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Effect of Forchlorfenuron on Somatic Embryo Proliferation and Plantlet Regeneration in Oil Palm SUP-PSU1

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Abstract

In vitro propagation of palm oil is still an obstacle for mass propagation due to the low plant regeneration frequency. The objective of this study was to investigate the effect of N-(2-chloro-4-pyridyl)-N-phenylurea (CPPU) on a somatic embryo (SE) proliferation and plant regeneration of oil palm SUP-PSU1. At an approximate size of 5 mm, SE from chopping with a sharp razor blade at 100 times was cultured on oil palm culture medium (OPCM) with 0.1 mg/L dicamba and different concentrations CPPU. The results showed that 0.3 mg/L CPPU gave the highest SE proliferation at 100 % and the number of SEs at 5.16 embryos/tube after 4 weeks of culture. SE was transferred to Murashige and Skoog (MS) medium supplemented with 0.2M sorbitol, a secondary somatic embryo (SSE), which was induced at 66.67 % with 21.2 embryos/tube after 8 weeks. Furthermore, cultured SSE on PGR-free MS medium for 8 weeks gave the highest germination rate at 65 %, with the number of shoots at 2.29 shoots. Thus, this study provides new information for improving the plantlet regeneration system through somatic embryogenesis in oil palm.

Keywords: Oil palm SUP-PSU1, N-(2-chloro-4-pyridyl)-N-phenylurea (CPPU), Somatic embryo, Plantlet regeneration

Introduction

Somatic embryogenesis is an alternative micro-propagation method that provides a high multiplication rate in many plant species in monocotyledonous and dicotyledonous plants. It has several distinct advantages compared with organogenesis [1]. Somatic embryogenesis is the process by which somatic cells differentiate into somatic embryos (SEs). SEs are morphologically resembled zygotic embryos. The process started from a single cell with a high meristematic activity, which developed into globular, heart, torpedo, and cotyledonary embryos [2]. They are bipolar and bear typical embryonic organs, radicle, hypocotyl, and cotyledons [3]. Most SEs in the woody plant species pass through the same stages of development as that zygotic embryo does [4].

Oil palm (*Elaeis guineensis* Jacq.) is a perennial monocotyledonous plant that yields up to 7 - 10 times higher than any other annual oil seed crop [5]. Plant regeneration via somatic embryogenesis in oil palm has been reported using several asexual explants such as young leaf [6], young inflorescence [7], and root [8]. However, the success of the process depends on several factors that need to study in individual plant species.

Plant growth regulator (PGR) is a major factor in improving somatic embryogenesis in monocotyledonous and dicotyledonous plants. There are 2 types of PGRs commonly used in tissue cultures, such as auxin and cytokinin. The success of somatic embryogenesis by the combination of those PGRs has been reported. Cytokinin is categorized into 2 classes; naturally occurring N6-substituted

adenine derivatives and the synthetic phenylurea derivatives such as diphenylurea (DPU), N-phenyl-N'-1,2,3-thiadiazol-5-ylurea (thidiazuron, TDZ) and N-(2-chloro-4-pyridyl)-N-phenylurea (CPPU) [9]. CPPU, commonly known as forchlorfenuron is 1 type of cytokinin in the form of DPU with higher activity induction in the induction of somatic embryogenesis than adenine type derivatives [9]. This chemical was reported to promote a high induction of somatic embryogenesis in citrus [10], grape [11], and peanut [12]. In addition, this cytokinin has been reported to cause the accumulation of other cytokinins in plant tissue [9]. To date, there is no report on somatic embryo induction using CPPU in oil palm. Thus, this study is the first report on the chopping of SE, followed by culturing on culture medium containing CPPU in combination with dicamba to increase SE's high proliferation after plant regeneration of oil palm SUP-PSU1.

Materials and methods

Plant material

Embryogenic callus (EC) induced from culturing zygotic embryo (ZE) of superior oil palm SUP-PSU1 (25) C3/77 at Crop Biotechnology Laboratory, Agricultural Innovation and Management Division, Faculty of Natural Resources, Prince of Songkla University was used as starting plant material. For EC proliferation, the callus was transferred to oil palm culture medium (OPCM) supplemented with 0.1 mg/L dicamba and 200 mg/L ascorbic acid and subcultured at monthly intervals and maintained at 26 ± 2 °C under 14 h photoperiod (15 μmol/m^2/s) provided by cool-white fluorescent lamps for 4 weeks.

Culture media and conditions

Culture medium used in all the experiments was OPCM supplemented with 200 mg/L ascorbic acid, 3 % (w/v) sucrose and 0.75 % (w/v) agar. The medium was adjusted to pH 5.7 prior to autoclaving at 1.05 kg/cm^2, 121 °C for 15 min. All cultures were placed at 26 ± 2 °C under 14 h photoperiod (15 μmol/m^2/s) provided by cool-white fluorescent lamps.

Effect of concentrations of CPPU on SE formation

One gram fresh weight (gFW) of SE was chopped with sterile scalpel at 100 times. Then, 0.1 gFW tissues of each sample was transferred to OPCM supplemented with different concentrations of CPPU (0-0.5 mg/L) in combination with 0.1 mg/L dicamba. After 4 weeks of culture, fresh weight of EC, induction frequency of SE, numbers and characteristics of SEs were recorded. Completely randomized design (CRD) with 5 replications was performed. Each replication consists of 5 test tubes and means among treatments were compared by Duncan's multiple range test (DMRT) at 1 or 5 % probability.

Secondary somatic embryo (SSE) induction

SEs at approximate size of 5 mm diameter derived from medium with or without CPPU in experiment 1 were excised and transferred to MS medium supplemented with 0.2M sorbitol for 8 weeks. At this period of culture, frequency of SSE induction and number of SSEs per cultured SE were recorded. CRD was designed with 5 replications. Each replication consists of 10 test tubes and means between treatments were separated by T-test at 1 or 5 % probability.

Conversion of SSE to plantlet

SEs from only 0.1 mg/L dicamba or in combination with 0.3 mg/L CPPU containing OPCM obtained from experiment 1 were transferred to MS medium supplemented with 0.2M sorbitol for 8 weeks to induce SSEs. Induced SSEs were subsequently transferred to MS or OPCM without PGRs for 8 weeks to germinate. Conversion or germination rate of SSE and the number of shoots produced from SSE were recorded. The CRD with 5 replications was designed. Each replication consists of 5 test tubes and means among treatments were separated by DMRT at 1 or 5 % probability.

Statistical analysis

The experimental data were analyzed using R 2.14.0 software. The data were subjected to one-way analysis of variance (ANOVA).

Table 1 Effect of different concentrations of CPPU on SE proliferation from chopped SE of oil palm SUP-PSU1 cultured on OPCM with 0.1 mg/l dicamba and 200 mg/l ascorbic acid for 4 weeks.

Concentrations of CPPU (mg/L)	Avg. EC FW (mg/tube)	SE proliferation (%)	Avg. no. of SEs (embryos/tube)	Avg. size of SEs (mm)
0	366 ± 12.03	75 ± 0.00c	1.64 ± 0.36c	3.1 ± 0.05
0.1	360 ± 20.21	100 ± 0.00a	3.09 ± 0.45abc	3.1 ± 0.01
0.2	335 ± 5.69	91 ± 0.57b	4.39 ± 0.96ab	3.7 ± 0.01
0.3	315 ± 48.07	100 ± 0.00a	5.16 ± 1.20a	3.8 ± 0.02
0.4	336 ± 27.67	90 ± 0.33b	2.02 ± 0.50bc	3.4 ± 0.04
0.5	317 ± 24.70	100 ± 0.00a	2.48 ± 0.38bc	3.3 ± 0.02
F-test	ns	**	*	ns
C.V. (%)	13.65	0.5	39.81	17.55

Ns = not significantly different
* significantly different ($p \leq 0.05$), ** significantly different ($p \leq 0.01$)
Mean values followed by the same letter within column are not significantly different according to DMRT.

Results and discussion

Effect of concentrations of CPPU on SE formation

Chopping SE with a sharp razor blade at 100 times subsequent to culture on OPCM without CPPU gave the highest FW of EC at 366 mg/tube. However, the highest SE proliferation at 100 % was obtained on 0.1, 0.3 and 0.5 mg/L CPPU containing medium, significantly different with 0.2, 0.4 and 0 mg/L CPPU that gave the SE proliferation at 91, 90 and 75 %, respectively (**Table 1**). Medium containing 0.3 mg/L CPPU gave the highest number and size of SEs at 5.16 embryos/tube and 3.8 mm after 4 weeks of culture, respectively (**Table 1**). The characteristics of SEs were dark green, while EC showed friable yellow with some browning (**Figure 1**).

Chopped SEs could develop into new SEs while some tissues developed into callus after 4 weeks of culture. Unfortunately, CPPU containing medium caused browning of tissue or callus (**Figure 1**). Interestingly, 0.3 mg/L CPPU in the culture medium produced the least of browning compared to other concentrations.

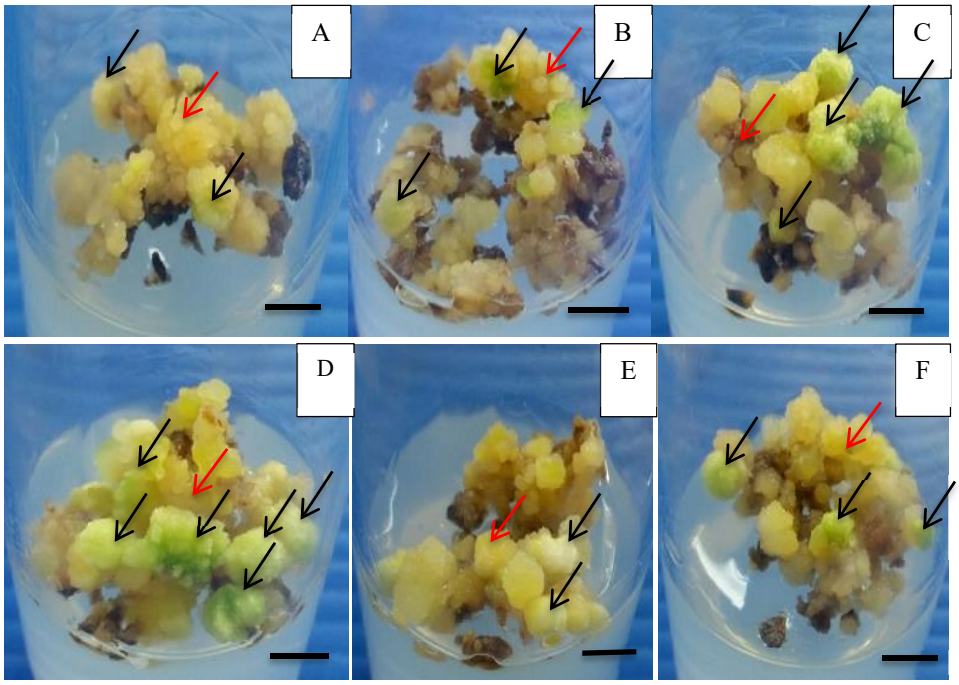

Figure 1 Characteristics of EC (red arrows) and SEs (black arrows) obtained from chopped SEs of oil palm SUP-PSU1 cultured on OPCM containing 0.1 mg/L dicamba, 200 mg/L ascorbic acid and different concentrations of CPPU for 4 weeks (bar = 0.5 cm).
A. 0 mg/L CPPU B. 0.1 mg/L CPPU C. 0.2 mg/L CPPU
D. 0.3 mg/L CPPU E. 0.4 mg/L CPPU F. 0.5 mg/L CPPU

Effect of CPPU on SSE induction

After transferring SE from OPCM containing 0.1 mg/L dicamba with or without 0.3 mg/L CPPU to MS medium with 0.2M sorbitol, significant difference ($p \leq 0.01$) in both induction frequency and number of SSEs were observed. CPPU at 0.3 mg/L containing OPCM medium gave the highest SSE induction frequency at 66.67 % and number of SSEs at 21.2 embryos/tube. In contrast, only 53.33 % of SSE induction and 8.13 embryos/tube were obtained from medium without CPPU (**Table 2** and **Figure 2**).

Table 2 Effect of CPPU on SSE formation of oil palm SUP-PSU1 cultured on MS medium supplemented with 0.2 M sorbitol after 8 weeks of culture.

Treatments	**SSE induction (%)**	**Avg. no. of SSEs (embryos/tube)**
0.1mg/L dicamba + 0 mg/L CPPU	53.33 ± 3.33	8.13 ± 0.67
0.1mg/L dicamba+ 0.3 mg/L CPPU	66.67 ± 0.00	21.2 ± 0.96
T-test	**	**
C.V. (%)	8.78	12.73

** significantly different ($p \leq 0.01$) according to T-test.

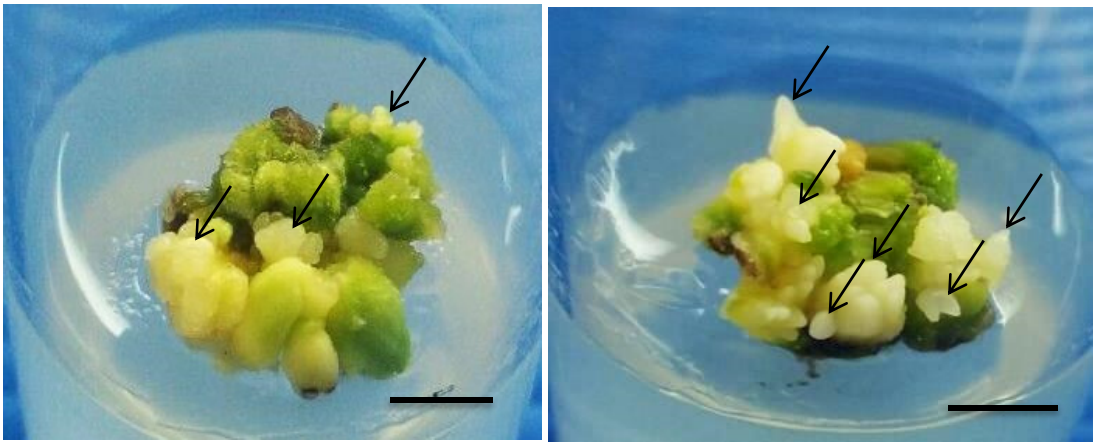

Figure 2 Characteristics of SSEs (arrows) derived from culturing SE of oil palm SUP-PSU1 on OPCM supplemented with 0.1 mg/L dicamba (left) or in combination with 0.3 mg/L CPPU (right) for 4 weeks and subsequent to MS medium with 0.2M sorbitol for 8 weeks (bar = 0.5 cm).

SSE germination

SSEs derived from sorbitol containing MS medium were germinated at different germination rate on the MS and OPCM. SSEs induced from CPPU containing medium gave higher germination rate than those from culture medium without CPPU. MS medium without PGRs promoted higher germination rate than that of OPCM. SSEs induced from transferring SEs on CPPU containing OPCM to sorbitol containing MS germinated gave the highest germination rate at 65 % on PGRs-free MS medium, significantly different ($p \leq 0.01$) with another treatment. Similar result was also obtained from average number of shoots. PGR-free MS medium gave the highest number of shoots at 2. 29 shoots/ tube, significantly different ($p \leq 0.05$) with another treatment (**Table 3**). Unfortunately, SSEs germinated only shoots without roots as shown in **Figure 3**.

Table 3 Effect of CPPU containing OPCM in previous culture on SSE germination on different PGR-free culture media for 8 weeks.

Previous OPCM with	PGR-free culture media	Germination rate (%)	Avg. no. of shoots (shoots/tube)
0.1 mg/L dicamba+ 0 mg/L CPPU	MS	25.00 ± 0.00b	1.33 ± 0.33ab
0.1 mg/L dicamba+ 0 mg/L CPPU	OPCM	16.67 ± 8.33b	0.66 ± 0.33b
0.1 mg/L dicamba+ 0.3 mg/L CPPU	MS	65.00 ± 6.12a	2.29 ± 0.41a
0.1 mg/L dicamba+ 0.3 mg/L CPPU	OPCM	30.00 ± 5.00b	1.00 ± 0.00b
F-test		**	*
C.V. (%)		31.42	44.54

*significantly different ($p \leq 0.05$), ** significantly different ($p \leq 0.01$)
Mean values followed by the same letter within column are not significantly different according to DMRT.

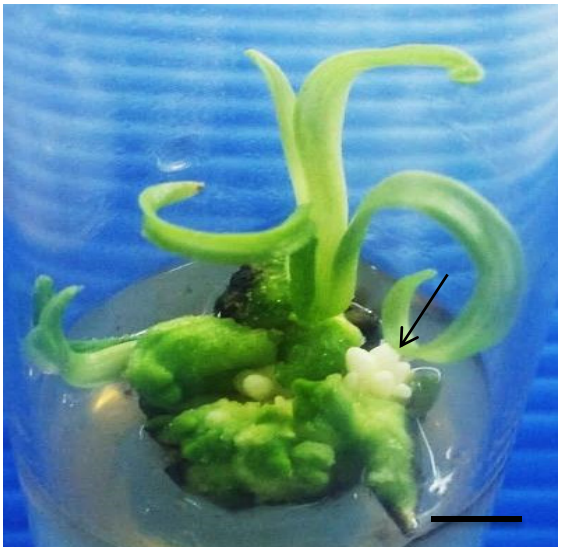

Figure 3 Conversion of SSEs (arrow) into plantlets on PGR-free MS medium for 8 weeks (bar = 0.5 cm).

Discussion

Two cycles occurred during the somatic embryo formation process. In the first cycle, auxins are required to induce and develop embryogenic cells [13]. The second cycle reduces the concentration or eliminates auxins in the culture medium to develop pro-embryos [14]. The highest result in somatic embryogenesis in the present study was obtained from 0.1 mg/L dicamba in oil palm SUP-PSU hybrid clone number 77, similar to those obtained by Jayanthi *et al.* [15]. In contrast, Karup *et al.* [16] reported that a combination of auxin (0.5 mg/L NAA) and cytokinin (6 mg/L BAP) containing MS medium was effective in SE induction of date palm. This study found that 0.3 mg/L CPPU combined with 0.1 mg/L dicamba containing MS medium gave the highest SE induction at 100 % and number of SEs at 5.16 embryos/tube. At the same time, Jayanthi *et al.* [5] showed that a very high concentration of 2,4-D (150 µM or 35 mg/L*)* together with picloram at the same concentrations was used to increase SE formation of oil palm at 6.8 - 9.35 %.

CPPU is a diphenylurea (DPU) derivative that promotes the accumulation of cytokinin in plant tissue. In this present study, a combination of this PGR with dicamba at a suitable ratio promoted a large number of SEs. The best results in SE proliferation (100 %) and No. of SEs (5.16 embryos/tube) were obtained from 0.3 mg/L CPPU combined with dicamba containing medium due to the lowest secretion of phenolic compound. However, a CPPU concentration higher than 3 mg/ L inhibited somatic embryogenesis due to toxicity to the cell. On the other hand, CPPU at 0.4 mg/L combined with 1 mg/L 2,4-D gave better results in callus and somatic embryo induction than 2,4-D alone in carnation [17]. The application of CPPU via somatic embryogenesis is affected by several factors, especially types of explant and endogenous plant growth regulators in tissue. In the previous study, CPPU has been used to improve dragon fruit qualities because it has the ability for cell enlargement [18]. The function of these molecules is still unclear, but some studies confirmed that it inhibits cytokinin oxidase (CKOx) activity and changes in CKOx activity or alters the cytokinin concentrations in plant tissues [17]. CKOx is a plant enzyme that acts as inactivation of naturally occurring cytokinins, which are considered crucial regulatory points in the control of endogenous cytokinin levels and the distribution of native cytokinins during the development of plants [17]. The different explants and genotypes responded differently after somatic embryogenesis. This needs to be investigated.

Upon transferring SE to medium without plant growth regulators for shoot induction, they could not easily be germinated into seedlings or shoots. Firstly, it needs to be transferred to the same medium

supplemented with sorbitol to induce secondary somatic embryos (SSEs) [19,20]. Those SSEs are more capable of germinating into new plantlets because it contains bipolar organization, high meristematic appearance characterized by darkly stained cytoplasm, nuclei, and high starch accumulation [21]. Sorbitol is a sugar alcohol considered stressing agent that can adjust osmotic potential leading to the development of SEs to SSEs in oil palm. It suggested that sorbitol might cause the increment and accumulation of some amino acids and polysaccharides under osmotic stress conditions [22]. Sorbitol at a concentration of 0.2M was reported to give the best result in SSE induction in oil palm [22]. SEs obtained from CPPU containing medium is more effective in SSE induction and germination than those from dicamba alone (without CPPU) . It is possible that CPPU might affect growth and development in the somatic embryogenesis of oil palm. Then, those SSEs could germinate into shoot on PGR-free MS medium within 8 weeks of culture.

Conclusions

These results indicate that 0.3 mg/L CPPU combined with 0.1 mg/L dicamba containing OPCM medium gave the best results in SE induction at 100 %, No. of SEs at 5.16 embryos/tube and size of SE at 3.8 mm after 4 weeks of culture. After transferring SE from OPCM containing 0.3 mg/L CPPU combined with 0.1 mg/L dicamba to MS medium supplemented with 0.2 M sorbitol for 8 weeks, the highest SSE induction at 66.67 % and No. of SSEs at 21.2 embryos were obtained. The highest germination rate at 65 % and no. of shoots at 2.29 shoots were obtained on PGR-free MS medium after 8 weeks of culture, as shown in **Figure 4**.

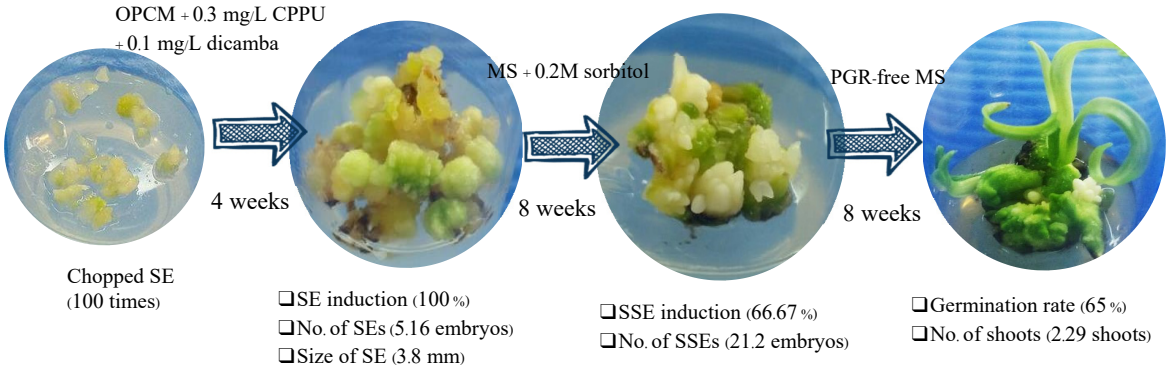

Figure 4 Summary of plantlet regeneration of oil palm hybrid tenera SUP-PSU1 through SSE formation.

Acknowledgements

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