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THE EFFECT OF PICLORAM AND LIGHT ON SOMATIC EMBRYOGENESIS REGENERATION OF PINEAPPLE

Pengaruh Pikloram dan Pencahayaan terhadap Regenerasi Nenas secara Embriogenesis Somatik

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ABSTRACT

Smooth Cayenne is the largest pineapple type cultivated in Indonesia, but its vegetative planting materials for mass propagation are limited. Somatic embryogenesis is a potential method to be applied. The aim of this study was to investigate the somatic embryogenesis regeneration under the effect of picloram and light. Callus formation was induced by picloram (21, 41 and 62 µM) added with 9 µM thidiazuron. The calli were transferred onto MS or Bac medium enriched with N-organic compounds with or without addition of 21 µM picloram under dark or light condition. The compact calli were subcultured onto MS medium supplemented with 4.65 µM kinetin, while the friable calli were transferred onto BIG medium (modified MS + 1.1 µM benzyl adenine + 0.9 µM indole butyric acid + 0.09 µM giberelic acid) or B medium (MS + 0.018 mM benzyl adenine). The results showed that the events of somatic embryogenesis were started from cell polarization, asymmetrical division, proembryo formation as embryogenic tissues and friable embryogenic tissues, and embryo development. The best treatment for callus induction was 21 µM picloram. The addition of 21 µM picloram on N-organic enriched medium and the use of light condition proliferated embryogenic calli. The N-organic enriched Bac medium and light condition yielded the highest number of mature somatic embryos (17 embryos per explant in 2 months). The B medium was better than BIG medium to develop somatic embryos from friable embryogenic tissues. The somatic embryogenesis method presented is potential for pineapple mass propagation and artificial seed production.

[***Keywords***: Pineapple, somatic embryogenesis, plant regeneration, picloram, light]

ABSTRAK

Smooth Cayenne merupakan kultivar nenas yang banyak dibudidayakan di Indonesia, namun ketersediaan benih untuk perbanyakan massal masih terbatas. Embriogenesis somatik adalah metode yang potensial untuk produksi bibit secara massal. Tujuan penelitian adalah untuk mempelajari pengaruh pikloram dan pencahayaan terhadap regenerasi embriogenesis somatik nenas. Kalus diinduksi menggunakan pikloram (21, 41, dan 62 µM) dan penambahan thidiazuron 9 µM. Selanjutnya, kalus dipindahkan ke media MS atau Bac yang diperkaya dengan senyawa N-organik dengan atau tanpa penambahan pikloram 21 µM dalam kondisi gelap atau dengan pencahayaan. Kalus kompak disubkultur pada media MS yang mengandung kinetin 4,65 µM, sedangkan kalus remah dipindahkan ke media BIG (MS modifikasi + bensil adenin 1.1 µM + indole butyric acid 0,9 µM + giberelic acid 0,09 µM) atau media B (MS + bensil adenin 0,018 µM). Hasil penelitian menunjukkan bahwa tahapan embriogenesis somatik diawali dengan polarisasi sel, pembelahan asimetris, pembentukan proembrio sebagai jaringan embriogenik dan jaringan embriogenik remah, serta perkembangan embrio. Perlakuan terbaik untuk induksi kalus adalah pikloram 21 µM. Penambahan pikloram 21 µM pada media yang diperkaya dengan senyawa N-organik mampu meningkatkan jumlah kalus embriogenik. Media Bac yang diperkaya senyawa N-organik dan kondisi pencahayaan menghasilkan jumlah embrio somatik dewasa terbanyak (17 embrio per eksplan dalam 2 bulan). Media B lebih baik daripada media BIG untuk regenerasi embrio somatik dari jaringan embriogenik remah. Metode embriogenesis somatik yang dihasilkan dari penelitian ini berpotensi diterapkan untuk perbanyakan massal dan produksi benih nenas.

[***Kata kunci***: *Nenas, embriogenesis somatik, regenerasi tanaman, pikloram, pencahayaan*]

INTRODUCTION

Pineapple is one of the most commercial fruits in the tropical and subtropical regions such as Thailand, the Philippines, Brazil, China, India, Nigeria, Mexico, Costa Rica, Colombia, Indonesia, USA and Australia with total areas of more than 600 thousand hectares (Rohrbach *et al.* 2003). Recently, this fruit is the

second most important tropical fruit worldwide after banana.

Pineapple can be propagated from various kinds of planting materials such as crown, sucker, butt or stump, hapas, ratoon and slip (IBPGR 1991; Coppens d'Eeckenbrugge and Leal 2003), however, their reproductive times are not uniform. Time harvesting of sucker is 18-20 months, ratoon is 12-14 months, slip is 20 months, and crown is 22-24 months (Department of Health and Ageing 2008). Therefore, these conventional methods are not appropriate for mass propagation of pineapple. This is especially important when the vegetative planting materials are limited such as for the Smooth Cayenne cultivar. This cultivar is the largest commercial pineapple type cultivated in Indonesia.

One potential method in mass propagation of pineapple is somatic embryogenesis technique which could provide a large number of seedlings in a short time with high uniformity. Somatic embryogenesis is a process in which a bipolar structure resembling a zygotic embryo develops from a somatic cell without vascular connection with the original tissue (Arnold *et al.* 2002).

Somatic embryogenesis regeneration technique has been applied broadly in many species of plants, such as coconut (Buffard-Morel *et al.* 1995), banana (Sidha *et al.* 2007), pruatjan (Roostika *et al.* 2007), strawberry (Kordestani and Karami 2008), coffee (Oktavia *et al.* 2003; Gatica *et al.* 2008), ginger (Lincy *et al.* 2009), longan (Roostika *et al.* 2009), orchid (Rianawati *et al.* 2009), and pineapple (Sripaoraya *et al.* 2003; Firoozabady and Moy 2004). However, information on the whole step of somatic embryogenesis regeneration on pineapple is limited, especially at the early stage of embryogenic cell initiation. According to Corredoira *et al.* (2006), an understanding of plant embryogenesis is important to provide an excellent morphogenic system by investigating the cellular process underlying differentiation. It will be benefecial to provide an established method of somatic embryogenesis that can be applied for large-scale clonally propagation, cryopreservation and genetic transformation.

In Indonesia, *in vitro* culture techniques on pineapple are still limited. Few studies to discuss were shoot proliferation (Nursandi *et al.* 2003; Purnamaningsih *et al.* 2009), shoot etiolation (Nursandi *et al.* 2003) and mutagenesis (Suminar 2010), while the somatic embryogenesis regeneration has not been studied.

Auxin influences plant development by causing some changes at the cellular level, including to affect the embryogenesis process (Kelley and Riechers 2007; Vanneste and Frim 2009). At low doses, auxinic herbicides possess similar hormonal properties to natural auxin (Kelley and Riechers 2007). Picloram is an auxinic herbicide (group of pyridine) and commonly used at low concentration as plant growth regulator. For instance, this compound is more effective as inducer for cell proliferation in tissue and cell culture than indole acetic acid (IAA) (Firoozabady and Moy 2004; Ahmed *et al.* 2011; Noormi *et al.* 2012). For that reason, this compound was used in this study to induce pineapple callus formation.

Beside the auxin, light condition seems to plays an important role in somatic embryogenesis regeneration. Uozumi *et al.* (1993) reported that lighting affected the celery embryo regeneration and development. Gatica *et al.* (2008) also reported the important role of lighting during somatic embryogenesis of coffee.

The objectives of this research were to study the complete event of somatic embryogenesis on pineapple started from embryogenic cells initiation to somatic embryos development under the effects of 4-amino 3,5,6-trichloropicolinic acid (picloram) and light.

MATERIALS AND METHODS

Plant Materials and Culture Conditions

The plant material used was *in vitro* cultures of pineapple cv. Smooth Cayenne collected from Subang, West Java. The cultures were established from axillary bud explants excised from crown of the plant growing under field condition. They were planted on MS (Murashige and Skoog 1962) salt and vitamin medium supplemented with 2 mg l^{-1} benzyl adenine (BA) and 2 mg l^{-1} kinetin (Kn) and maintained on MS salts and vitamin medium, supplemented with 0.5 mg l^{-1} BA and 1 mg l^{-1} Kn. The pH of the medium was adjusted to 5.7 ± 0.1 before autoclaving. The cultures were illuminated for 16 hour per day with 1000 lux irradiance and exposed in a culture room at a temperature of 25 ± 2°C. This study was divided into three steps: (1) callus induction, (2) embryogenic callus formation, and (3) somatic embryo development.

Callus Induction

In vitro shoots were cut longitudinally into 2-4 sections, depended on the size of the core. The sections were placed horizontally on callus induction media (8-10 sections per bottle) and incubated under dim light (below 500 lux). Callus induction media

were MS basal media containing 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid or 4-amino-3,5,6-trichloropicolinic acid (picloram) at the rate of 21, 41 and 62 µM (or 5, 10 and 15 mg l^{-1}) with addition of 9 µM (2 mg l^{-1}) thidiazuron (TDZ). They were incubated under 25 ± 2°C in the dark for 3 weeks. After 3 weeks (explants grew larger), leaves were isolated individually and planted on the same medium and incubated at the same condition as in the previous step.

The experiment was arranged in completely randomized design with five replications, where each bottle consisted of 15 leaf base explants. The variables observed were the percentage of callus formation at 3, 6, 8 and 12 weeks after planting (WAP) and the fresh weight of the cultures at 12 WAP. The data were analyzed by Program SPSS V.19 (IBM Company).

Embryogenic Callus Formation

The calli were subcultured to different compositions of media to induce embryogenic callus formation. The experiment was arranged as factorial in completely randomized design with eight replications. The first factor was the use of picloram (with or without addition of 21 µM picloram), whereas the second factor was basal media (MS and Bac). All of those media contained 1 mg l^{-1} glutamine (Gln), 500 mg l^{-1} casein hydrolysate (CH), 120 mg l^{-1} arginine (Arg), and 2 mg l^{-1} glycine (Gly). The hormone-free MS basal medium was also tested. The composition of macronutrients, micronutrients, vitamins and sucrose of MS and Bac media in this study is shown in Table 1. The treatment was replicated eight times. The calli, including original explants, were placed in a culture room at 25 ± 2°C, in the dark and light conditions (800-1000 lux for 16 hours per day) for 3 weeks.

The data were analyzed by Program SPSS V.19 (IBM Company). The variables observed were the percentage of embryogenic callus formation, percentage of premature embryo, percentage of root formation, and the visual performance of the callus (compact or friable, white or yellow or green, easy to disperse or not, watery or vigorous, and nodular or globular). Microscopical observation was conducted by a converted stereomicroscope (Olympus CK40-F200) at 400x magnification to confirm the type of cell division.

Somatic Embryo Development

This step consisted of two experiments. The first experiment was regeneration of compact calli. They were transferred onto MS basal media containing 1 mg l^{-1} (4.65 µM) Kn for premature embryo development. The experiment was arranged as factorial in completely randomized design with eight replications. The first factor was embryogenic callus induction media, and the second factor was regeneration media. They were placed in the culture room with 25 ± 2°C, in the light condition for 16 hours per day with 800-1000 lux irradiance. The variables observed were the percentage of regenerated explants, the number of premature somatic embryos, and the performance of the calli. The data were analyzed by Program SPSS V.19 (IBM Company).

Table 1. Composition of macronutrients, micronutrients, vitamins, and sucrose in the MS and Bac basal media.

Compounds	MS (mg l^{-1})	Bac[1)] (mg l^{-1})
Macronutrients		
KNO_3	1900	2528
NH_4NO_3	1650	-
NH_4Cl	-	535
$CaCl_2.2H_2O$	440	440
$MgSO_4.7H_2O$	370	370
KH_2PO_4	170	170
Micronutrients		
$MnSO_4.\ 4H_2O$	18.9	18.9
$ZnSO_4.7H_2O$	10	10
H_3BO_3	10	10
KI	0.83	0.83
$Na_2MoO_4.2H_2O$	0.25	0.25
$CuSO_4.5H_2O$	0.025	0.025
$CoCl_2.6H_2O$	0.025	0.025
$FeSO_4.7H_2O$	27.85	27.85
$Na_2EDTA.2H_2O$	37.25	37.25
Vitamin		
Inositol	100	100
Thiamine-HCl	0.1	0.4
Nicotinic acid	0.5	0.5
Prydoxine-HCl	0.5	0.5
Carbon source		
Sucrose	30,000	30,000

1)Modified by Valverde and Arias (1989) *in* Firoozabady and Moy (2004).

The second experiment was regeneration of friable calli. They were transferred into two different media. The first was basal medium supplemented with 4 mg l^{-1} (17.7 µM) BA (Firoozabady and Moy 2004). This medium was coded as B. The second growth medium was MS basal medium with slight modification in vitamin (without nicotinic acid and pyridoxine HCl) enriched with 1.1 µM (0.25 mg l^{-1}) BA, 0.9 µM (0.18 mg l^{-1}) IBA, and 0.09 µM (0.03 mg l^{-1}) giberelic acid (GA_3) (Perez *et al.* 2009). This later growth medium was

coded as BIG. They were placed in the culture room with 25 ± 2°C, in the light condition for 16 hours per day with 800-1000 lux irradiance. The variables observed were the percentage of regenerated explants, the number of mature somatic embryos, and the performance of the calli. The data were presented as means and standards of deviation.

RESULTS AND DISCUSSION

Callus Induction

The callus formation was initiated at 3 WAP. Almost all of the explants initiated calli at the leaf base. It was believed that the leaf base contains meristematic regions or possesses newly developed tissue with rapidly dividing cells that are amenable to morphogenesis in the tissue culture (Firoozabady and Moy 2004). Suryowinoto (1996) reported that Bromeliaceae has adventitious meristem in the leaf base.

The results showed that the calli were formed up to 8 weeks of observation. At 12 weeks of incubation period, the callus growth was stagnant. This means that the calli stopped to grow after 8 weeks of incubation period. The higher callus formation (90%) was resulted from the treatment with 21 µM picloram. However, it was not significantly different with the other treatments as well as the fresh weight of the calli (Fig. 1). This result is better than those previously reported. Sripaoraya *et al.* (2003) showed the low frequency of callus formation (58%) using 3 mg l^{-1} picloram on inducing embryogenesis of pineapple cv. Phuket. Firoozabady and Moy (2004) found callus formation of 55% by applying 41µM picloram on embryogenesis of pineapple cv. Smooth Cayenne.

Visual observation showed that globular and yellowish calli were produced from the leaf bases. Microscopical examination demonstrated that the callus was mixed by non-embryogenic and embryogenic cells. The embryogenic cells were preceded with polarization and then followed by asymmetrical division to become apical and basal cells (Fig. 2). Abrash and Bergmann (2009) reported that asymmetrical division produces distinct daughter cells which morphologically different. Furthermore, Paciorek and Bergmann (2010) illustrated the detail process of asymmetrical division. It is started with symmetry breaking and followed by polarity establishment through the formation of preprophase band (PPB) and mitotic spindle to define the orientation of cell division. After phragmoplast formation, the cell divides into two different daughter cells. It was

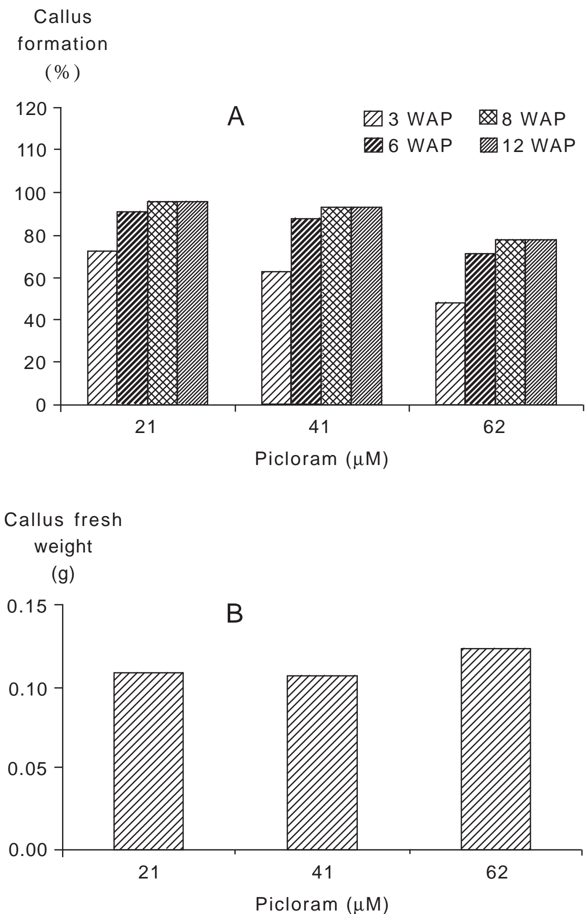

Fig. 1. The effect of picloram on the growth of pineapple leaf base explants; callus formation (A) and callus fresh weight at 12 weeks after application (B).

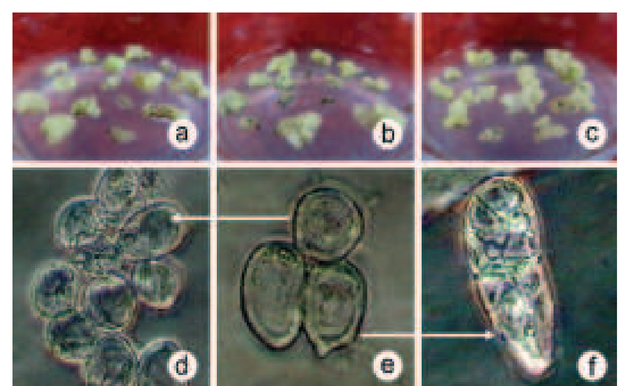

Fig. 2. The response of pineapple leaf bases on picloram containing media. The upper row showed the calli produced by media containing different picloram concentrations, i.e. 21 µM (a), 41 µM (b), 62 µM (c), and the lower row showed microscopical observation of different cell types, viz. non-embryogenic cell cluster (d), polarized and non-polarized cells (e), and embryogenic cells consisted of basal and apical areas (f). The arrows indicated further development of each cell.

suggested that auxin contributes in this division through its polar transport. Vanneste and Frim (2009) reported that auxin is a trigger to change plant development through its polar transport.

The calli yielded in this study were globular and yellowish calli consisted of polarized (embryogenic) and not polarized (non-embryogenic) cells. The cluster of embryogenic cells was assumed as pre-embryogenic mass (PEM). A similar structure was also reported by Firoozabady and Moy (2004). They named the structure as embryogenic cell clusters (ECCs). ECCs were highly friable tissues containing cell clusters mixed with elongated and large vacuolated cells together with individual globular embryos. They further reported that the structures are very potential to be used for somatic embryo production. Unfortunately in this study, the embryogenic character was lost when the ECCs were let to grow continuously in the callus induction medium containing picloram for 2 months.

Embryogenic Callus Formation

The results showed that embryogenic character of pineapple calli could be stimulated by subculturing onto the N-enriched medium. The embryogenic cell initiation was started from cell polarization, followed by asymmetrical division and cell elongation (Fig. 3) Arnold *et al.* (2002) stated that the important mechanisms during the process of somatic embryogenesis are asymmetric cell division and cell elongation.

The events of somatic embryogenesis and non-embryogenesis process of pineapple cells at pre-embryo stage could be observed microscopically (Fig. 3). Figure 3 shows that the early embryogenic cells (the -4 cells) were divided through periclinal division during differentiation. This indicates the occurrence of unicellular origin of somatic embryogenesis. The similar result was observed on *Quercus robur* (Corredoira *et al.* 2006), excepted in the transversal division. This suggests that the pineapple embryogenic calli are potential to be developed as cell suspension culture for mass propagation. Corredoira *et al.* (2006) reported that the determination of somatic embryo origin is essential to the genetic uniformity of the regenerated plants. The unicellular origin of somatic embryos is desirable for genetic transformation.

Based on visual observation, two different structures were obtained from this step, namely the greenish compact calli and the yellowish dispersable calli. The compact calli contained nodular structures or some premature embryos, whereas the friable calli did not contain such structures (Table 2). Firoozabady and Moy (2004) distinguished those structures. The first structure was called embryogenic tissue (ET) and the second one was termed as friable embryogenic tissue (FET). ET was defined as non-dispersible organized structure produced by proliferation of premature somatic embryos or a cluster of premature

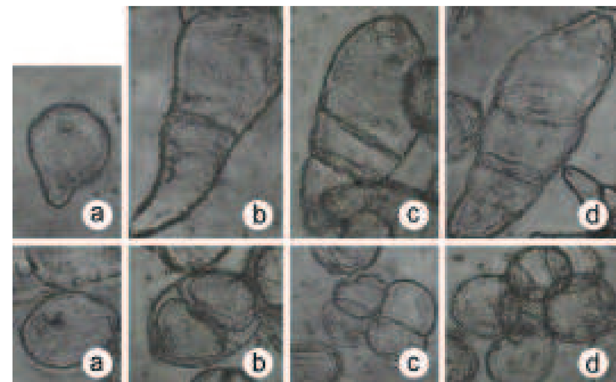

Fig. 3. Microscopical observation (400 x magnifications) of pineapple embryogenic cell clusters (ECCs) after planted on embryogenic callus proliferation media. The upper panel is the development of embryogenic cell and the lower panel is non-embryogenic cell: single cell (a), two cells (b), three or four cells (c), and further development (d).

Table 2. The response of pineapple cultures to different types of embryogenic callus proliferation media and light condition at 1 month after subculture.

Light condition	Media	Visual performance of the cultures	Structure observed
Light	MS0	Nodular, compact, greenish, and contained premature embryos	ET
	MS+	Compact, greenish, and contained premature embryos	ET
	MS21	Globular, dispersible, and yellowish	FET
	Bac+	Nodular, compact, greenish, and contained premature embryos	ET
	Bac21	Globular, semidispersible, and yellowish	FET
Dark	MS0	Compact and browning	ET
	MS+	Semi dispersible, nodular, and yellowish	ET
	MS21	Globular, semidispersible, and yellowish	FET
	Bac+	Compact and browning	ET
	Bac21	Globular, semidispersible, and yellowish	FET

MS0 = hormone-free MS basal medium, MS+ = N-organic enriched MS medium, MS21 = N-organic enriched MS + 21 µM picloram, Bac+ = N-organic enriched Bac medium, Bac21 = N-organic enriched Bac + 21 µM picloram, ET = embryogenic tissue, FET = friable embryogenic tissue.

somatic embryos fused together. FET was defined as dispersible tissue containing individual globular embryos in a matrix. Figure 4 shows the performance of ETs and FETs of pineapple.

There was no interaction between embryogenic callus induction media and light condition to the percentage of browning, nodule formation, and premature embryo formation (Fig. 5). Qualitatively, the cultures incubated in the dark condition were more severe from browning than the cultures incubated in the light condition. Browning is caused by the oxidation of phenols after cellular disorganization and eventual cell death that occurred non-enzymatically or enzymatically and catalyzed by phenol oxidases or peroxidases producing dark brown quinones. Peroxidases were associated with chlorophyll degradation in senescent plant tissues (Laukkanen *et al.* 2000). When they were exposed under light condition, almost all treatments yielded premature embryos (ETs) as they were in the state of higher cell differentiation events. In contrast to those grown on MS medium supplemented with 21 μM picloram which proliferated callus without formation of premature embryos because they were in the state of cell proliferation events. This suggested that picloram induced cell proliferation. Arteca (1996) reported that picloram is readily absorbed by all parts of the plant and transported to area of rapid growth, therefore it causes highly cell proliferation.

Usually, the light exposure gives a negative effect on growth inhibition on the auxin treated explants. Uozumi *et al.* (1993) and Mesenes *et al.* (2005) showed that light was not obligatory in embryo formation. They strongly suggested that illumination should be reduced or omitted at the beginning of the first regeneration stage. However, illumination is required after the late embryo development has progressed. The similar result was reported by Gatica *et al.* (2008) where dark condition was more suitable in producing coffee embryoids. However, this study likely showed a different result, where dark condition yielded growth inhibition and cell necrotic, marked with browning possibly caused by the high accumulation of picloram in the cultures under dark condition. Since picloram is an herbicide, high accumulation of this compound caused phytotoxic to the cultures and inhibited culture growth. Picloram was stable during dark condition, but it was degraded under light condition (Hagen *et al.* 1991). Furthermore, Arteca (1996) reported that picloram persists in the cells and cannot be metabolized.

The result indicated that the use of media formulation treatment demonstrated a more significant effect than light condition treatment on the ETs proliferation. This is especially true for calli grown on media containing picloram (Fig. 5). As previously described, picloram is a kind of auxinic herbicide and may act as an effective inducer for cell differentiation and cell proliferation (Kelley and Riechers 2007) through polar transport auxin (Vanneste and Frim 2009). The use of picloram has also been reported in many plant species. Kordestani and Karami (2008) used picloram on strawberry. Furthermore, Sudarmonowati and Henshaw (1996) reported that picloram could induce somatic embryogenesis of cassava cultivars that failed to produce somatic embryos when they were cultured on 2,4-D containing media. Sidha *et al.* (2007) reported that among the auxins tested, picloram and

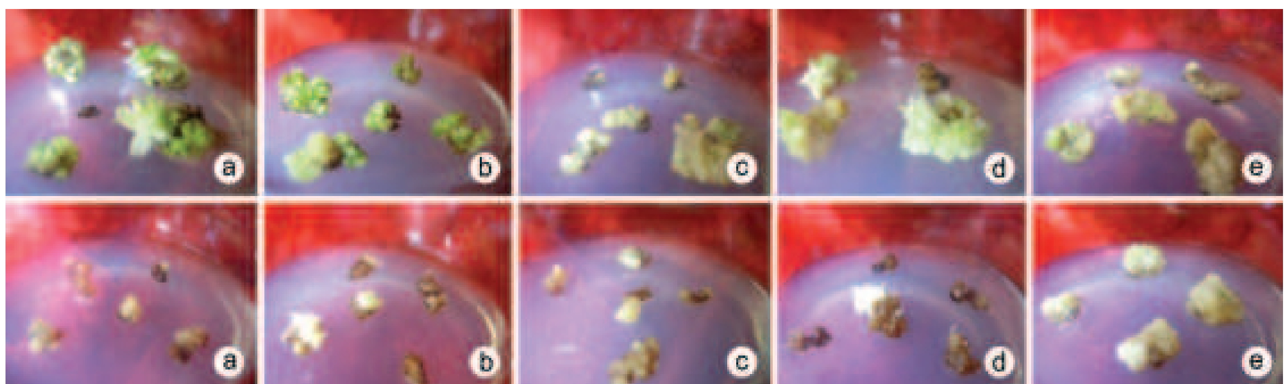

Fig. 4. Performance of the embryogenic tissues (ETs) and friable embryogenic tissues (FETs) of pineapple cultures planted on different kinds of embryogenic callus induction media: hormone-free MS basal medium (a), N-organic enriched MS medium (b), N-organic enriched MS + 21 μM picloram (c), N-organic enriched Bac medium (d), and N-organic enriched Bac + 21 μM picloram (e). Pictures on the upper panel are those cultured in light condition, whereas on the lower panel are those in dark condition.

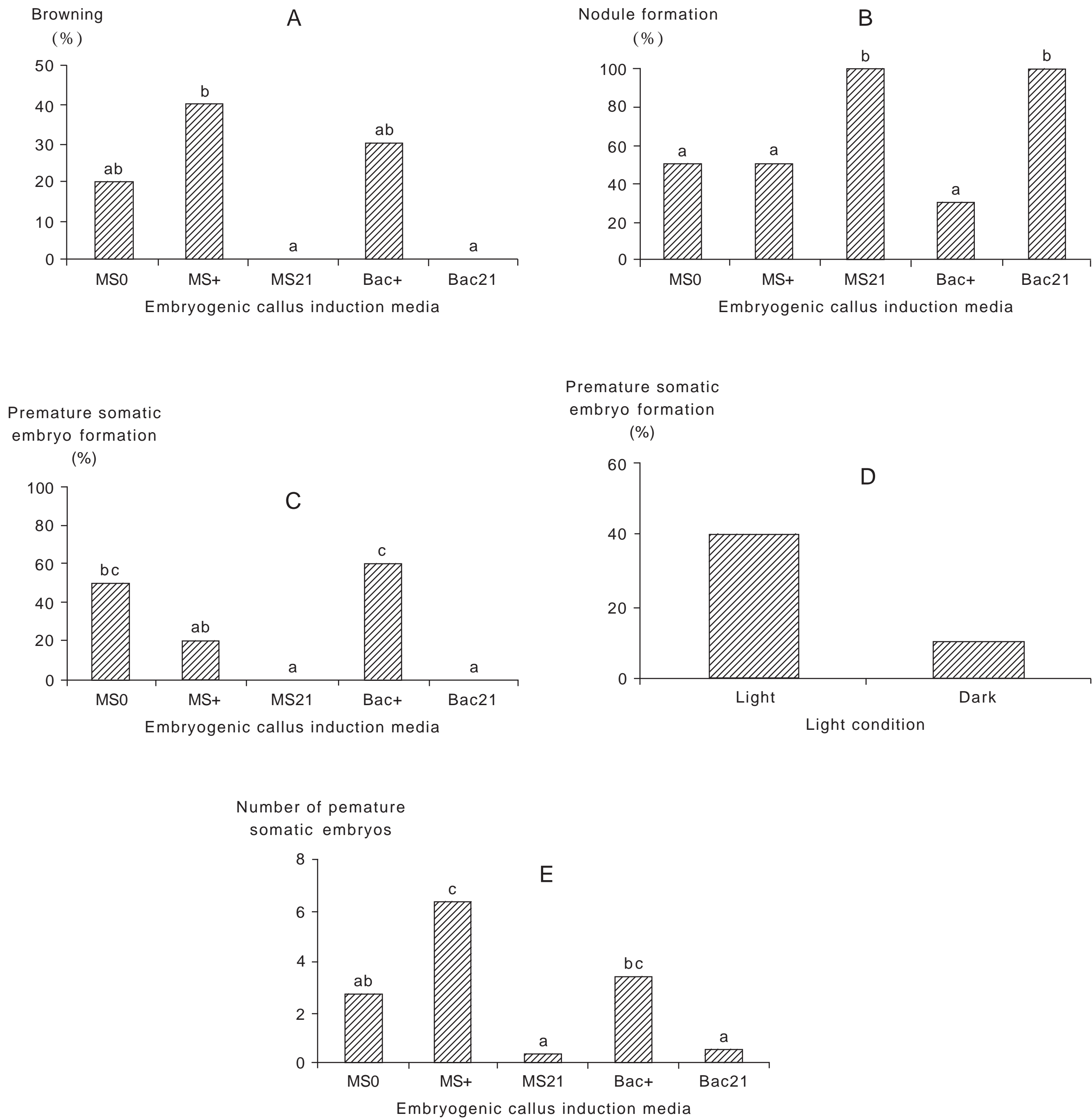

Fig. 5. The effect of single factor (media or lighting) on the percentage of browning (A), nodule formation (B), premature somatic embryo formation (C and D), and number of premature somatic embryos (E) of pineapple; MS0 = hormone-free MS basal medium, MS+ = N-organic enriched MS medium, MS21 = N-organic enriched MS + 21 μM picloram, Bac+ = N-enriched Bac medium, Bac21 = N-organic enriched Bac + 21 μM picloram.

3,6-dichloro-o-anisic acid (dicamba) showed the highest rate of callus proliferation on banana.

In this study, the emergence of premature embryos might be caused by the activity of endogenous cytokinin. As reported by several researchers, the high ratio of cytokinin versus auxin will change to growth into shoot formation (Srivastava 2002; Moubayidin *et al.* 2009). Based on this result, the ETs and FETs structures were then subcultured into different media. The ETs were subcultured onto MS medium with addition of 1 mg l^{-1} Kn to regenerate the premature somatic embryos, whereas the non-FETs were subcultured into B or BIG medium to develop the somatic embryos.

Somatic Embryo Development

In the case of ETs, the highest number of mature somatic embryos was obtained from N-enriched Bac medium in light condition (Table 3). The performance of ETs before and after regeneration is presented in Figure 6. It showed that the structures of cultures of N-enriched Bac and N-enriched MS media became dispersible. The percentage of regenerated ETs was 100% and yielded more than 17 mature embryos per ET in 2 months (Table 3). This result was higher than that obtained on pineapple cv. Phuket (65.7% with three embryos per ET) (Sripaoraya *et al.* 2003). This result was also higher than the study reported by Firoozabady and Moy (2004) which obtained 63% regenerated ETs using pineapple cv. Champaka.

Table 3. The development of embryogenic tissues (ETs) of pineapple on MS medium containing 1 mg l^{-1} Kn, 2 months after subculture.

Original light condition	Original media	Regenerated ETs (%)	Number of mature embryos
Light	MS0	87.5	9.1 ± 5.7
	MS+	100	10.3 ± 7.4
	Bac+	100	17.3 ± 7.5
Dark	MS0	77.8	10.6 ± 8.9
	MS+	100	9.5 ± 6.1
	Bac+	100	6.4 ± 4.3

MS0 = hormone-free MS basal medium, MS+ = N-organic enriched MS medium, and Bac+ = N-organic enriched Bac medium.

In this study, the globular and coleoptilar stage could be observed visually (Fig. 7). However the scutellar stage could not be detected since the premature somatic embryos had fused together in ETs or the embryos were in matrix in FETs. Root formation emerged spontaneously without root induction and the roots structure of the somatic embryos typically looked like that derived from zygotic embryos, where root and shoot initiation took place simultaneously in the bipolar structure of embryo. Kordestani and Karami (2008) also reported the same phenomenon. Due to the presence of well-developed root and shoot primordia, somatic embryos of strawberry germinated easily to produce plantlets without an additional step of rooting. Jimenez (2005) stated that the development of embryos in monocots passes through several stages, namely globular, scutellar, and coleoptilar.

The same as ETs, the browning event of FETs was also higher in dark condition compared to that grown under light condition. The highest rate of browning (68%) was resulted from MS medium containing 21 μM picloram incubated in dark condition and regenerated on B regeneration medium (Tabel 4). The FETs needed longer time to develop the embryos (2.5 months) than ETs (2 months), however the percentage of regenerated FETs was also higher (83-100%).

The B medium was more suitable for developing somatic embryos originated from light condition treatment. On the contrary, the BIG medium was more appropriate for developing somatic embryos originated from dark condition treatment. Generally the

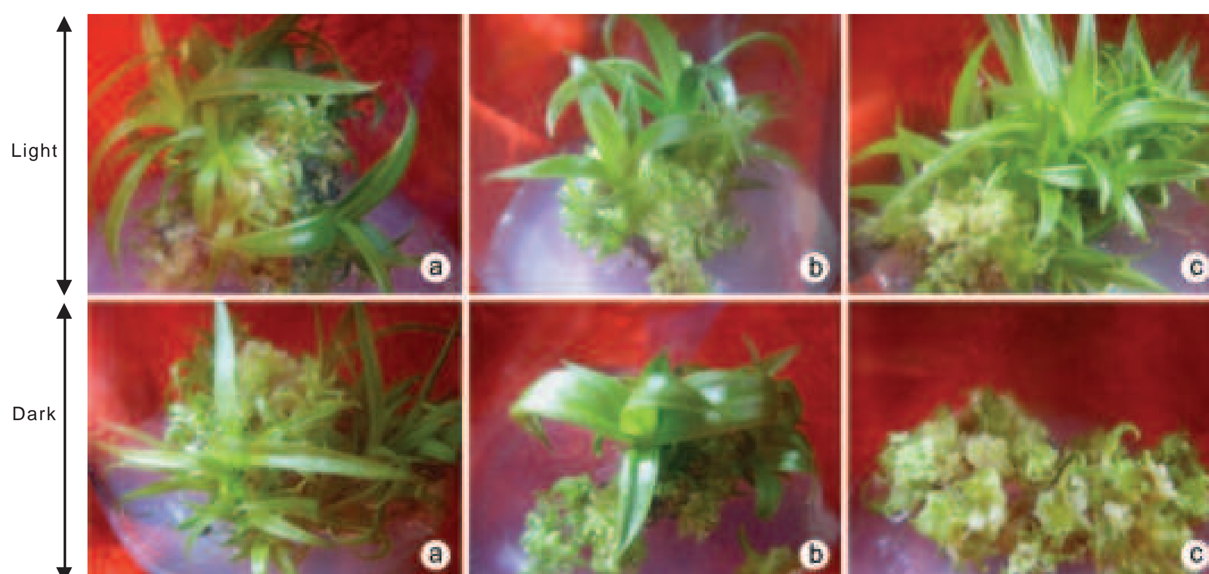

Fig. 6. The performance of pineapple embryogenic tissues (ETs) after regeneration on MS medium enriched with 1 mg l^{-1} Kn. The original media were hormone-free MS basal medium (a), N-organic enriched MS medium (b), and N-organic enriched Bac medium (c).

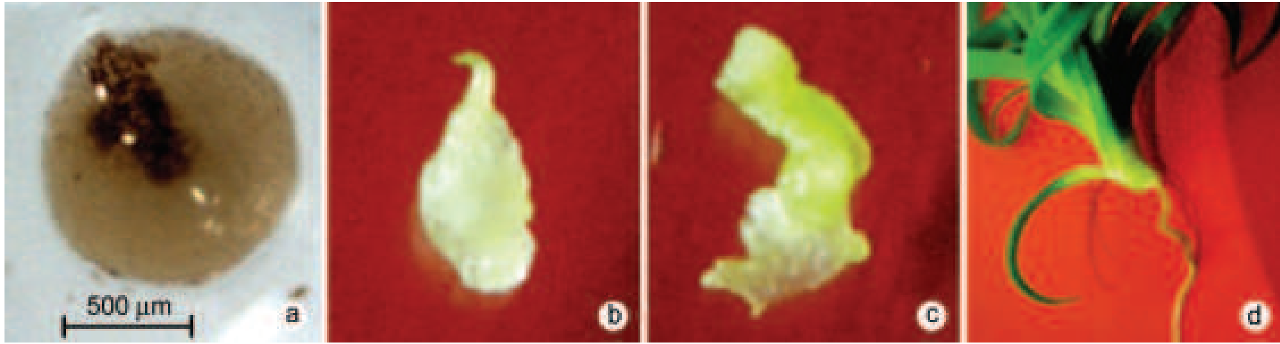

Fig. 7. The developmental stages of pineapple somatic embryos from friable embryogenic tissues (FETs) structure: globular (a), late coleoptilar stage (b), germinated embryo (c), and plantlet (d).

best treatment was obtained from N-enriched MS medium containing 21 µM picloram and grown under light condition followed by transferring the FETs into MS medium containing 4 mg l^{-1} BA (Tables 4, 5 and 6).

Application of somatic embryogenesis on pineapple via ETs was more recommended than via FETs. The mature somatic embryos formated through ETs were higher (17.3 embryos per explant) than that through FETs (14.8 embryos per explant) and the period for regenerating ETs was shorter (2 months) than FETs (2.5 months). Generally, this study provides a baseline for mass propagation of pineapple, especially artificial seed production.

Table 4. The browning level of pineapple friable embryogenic tissues (FETs) on different media and light conditions at 1 month after subculture.

Regeneration media	Browning level (%)			
	MS21		Bac21	
	Light	Dark	Light	Dark
BIG	14	26	38	36
B	12	68	42	46

MS21 = N-organic enriched MS + 21 µM picloram, Bac21 = N-organic enriched Bac + 21 µM picloram, BIG = MS without nicotinic acid and pyridoxine HCl + 1.1 µM BA + 0.9 µM IBA + 0.09 µM GA_3, and B = MS + 0.018 mM BA.

Table 5. The regeneration of pineapple friable embryogenic tissues (FETs) on different kinds of media and light conditions at 2.5 months after subculture.

Regeneration media	Regenerated FETs (%)			
	MS21		Bac21	
	Light	Dark	Light	Dark
BIG	100	83	100	100
B	100	100	100	100

MS21 = N-organic enriched MS + 21 µM picloram, Bac21 = N-organic enriched Bac + 21 µM picloram, BIG = MS without nicotinic acid and pyridoxine HCl + 1.1 µM BA + 0.9 µM IBA + 0.09 µM GA_3, and B = MS + 0.018 mM BA.

Table 6. The number of pineapple somatic embryos yielded from friable embryogenic tissues (FETs) on different kinds of media and light conditions at 2.5 months after subculture.

Regeneration media	Number of mature somatic embryos			
	MS21		Bac21	
	Light	Dark	Light	Dark
BIG	12.7 ± 6.8	10.5 ± 6.0	2.5 ± 1.3	11.4 ± 5.5
B	14.8 ± 6.0	8.8 ± 3.9	9.2 ± 3.3	4.8 ± 2.6

MS21 = N-organic enriched MS + 21 µM picloram, Bac21 = N-organic enriched Bac + 21 µM picloram, BIG = MS without nicotinic acid and pyridoxine HCl + 1.1 µM BA + 0.9 µM IBA + 0.09 µM GA_3, and B = MS + 0.018 mM BA.

CONCLUSION

The events of pineapple somatic embryogenesis were started from cell polarization, asymmetrical division, proembryo formation as embryogenic tissues (ETs) and friable embryogenic tissues (FETs), and embryo development (globular and coleoptilar stage). The best medium for callus induction was MS medium supplemented with 21 µM picloram. The effect of 21 µM picloram was more dominant than that of light condition to proliferate embryogenic calli. The highest number of mature ETs (17 embryos per explant in 2 months) and mature FETs (14 embryos per explant in 2.5 months) were obtained on the N-organic enriched MS medium supplemented with

0.018 mM BA. This method is potential to be applied for mass propagation or artificial seed production on pineapple.

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