enzymatic antioxidant, carotenoids protect cells from excess damage during the stress (Ghobadi *et al*., 2013). Chorophyll and carotenoids also reported as useful traits for identification the drought tolerance level of plants (Talebi *et al*., 2013).

The availability of drought tolerant of oil palm plant material is needed. One of strategy that can be conducted to obtain drought tolerant of oil palm is *via* genetic engineering. Transformation and genetic engineering in plants using the *P5CS* gene has been carried out on various plants, i.e. tobacco (Riduan *et al*., 2010; Zarei *et al*., 2012), sugarcane (Minarsih *et al*., 2015), wheat (Pavei *et al*., 2016), and oil palm (Budiani *et al*., 2019). Furthermore, Budiani *et al.* (2019) successfully developed *P5CS*-transformed oil palm embryonic calli into plantlets but these plantlets have not been evaluated yet under certain stress conditions, especially to drought stress. In the present study, we evaluated the physiological responses and *P5CS* gene expression of transgenic oil palm plantlets under drought stress using PEG-6000. The findings could be useful and become a basis knowledge for improving oil palm productivity under related water deficit stress.

Materials and Methods

Plant materials

The *P5CS*-transgenic oil palm (Tenera) plantlets on Murashige-Skoog culture medium under room with controlled temperature of 25 °C, with 20-25 cm height and 4-6 leaves were used in this study. The pBI-P5CS construct containing *P5CS* gene-encoded Δ^1-pyrroline-5-carboxylate synthetase enzyme from *Vigna aconitifolia* (Figure 1) was kindly provided by Dr. Desh Pal S. Verma (Department of Molecular Genetics and Plant Biotechnology Center, The Ohio State University, USA) (Minarsih *et al*., 2001) and were then inserted to oil palm (*Elaeis guineensis* Jacq.) by Budiani *et al*. (2019).

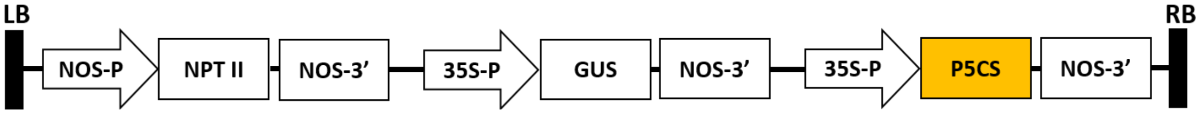


Figure 1. Construct map of pBI plasmid containing *P5CS* gene (*pBI-P5CS*)
Gambar 1. Peta konstruksi plasmid pBI dengan gen P5CS (pBI-P5CS)

Experiment conditions

The experiment was conducted using Murashige-Skoog culture medium containing PEG-6000 as a drought treatment. There were three treatments in this study, i.e. 0% PEG-6000 (-0.24 MPa) as control, 2% PEG-6000 (-0.98 MPa), and 4% PEG-6000 (-2.52 MPa) as stress conditions according to Cha-um *et al.* (2010). These treatments were exposed to non-transgenic and *P5CS* transgenic plantlets with the criteria as described above. The experiment was arranged as a factorial completely randomized design with three replications (n = 3). Drought level score, total chlorophyll-, carotenoid-, proline content, and *P5CS* gene expression were measured in this study. These parameters were observed at 7 and 14 DAT.

Drought level score determination of oil palm under PEG-6000-induced stresses

The level of PEG-6000-induced drought was represented as a score of 0 (without drying area on the leaves) to 9 (the plant died) according to the leaf drying area at the vegetative stage (IRRI, 2002). The drought level score determination was done at 1, 7, and 14 DAT.

Total chlorophyll and carotenoid content determination

The total chlorophyll and carotenoid contents were extracted by acetone 80%. Briefly, 100 mg of fresh leaf from treated plants were ground in 10 mL of cold acetone 80% (v/v) for total chlorophyll and carotenoid pigments extraction. The extract was centrifuged at 3000g at 4 °C for 15 minutes (Turhadi *et al.*, 2019). The supernatant was read at 470, 663, and 646 nm using Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific Inc., USA). The chlorophyll and carotenoid were determined according to a formula by Lichtenthaler (1987) at 7 and 14 DAT in three plantlets in each treatments.

Proline content determination

Proline content was determined according to Bates *et al.* (1973). Briefly, 0.25 g of fresh leaf were ground using 5 mL of sulfosalicylic acid 3% (w/v). The extracts were centrifuged at 10000 rpm at 25 °C for 10 minutes. About 2 mL of supernatant was mixed with 2 mL of acid-ninhydrin reagent (1.25 g ninhydrin in 30 mL of glacial acetic acid and 20 mL of 6 M phosporic acid) and 2 mL of glacial acetic acid. The mixture was then incubated at 100 °C for 1 hour and immediately soaked in ice water. The filtrate was extracted using 4 mL toluene and vortexed. The proline content of leaves which harvested on 7 and 14 DAT was determined at λ 520 nm using Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific Inc., USA).

RNA isolation and quantitative real time-polymerase chain reaction (qRT-PCR)

P5CS gene expression level was done at 7 and 14 DAT. Total RNA was isolated from leaf tissues of treated plants using TRIzol™ *reagent* (Invitrogen, USA) according to the manufacturer's protocols. RNAs were treated with the DNase I kit (Sigma-Aldrich®, USA) to remove contaminating genomic DNA according to the manufacturer's protocols. The cDNAs were then synthesized using AccuPower® CycleScript RT Premix (dT20) (BIONEER, USA) according to the manufacturer's protocols. The synthesized cDNAs were used for the *P5CS* gene expression level by qRT-PCR. The *P5CS* primers used in the expression analysis was designed based on *P5CS* gene sequence of *Vigna aconitifolia* with Genbank accession no. M92276.1. The qRT-PCR in each sample was performed in triplicates using gene-specific primers (Table 1) and SensiFAST™ SYBR® Hi-ROX (Bioline, USA) according to the manufacturer's protocols. The *Actin* primer used for this quantification as a reference gene (Table 1). The expression level of the *P5CS* gene was quantified by $2^{-\Delta\Delta Ct}$ formula (Schmittgen & Livak, 2008).

Statistical analysis

Data were statistically analyzed using analysis of variance (ANOVA) (α=0.05) and correlation analysis (α=0.05). Analysis of variance was performed in SPSS 16.0 program (SPSS Inc., Chicago, IL, USA), while correlation analysis was performed and visualized in R-Studio program using corrplot package.

Table 1. Primers used in this study
Tabel 1. Primer yang digunakan pada penelitian ini

Primer identity *Identitas primer*	Sequences *Sekuen*
Actin-F	CCCACCTGAACGGAAATACA
Actin-R	CGGATGGCACCTCAGTCTTA
qRT-P5CS-F	CGGTTGGAAGATTGGGAGCT
qRT-P5CS-R	TTGGGGTTTCTGAAGGTCGG

Results and Discussion

Drought level score of non- and transgenic oil palm under polyethylene glycol 6000-induced stresses

Non- and transgenic plantlets demonstrated different responses to drought stress treatment using PEG-6000 in this present study. Leaves of non-transgenic plantlets showed drought response in 4% PEG-6000 at 7 DAT. The increase of PEG-6000 concentration and longer duration of treatment caused an increase of drought symptom especially in non-transgenic oil palm plantlets as shown in Figure 2.

The drought level between transgenic and non-transgenic plantlet leaves showed clearly different at 14 DAT under 4% PEG-6000 treatment. The leaves of the transgenic plantlets showed mostly green (average score = 1.0), while non-transgenic plantlets showed brown color or dry (average score = 5.0) (Figure 3). Lower scores indicated low level of drought stress and *vice versa*. These results indicated that *P5CS*-transgenic oil palm had higher tolerance level to drought stress than non-transgenic.

Total chlorophyll, carotenoids, and proline content in transgenic oil palm

The response of *P5CS*-transgenic oil palm to drought stress conditions was demonstrated by measuring several physiological parameters, i.e. total chlorophyll-, carotenoids-, and proline content in leaf tissues (Figure 4a-d & 5). Total chlorophyll content significantly ($p<0.05$) decreased within interaction between genotypes and PEG-6000 concentration (Figure 4a). Transgenic plantlets showed higher total chlorophyll content than that of non-transgenic under 2 and 4% PEG-6000 (Figure 4a). Total chlorophyll content of non-transgenic plantlets decreased by 61% in compared to the control at 14 DAT (Figure 4b). Conversely, the transgenic plantlets were able to maintain the total chlorophyll content even under PEG-6000 stresses. The total chlorophyll content in transgenic plantlets under 4% PEG-6000 showed an increase of 31% and 15% at 7 and 14 DAT, respectively. Based on these responses indicated that *P5CS* transgene from *Vigna aconitifolia* increased the oil palm tolerance level to drought stress.

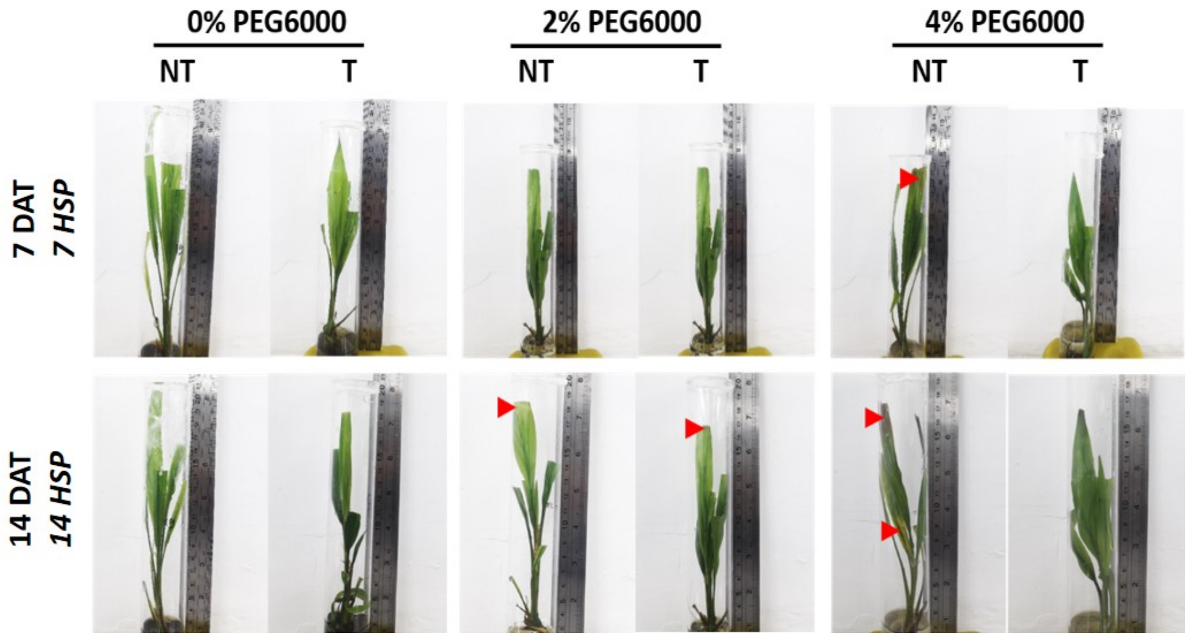


Figure 2. Leaf profiles of oil palm plantlets in 0, 2, and 4% PEG-6000 during 14 days of *in-vitro* treatment. NT = non-transgenic; T = transgenic. DAT = day after treatment; 7 and 14 = duration of treatment. Red arrows showed a drought leaf symptom

Gambar 2. Profil daun planlet kelapa sawit pada PEG-6000 0, 2, dan 4% selama 14 hari perlakuan secara in-vitro*. NT = non-transgenik; T = transgenik. HSP = hari setelah perlakuan; 7 dan 14 = lama perlakuan. Tanda panah warna merah menandakan gejala daun yang mengering*

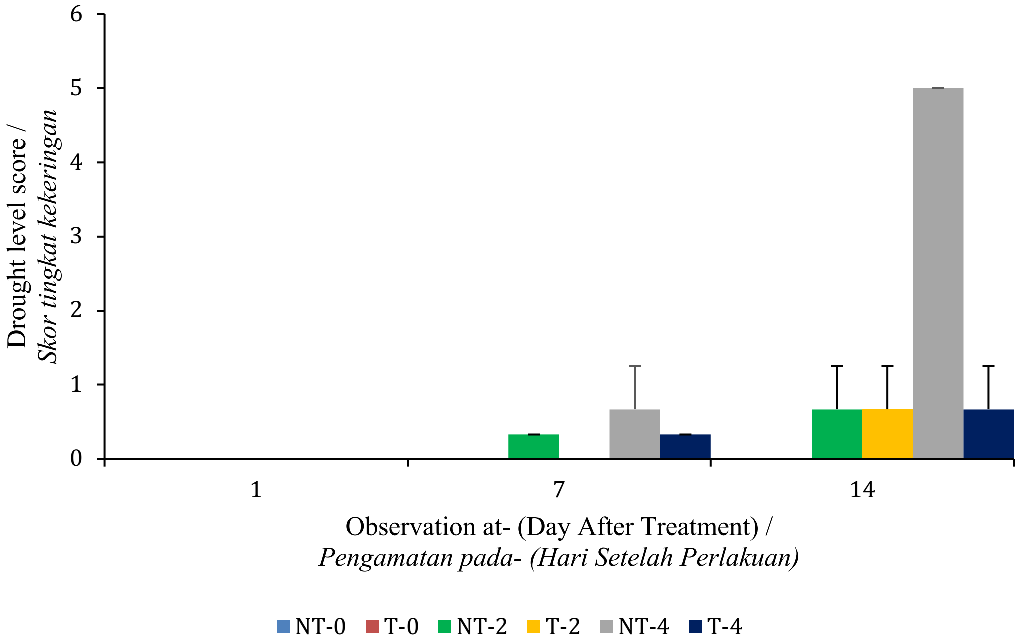


Figure 3. Drought level score of oil palm plantlets in 0, 2, and 4% PEG-6000 during 14 days of *in-vitro* treatment. NT = non-transgenic; T = transgenic. 1, 7, and 14 = duration of treatment

Gambar 3. Skor tingkat kekeringan planlet kelapa sawit pada PEG-6000 0, 2, dan 4% selama 14 hari perlakuan secara in-vitro. NT = non-transgenik; T = transgenik. 1, 7, dan 14 = lama perlakuan

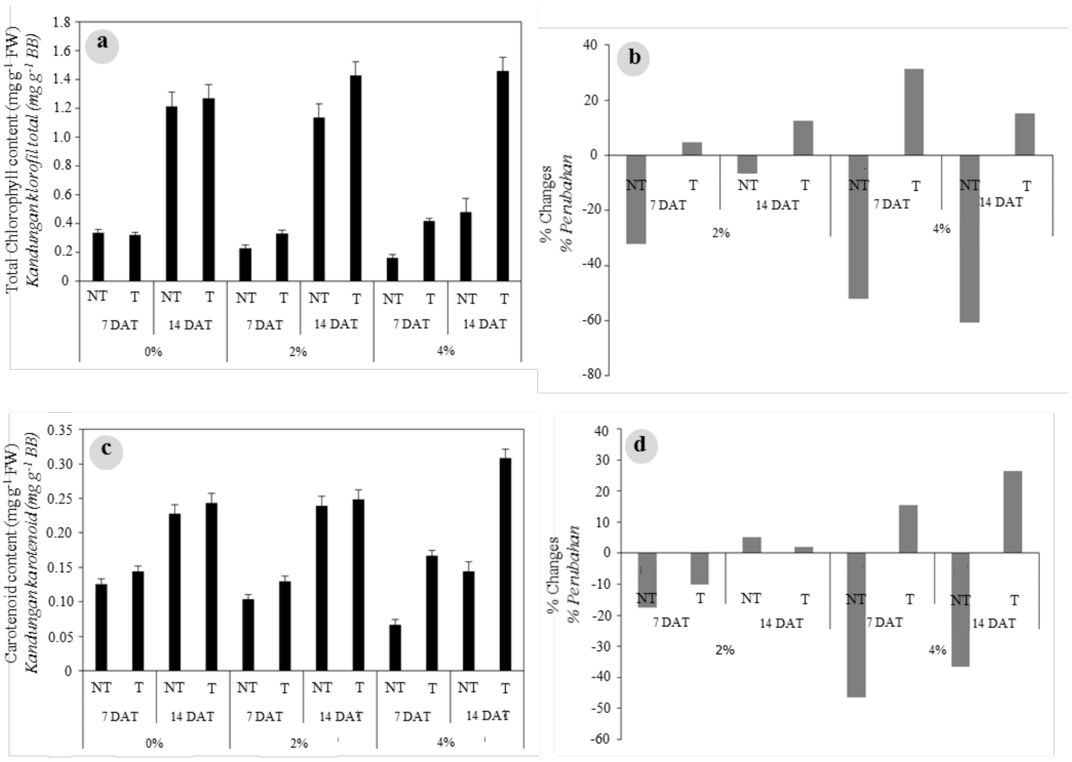


Figure 4. Total chlorophyll (a-b) and carotenoids (c-d) contents of oil palm plantlets in 0, 2, and 4% PEG-6000 during 14 days of *in-vitro* treatment. NT = non-transgenic; T = transgenic. DAT = day after treatment; 7 and 14 = duration of treatment. % changes means percentage of increase/decrease in each parameter that calculated using formula = (PEG treatment – control)/control x 100%

Gambar 4. Kandungan klorofil total (a-b) dan karotenoid (c-d) planlet kelapa sawit pada PEG-6000 0, 2, dan 4% selama 14 hari perlakuan secara in-vitro. *NT = non-transgenik; T = transgenik. HSP = hari setelah perlakuan; 7 dan 14 = lama perlakuan. % perubahan berarti persentase peningkatan/penurunan pada setiap peubah yang dihitung menggunakan rumus = (perlakuan PEG – kontrol)/kontrol x 100%*

The decreasing of oil palm's chlorophyll content under PEG-6000 treatment using *in-vitro* conditions also reported in previous research studies (Cha-um *et al.,* 2010, 2012). Moreover, the decreasing of chlorophyll content under drought stress also reported in oil palm using watering intensity treatments (Cha-um *et al*., 2013; Azzeme *et al*., 2016). In this present study, the decreasing of total chlorophyll content under PEG-6000 treatment was suggested due to the damage of chloroplast organelle during stress.

Beside of total chlorophyll content, the increase of tolerance level of transgenic oil palm plantlets was also shown in carotenoids profile content. There was significantly ($p<0.05$) interaction between genotypes and PEG-6000 concentration to carotenoids content of oil palm plantlets. The carotenoids content of transgenic plantlets under 4% PEG-6000 higher than that control and 2% PEG-6000 (Figure 4c). Our result showed that carotenoids content of transgenic plantlets under 4% PEG-6000 increased by 16% and 26% at 7 and 14 DAT, respectively. Conversely, the carotenoids content of non-transgenic plantlets under 4% PEG-6000 decreased by 47% and 37% at 7 and 14 DAT, respectively (Figure 4d).

The increase of carotenoids content in transgenic plantlets suggested of having a role in the tolerance mechanism of oil palm to drought stress. Besides as light-harvesting pigment, carotenoids also have a role as chlorophyll protecting pigment and protect the chloroplast from photooxidative damage as well (Wang *et al*., 2014; Das & Roychoudhury, 2014). Moreover, drought is an abiotic stress that produces reactive oxygen species (ROS). According to Das & Roychoudhury (2014) carotenoids as pigment that produced in chloroplast and other organelles (non-green plastids) involved in the non-enzymatic detoxification of ROS.

Oil palm transgenic significantly ($p<0.05$) had higher proline content than that non-transgenic plantlets (Figure 5a). In addition, the proline content also increased during the stress especially, in the transgenic plantlets. A higher increase of proline content was shown by transgenic compared to non-transgenic plantlets (Figure 5b). The proline content of transgenic under 4% PEG-6000 at 14 DAT increased by 273%, while in non-transgenic plantlets increased by 102%.

The presence of *P5CS* transgene that controlled by 35S-CaMV promoter caused the increase of proline content in oil palm transgenic plantlets during stress. Toruan-Mathius *et al.* (2004) stated that proline content is one of the biochemical marker of drought stress in oil palm. In our present study, the proline content in transgenic plantlets sharply increased during the stress. Borgo *et al.* (2015) stated that proline accumulation is related to the tolerance level of *Vigna aconitifolia* under drought stress conditions. Proline is suggested to be involved in the chloroplast protection by quenching reactive oxygen species in *Arabidopsis thaliana* (Moustakas *et al*., 2011).

Various studies found that proline content also increased after treated with drought stress. The *P5CS*-transgenic tobacco showed increase of proline content compared non-transgenic ranged 3618 - 4449 and 2044 µg/g fresh weight, respectively (Riduan *et al*., 2010). The increase of proline content in those transgenic plants improved the tolerance level to drought stress.

P5CS *gene expression in transgenic oil palm increased during stresses*

The increase of proline content was in line with the increase of *P5CS* gene expression. The increase of *P5CS* gene expression in transgenic plantlets under 2 and 4% PEG-6000 was higher than that of in non-transgenic plantlets (Figure 6). This result supported the hypothesis about the tolerance level of transgenic oil palm related to the increase of proline accumulation. According to Fichman *et al.* (2015) Δ^1-pyrroline-5-carboxylate synthetase which is encoded by *P5CS* is one of the key enzyme in proline biosynthesis. The increase of proline content also reported that no toxic effect to mitochondria and chloroplast ultrastructure of *VaP5CS129A* transgenic plants under 12 days of water deficit conditions (Borgo *et al*., 2015).

Relationship between physiological and P5CS *gene expression level in oil palm during PEG-6000 stress treatment*

Carotenoids significantly ($p<0.05$) and positively correlated with chlorophyll content at 7 and 14 DAT. In addition, the *P5CS* gene expression at 7 and 14 DAT also showed positive correlation with proline content (Figure 7). Bandurska *et al.* (2017) also reported positive correlation between proline content and activity of P5CS protein in barley (*Hordeum vulgare* L.) under drought stress. Our present study showed a significantly negative correlation ($p<0.05$) between leaf drought level score and chlorophyll content (Figure 7). As a consequence of drought level increased, the chlorophyll content of oil palm in leaves tissues decreased.

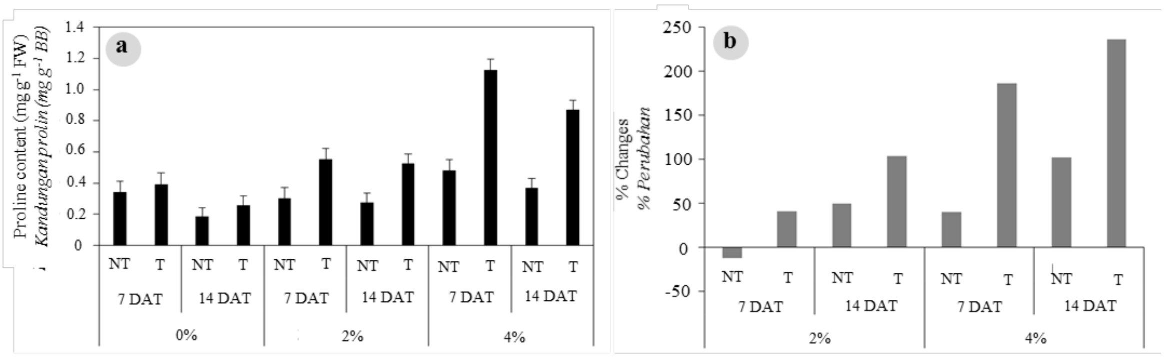


Figure 5. Proline content of oil palm plantlets in 0, 2, and 4% PEG-6000 during 14 days of *in-vitro* treatment. NT = non-transgenic; T = transgenic. DAT = day after treatment; 7 and 14 = duration of treatment. % changes means percentage of increase/decrease in each parameter that calculated using formula = (PEG treatment – control)/control x 100%

Gambar 5. Kandungan prolin planlet kelapa sawit pada 0, 2, dan 4% PEG-6000 selama 14 hari perlakuan secara in-vitro. *NT = non-transgenik; T = transgenik. HSP = hari setelah perlakuan; 7 dan 14 = lama perlakuan. % perubahan berarti persentase peningkatan/penurunan pada setiap peubah yang dihitung menggunakan rumus = (perlakuan PEG – kontrol)/kontrol x 100%*

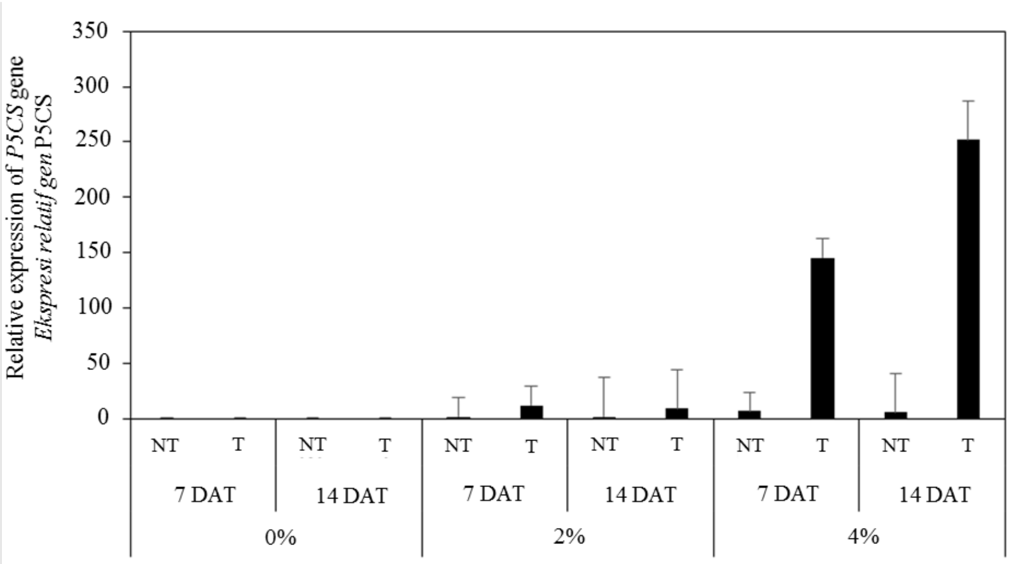
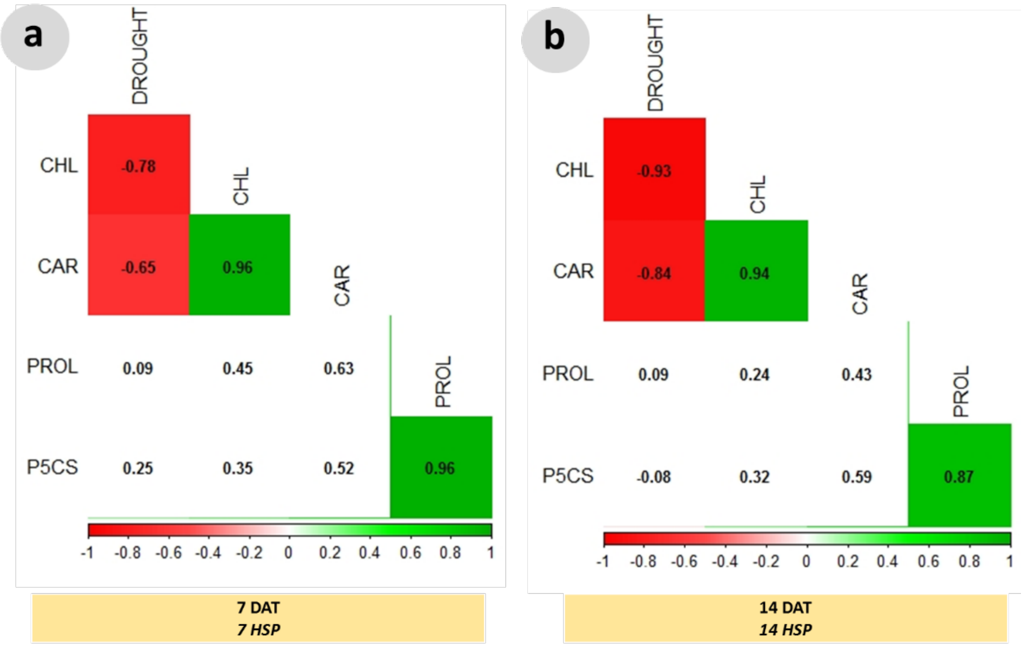


Figure 6. *P5CS* gene expression level of oil palm plantlets in 0, 2, and 4% PEG-6000 during 14 days of *in-vitro* treatment. NT = non-transgenic; T = transgenic. DAT = day after treatment; 7 and 14 = duration of treatment

Gambar 6. Ekspresi relatif gen P5CS *planlet kelapa sawit pada 0, 2, dan 4% PEG-6000 selama 14 hari perlakuan secara* in-vitro. *NT = non-transgenik; T = transgenik. HSP = hari setelah perlakuan; 7 dan 14 = lama perlakuan*



7 DAT
7 HSP

14 DAT
14 HSP

Figure 7. Pearson's correlation coefficient between physiological parameter and *P5CS* gene expression in oil palm plantlets under PEG-6000 treatment during 7 (a) and 14 (b) days. DROUGHT = drought level score; CHL = total chlorophyll content; CAR = Carotenoids content; PROL = proline content; P5CS = *P5CS* gene expression. Correlation analysis was performed at α = 0.05. Red colour indicates significantly negative correlated; Green colour indicates significantly positive correlated; Without colour indicates non-significantly correlated. DAT = day after treatment; 7 and 14 = duration of treatment

Gambar 7. Koefisien korelasi Pearson antara peubah fisiologi dan ekspresi gen P5CS planlet kelapa sawit pada perlakuan PEG-6000 selama 7 (a) dan 14 (b) hari. DROUGHT = skor tingkat kekeringan; *CHL = kandungan klorofil total; CAR = kandungan karotenoid; PROL = kandungan prolin; P5CS = ekspresi gen* P5CS. *Analisis korelasi dilakukan pada α = 0,05. warna merah menandakan signifikan berkorelasi secara negatif; warna hijau menandakan signifikan berkorelasi secara positif; tanpa warna menandakan tidak berkoreasi secara signifikan. HSP = hari setelah perlakuan; 7 dan 14 = lama perlakuan*

Conclusion

Drought stress induced by PEG-6000 resulted physiological changes in oil palm transgenic plantlets showing improved drought tolerance potential. The 4% PEG-6000 clearly showed different response between *P5CS*-transgenic and non-transgenic oil palm plantlets on their leaves drought level score. The drought stress during 14 days treatment significantly decreased chlorophyll and carotenoids content in non-transgenic plantlets, while in transgenic planlets. The *P5CS*-transgene increased the tolerance level of oil palm under PEG-6000 stress as shown by the increasing of proline accumulation and *P5CS* gene expression in leaf tissues.

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