Indirect Organogenesis and Somatic Embryogenesis of Pineapple Induced by *Dichlorophenoxy Acetic Acid*

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ABSTRAK

Organogenesis dan Embriogenesis Somatik Tak Langsung pada Nenas yang Diinduksi oleh *Dichlorophenoxy Acetic Acid*. Ika Roostika, Ika Mariska, Nurul Khumaida, dan Gustaaf A. Wattimena. Penelitian ini bertujuan untuk mengetahui pengaruh 2,4-D, AdS, dan media dasar dalam regenerasi tanaman nenas secara organogenesis dan embriogenesis somatik tak langsung. Induksi kalus dilakukan dengan perlakuan 2,4-D (21, 41, dan 62 µM) dengan penambahan TDZ 9 µM. Sel-sel yang non embriogenik diregenerasikan secara organogenesis dengan memindahkannya ke media yang mengandung kinetin 4,65 µM. Induksi kalus embriogenik dilakukan pada media MS atau Bac yang diperkaya dengan senyawa N-organik (Gln 6,8 µM, CH 500 mg l^{-1}, Arg 0,69 mM, dan Gly 0,027 µM) dengan penambahan AdS (0; 0,05; dan 0,1 µM). Kalus embriogenik diregenerasikan pada dua macam media. Media pertama adalah media MS modifikasi dengan penambahan IBA 0,9 µM, BA 1,1 µM, GA$_3$ 0,09 µM sedangkan yang kedua adalah media MS dengan penambahan BA 0,018 mM. Hasil penelitian membuktikan bahwa penggunaan 2,4-D secara tunggal tidak mampu menginduksi sel embriogenik sehingga sebagian kalus non embriogenik diregenerasikan secara organogenesis. Media terbaik untuk organogenesis adalah 2,4-D 21 µM karena menghasilkan persentase pembentukan kalus yang tinggi (80%), bobot basah kalus tertinggi (0,2 g/eksplan), dan jumlah tunas tertinggi (sekitar 25 tunas/eksplan/2 bulan). Kalus embriogenik terbentuk ketika kalus non embriogenik diperlakukan dengan senyawa N-organik. Media regenerasi merupakan faktor yang berpengaruh nyata terhadap tingkat pencoklatan. Media MS dengan penambahan BA 0,018 mM mampu menekan pencoklatan. Terdapat interaksi yang nyata antara media induksi kalus embriogenik dengan media regenerasi terhadap jumlah embrio somatik dewasa. Media MS yang diperkaya dengan senyawa N-organik dan AdS 0,05 µM yang dikombinasikan dengan media MS dengan penambahan BA 0,018 mM merupakan perlakuan terbaik untuk induksi kalus embriogenik dan regenerasi embrio somatik nenas (17 embrio dewasa/eksplan).

Kata kunci: Organogenesis, embriogenesis somatik, nenas, 2,4-D, adenin sulfat.

ABSTRACT

Indirect Organogenesis and Somatic Embryogenesis of Pineapple Induced by *Dichlorophenoxy Acetic Acid*. Ika Roostika, Ika Mariska, Nurul Khumaida, and Gustaaf A. Wattimena. This research aimed to study the effect of 2,4-D, AdS, and basal media to the regeneration of pineapple through indirect organogenesis and somatic embryogenesis, and to study the complete event of somatic embryogenesis. Callus formation was induced by 21, 41, and 62 µM 2,4-D with addition of 9 µM TDZ. The non embryogenic calli were transferred onto 4.65 µM Kn containing medium. Embryogenic callus formation was induced on MS or Bac basal media consisted of N-organic compounds with addition of AdS (0, 0.05 and 0.1 µM). The embryogenic calli were regenerated on modified MS medium with addition of 0.9 µM IBA, 1.1 µM BA, 0.09 µM GA$_3$ or MS medium supplemented with 0.018 mM BA. The result proved that the single auxin of 2,4-D was not enough to induce embryogenic cells. Therefore the non embryogenic calli were regenerated through organogenesis. The 21 µM 2,4-D yielded high level of callus formation (80%), higher fresh weight (0.2 g/explant) and higher number of shoot (25 shoots/explant in two months). Embryogenic calli were produced on N-organic compounds enriched media. The regeneration medium significantly affected the level of browning, where the MS medium with addition of 0.018 mM BA yielded lower level of browning. There was an interaction of embryogenic callus induction medium and regeneration medium to the number of mature somatic embryos. The embryogenic callus induction on MS medium enriched with N-organic compounds and 0.05 µM AdS followed by the regeneration of somatic embryos on MS medium with addition of 0.018 mM BA was the best treatment which yielded 17 mature somatic embryos/explant.

Keywords: Organogenesis, somatic embryogenesis, pineapple, 2,4-D, adenine sulphate.

INTRODUCTION

Pineapple is a tropical commercial fruit crop worldwide after banana, and potentially to be further developed in Indonesia. Conventionally, pineapple can be propagated from many propagules (IBPGR, 1991; Coppens d'Eeckenbrugge and Leal, 2003) such as crown, sucker, butt or stump, hapas, ratoon, and slip

but their reproductive time are not uniform. Unfortunately, the vegetative propagule availability is commonly limited in cultivar Smooth Cayenne then the conventional method need to be supported by the other method for large scale seedling production.

Today micropropagation is being used commercially in the pineapple industry in abroad (Smith *et al.*, 2003) but in Indonesia, the industry survives with conventional vegetative propagation such as Great Giant Pineapple Company. However, this method should be applied to provide a large number of seedlings of important new cultivars resulted from hybridization, selection, mutation, and genetic engineering (Firoozabady and Moy, 2004; Nursandi *et al.*, 2005). In practice, micropropagation is used for the establishment of multiplication blocks which then provide conventional planting material for larger production blocks. Furthermore, the micropropagation method allows screening of variants on fruit characteristic before conventional method of propagation is used (Smith *et al.,* 2003).

In Indonesia, the *in vitro* culture of pineapple cultivar Smooth Cayenne is still limited to discus shoot proliferation (Purnamaningsih *et al.*, 2009), shoot etiolation (Nursandi *et al.*, 2003), and mutagenesis (Suminar, 2010). There is no report concerning on organogenesis and somatic embryogenesis for mass propagation of cultivar Smooth Cayenne.

Organogenesis is a direct formation of adventitious shoot from the explant yielding unipolar structure, whereas somatic embryogenesis is a regeneration process of somatic cells without vascular connection with the original tissue that are developed by cell divisions to form bipolar structure as complete embryos. Unipolar structures contains of shoot or root, while bipolar structure of the somatic embryos contains of both shoot and root meristem (Phillips *et al.*, 1995; Arnold *et al.*, 2002). Unfortunately, there is no report of the complete event of pineapple somatic embryogenesis worldwide. An understanding of embryogenesis is important to provide an excellent morphogenic system by investigating the cellular process underlying differentiation (Corredoira *et al.*, 2006). It will be benefit to provide an establish method of somatic embryogenesis that can be applied for large-scale clonal propagation, cryopreservation, and tranformation.

Among synthetic auxins, 2,4-D is commonly used at relatively low concentration. According to Kelley and Riechers (2007), 2,4-D is an auxinic herbicide that possess similar hormonal properties to natural auxin. For instance Srivastava (2002) reviewed that this compound is an effective inducer for cell proliferation in tissue and cell culture.

The aim of this research was to study the effect of *dichlorophenoxy acetic acid* (2,4-D), *adenine sulphate* (AdS), and basal media to the regeneration of pineapple cultivar Smooth Cayenne through indirect organogenesis and somatic embryogenesis, and to study the complete event of somatic embryogenesis started from embryogenic cells initiation to somatic embryo development.

MATERIALS AND METHODS

The plant material used was *in vitro* culture of pineapple cultivar Smooth Cayenne clone Simadu from Subang, West Java. The cultures were maintained on MS (Murashige and Skoog, 1962) salts and vitamins, supplemented with 3% sucrose, 2.21 μM (0.5 mg l^{-1}) *benzyl adenine* (BA) and 4.65 μM (1 mg l^{-1}) *kinetin* (Kn). The pH of the medium was adjusted to 5.7$\pm$0.1 before autoclaving. The cultures were illuminated 16 h per day with 1.000 lux irradiance provided by neon light and exposed to a temperature of 25$\pm$2°C. The research was divided into two regeneration methods that were indirect organogenesis and indirect somatic embryogenesis.

Indirect Organogenesis Regeneration

In vitro shoots about 4-6 cm height were cut longitudinally become two sections. The sections were placed horizontally on callus induction media (10 sections per bottle) and incubated under dim light (below 500 lux). Callus induction media were MS basal media containing of 2,4-D at the rate of 21, 41, and 62 μM (4.6, 9.2, and 13.7 mg l^{-1}) with 9 μM (2 mg l^{-1}) *thidiazuron* (TDZ). The explants were incubated under 25$\pm$2°C in the dark condition. After 3 weeks (explants grew larger), leaves were isolated individually and they were plated on the same medium and incubated at the same condition as previous step. The experimental design was Completely Randomized Design with 5 replications (bottles). Each bottle contained of 15 leaf base explants. The callus formation was observed 4 times at 3, 6, 8, and 12 weeks of incubation periods. The fresh weight of the calli was measured at the end of observation. The data was analyzed using SPSS Version 19 (IBM Company) and the differences between the means were compared by Duncans's multiple range tests at the 0.05 level. Visual performance of the calli (compact or friable, white or yellow or green, easy to disperse or not, watery or vigorous) was also observed. Microscopical

examination was also conducted to check the type of cell division. If there was an asymmetrical division, the calli were then transferred onto somatic embryo regeneration medium but if there was a symmetrical division, the calli were subcultured onto shoot regeneration medium (MS basal media with addition of 4.65 uM (1 mg l^{-1}) Kn) to regenerate and elongate the shoots, at least 1 cm height. The variables observed were number of normal and abnormal shoots. The shoot abnormality was observed visually and determined as imbalance axiality. The data was presented as averages and deviation standards.

Indirect Somatic Embryogenesis Regeneration

Firstly, callus was induced from leaf base explants by planting the explants on MS basal medium containing of the best rate of 2,4-D, based on the previous experiment. After callus formation, the cultures were subcultured onto embryogenic callus induction media. The experimental design was factorial in Completely Randomized Design with 10 replications. The first factor was basal media (MS and Bac) and the second factor was *adenine sulphate* (AdS) at the rate of 0, 0.05, and 0.1 uM (0, 20, and 40 mg l^{-1}). Both of basal media consisted of N-organic compounds which were 6.8 μM (1 mg l^{-1}) *glutamine* (Gln), 500 mg l^{-1} casein hydrolysate (CH), 0.69 mM (120 mg l^{-1}) *arginine* (Arg), and 0.027 μM (2 mg l^{-1}) *glycine* (Gly). Bac medium is similar with MS medium excepted in the composition of nitrogen source in the form of KNO$_3$ (2528 mg l^{-1}), NH$_4$Cl (535 mg l^{-1}) which was used by Firoozabady and Moy (2004). The calli including original explants were placed in the room culture with 25±2°C, in the dark condition for 3 weeks. The variable observed were the longest and shortest diameter of the calli. Microscopical and histological examination was also conducted to check the type of the cell division and the development of somatic embryo. The histological analysis was modified method of Kiernan (1990), presented in Figure 1.

The factorial Completely Randomized Design was also used in the experiment of somatic embryo development. The first factor was embryogenic callus induction media and the second factor was regeneration media. The embryogenic callus induction media were MS+ or Bac+ with addition of AdS as mentioned above. The first regeneration medium was coded as Reg A which was MS basal medium without nicotinic acid and pyridoxine HCl with addition of 0.9 μM (0.18 mg l^{-1}) IBA, 1.1 μM (0.25 mg l^{-1}) BA, 0.09 μM (0.03 mg l^{-1}) GA$_3$ according to Perez *et al.* (2009). The second regeneration medium was coded as Reg B which was MS basal medium supplemented with

0.018 mM (4 mg l^{-1}) BA according to Firoozabady and Moy (2004). The treatments were replicated 6 times. The explants were illuminated 16 h per day with 1.000 lux irradiance provided by neon light and exposed to a temperature 25±2°C. The variables observed were percentage of browning and the number of mature somatic embryos.
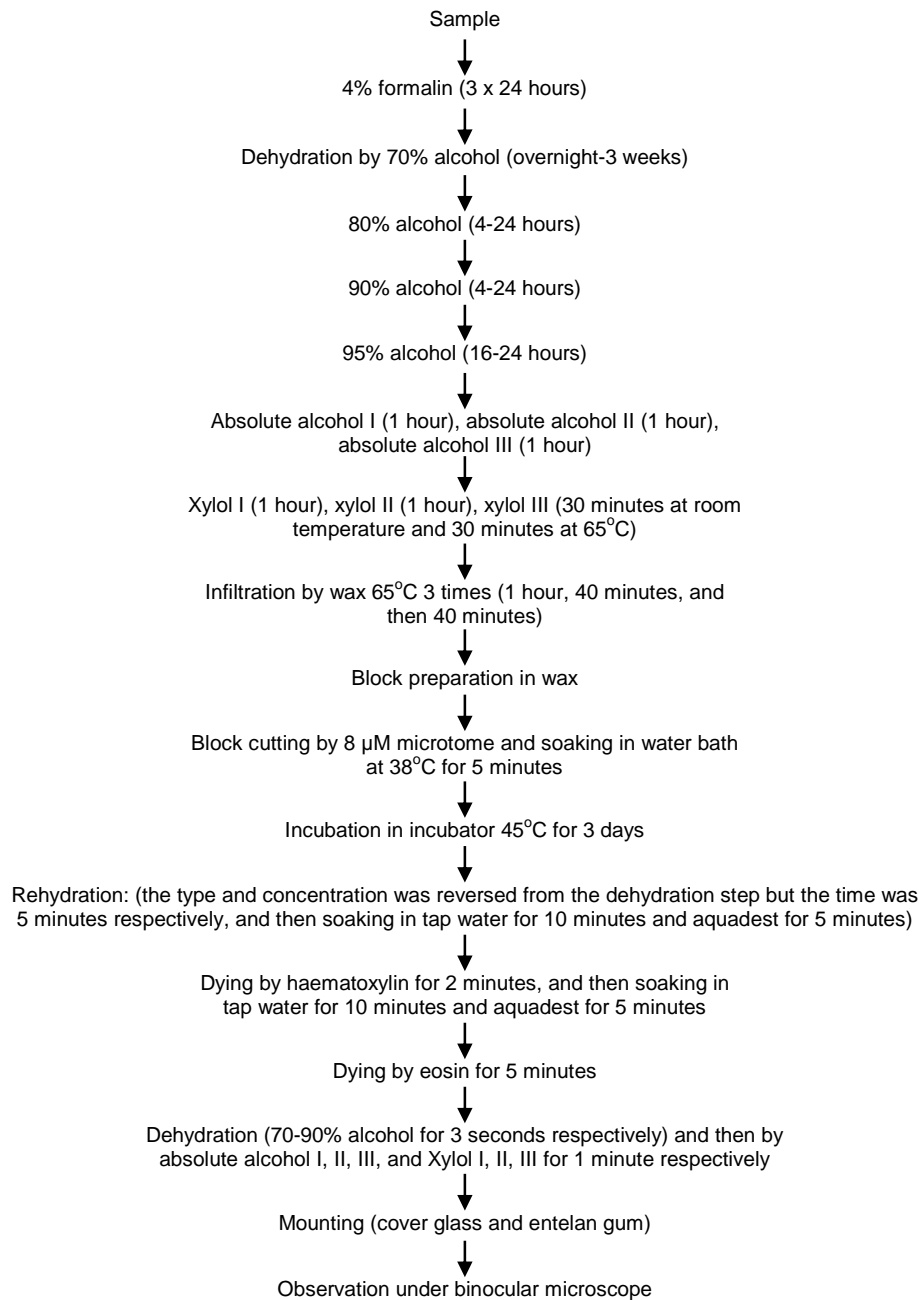
RESULT AND DISCUSSION

Indirect Organogenesis Regeneration

After 1-2 weeks, the shoot sections were swollen (Figure 2A), and then several leaves opened in consequence of the activity of plant growth regulator (Figure 2B). The callus formation was initiated 3 weeks after planting (Figure 2C). The most of calli initiated from leaf base area and partly initiated from injured leaf area near leaf base (Figure 2D-E). Firoozabady and Moy (2004) believed that leaf bases may either contain meristematic regions or possess newly developed tissue with rapidly dividing cells that are amenable to morphogenesis in the tissue culture. Suryowinoto (1996) explained that *Bromeliaceae* has adventitious meristem in the leaf base. The emergence of calli from injured leaf may be due to the effect of TDZ in the callus induction media. TDZ is a phenylurea-type compound which has high physiological activity like cytokinin (Sakakibara 2004).

Figure 3 showed that callus formation increased from 3 weeks to 8 weeks, and then the growth was stopped. The highest callus formation (90%) resulted from 41 μM 2,4-D but it was not significantly different with the result of 21 μM 2,4-D (Figure 3A). However, the 21 μM 2,4-D yielded the highest fresh weight significantly different with the other treatment (Figure 3B). It is indicated that the calli grew faster in 21 μM 2,4-D than the other treatments. Thus, 21 μM 2,4-D was the best treatment for inducing callus formation. This result was better than the previous research which conducted by Sripaoraya *et al.* (2003) who provided the lower frequency of callus formation of pineapple cultivar Phuket (58%) and also by Firoozabady and Moy (2004) who harvested 55% callus formation of pineapple cultivar Smooth Cayenne.

Visually, the 2,4-D induced compact and greenish calli (Figure 4A-C) rather than friable and yellowish calli which are usually observed in the embryogenic calli. Additionally, phenomenon of browning could be observed from the 41 and 62 μM 2,4-D treatments (Figure 4B-C). The highest concentration of (62 μM 2,4-D) supposed to caused cells necrotic since it is a herbicide agent.

Sample

↓

4% formalin (3 x 24 hours)

↓

Dehydration by 70% alcohol (overnight-3 weeks)

↓

80% alcohol (4-24 hours)

↓

90% alcohol (4-24 hours)

↓

95% alcohol (16-24 hours)

↓

Absolute alcohol I (1 hour), absolute alcohol II (1 hour),
absolute alcohol III (1 hour)

↓

Xylol I (1 hour), xylol II (1 hour), xylol III (30 minutes at room
temperature and 30 minutes at $65^{o}C$)

↓

Infiltration by wax $65^{o}C$ 3 times (1 hour, 40 minutes, and
then 40 minutes)

↓

Block preparation in wax

↓

Block cutting by 8 μM microtome and soaking in water bath
at $38^{o}C$ for 5 minutes

↓

Incubation in incubator $45^{o}C$ for 3 days

↓

Rehydration: (the type and concentration was reversed from the dehydration step but the time was
5 minutes respectively, and then soaking in tap water for 10 minutes and aquadest for 5 minutes)

↓

Dying by haematoxylin for 2 minutes, and then soaking in
tap water for 10 minutes and aquadest for 5 minutes

↓

Dying by eosin for 5 minutes

↓

Dehydration (70-90% alcohol for 3 seconds respectively) and then by
absolute alcohol I, II, III, and Xylol I, II, III for 1 minute respectively

↓

Mounting (cover glass and entelan gum)

↓

Observation under binocular microscope

**Figure 1.** The step of histological analysis of pineapple embryogenic callus.

The microscopical examination showed that 2,4-D-treated cells still bound in the tissue (Figure 4D) and the dispersed cells even did not show polarization (Figure 4E). The cell aggregate showed the symmetrical cell division (Figure 4F). This indicated that the cells were not embryogenic. It was explained that the non embryogenic cells can be determined by symmetrical division (Abrash and Bergmann, 2009; Paciorek and Bergmann, 2010). Furthermore, the non embryogenic cell aggregate grew to become nodular structure (Figure 4G) and finally to form plantlet which consisted of several roots and sometimes with little buds (Figure 4H), typically different with the characteristic of bipolar structure of somatic embryo.

On shoot regeneration step, there was a different response of the calli. The increase level of 2,4-D during callus formation caused the decrease shoot growth during regeneration on Kn containing media (Figure 5A-C). The necrotic tissue could not grow further as
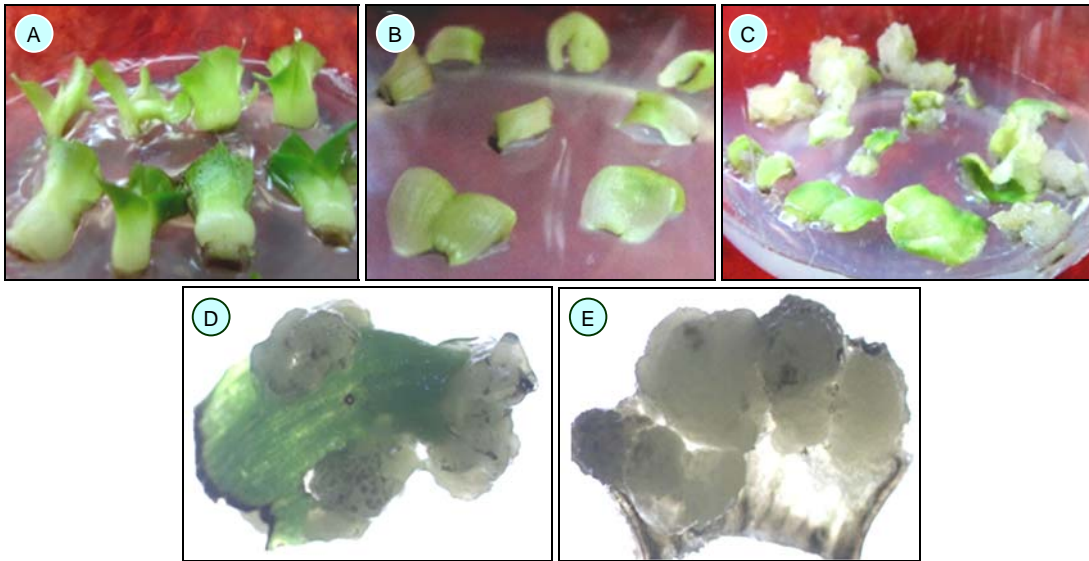
Figure 2. The steps of callus induction of pineapple leaf base explants treated by 2,4-D: half shoot sections (A), leaf base explants (B), callus initiation (C), callus emerged from injured leaf near leaf base (D), and callus emerged from leaf base area (E).
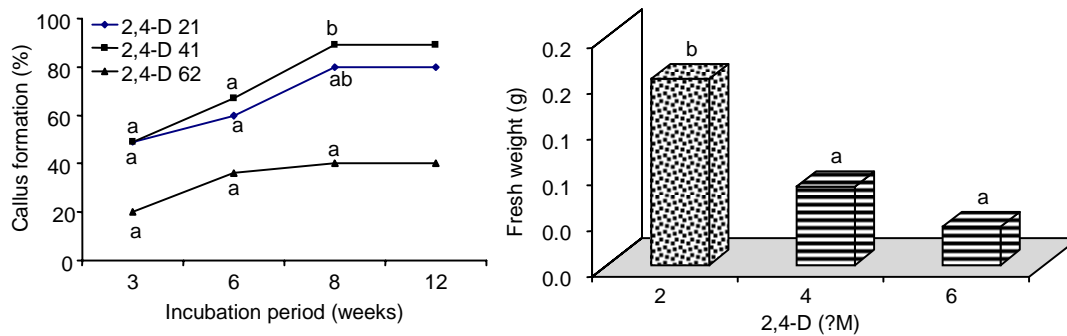
Post-hoc comparisons were performed by Duncan's multiple range tests (critical range p=0.05).

Figure 3. The effect of 2,4-D to the percentage of callus formation and fresh weight of calli.
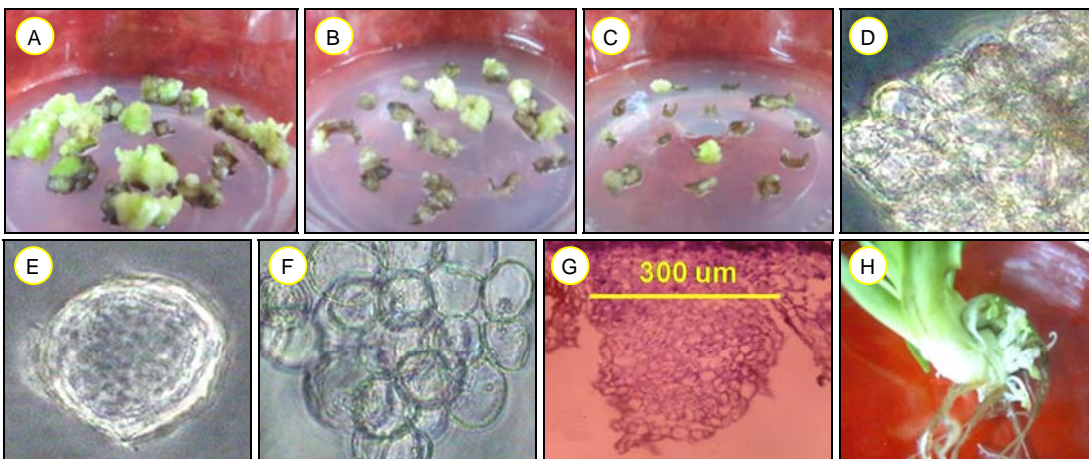
Figure 4. The response of pineapple explants to 2,4-D and microscopical observation of the cells: 21 µM (A), 41 µM (B), 62 µM (C), tissue (D), non polarized cell (E), non embryogenic cells aggregate (F), nodular structure (G), and plantlet (H).

shown in the treatment of 62 µM 2,4-D (Figure 5C). The high level of shoot regeneration (about 25 shoots/explant/2 months) resulted from 21 µM 2,4-D treatment (Figure 6A).

Figure 5 also showed the negative effect of high level of 2,4-D, especially at 62 µM which generated abnormal shoot (Figure 5F and Figure 6B). The abnormality could be observed visually where the axiality of the shoot was imbalance or the top of leaves were cleaved into two parts (Figure 5I). However, this phenotype was reversible. The abnormal shoots needed additional period for turning to normal morphology. After 3.5 months additional incubation, almost all of the abnormal shoots (more than 90%) changed to normal shoot (Figure 6C).

Indirect Somatic Embryogenesis Regeneration

Since the the single compound of 2,4-D could not induce embryogenic cells, the calli were then transferred onto N-organic compounds containing medium with or without of AdS. Firoozabady and Moy (2004) used the same amino acids and CH unless AdS for supporting embryogenic tissue formation.

Figure 7 showed that the globular embryos were bound in the tissue and they were not separated completely. This indicated that embryogenic calli
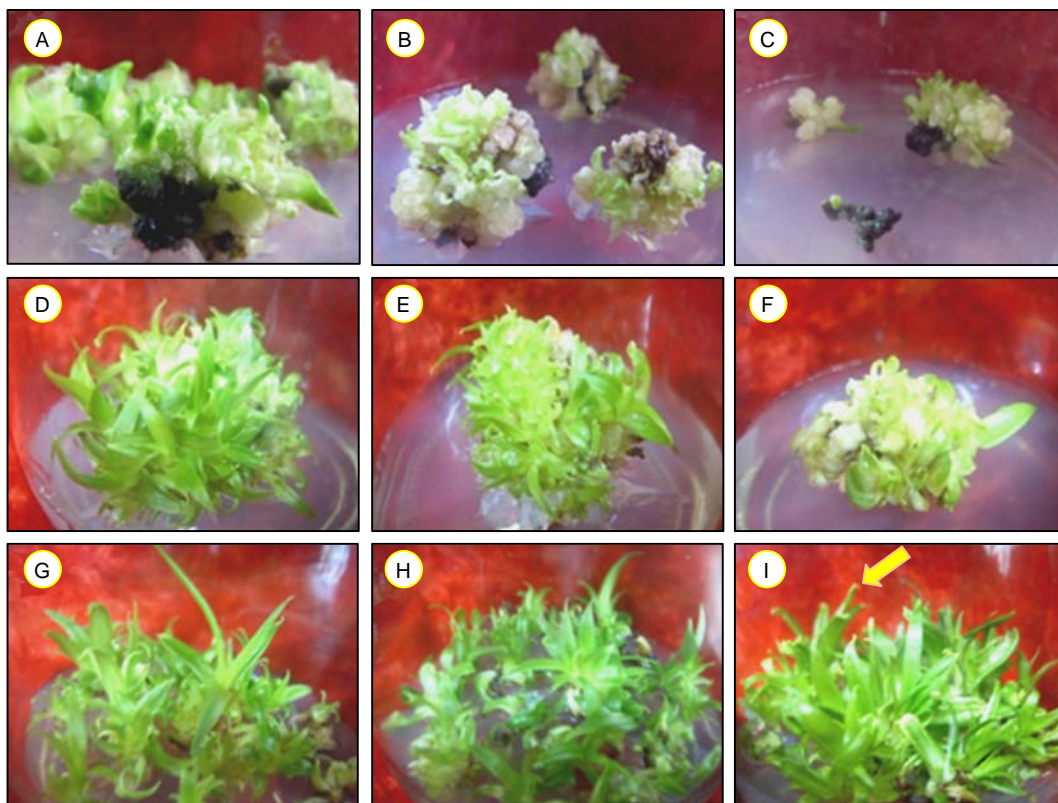
Figure 5. Performance of regenerated pineapple shoots during shoot regeneration (upper row) and shoot elongation (lower row): 21 µM 2,4-D (A and D), 41 µM 2,4-D (B and E), 62 µM 2,4-D (C and F). The arrow showed the cleaved leaf.
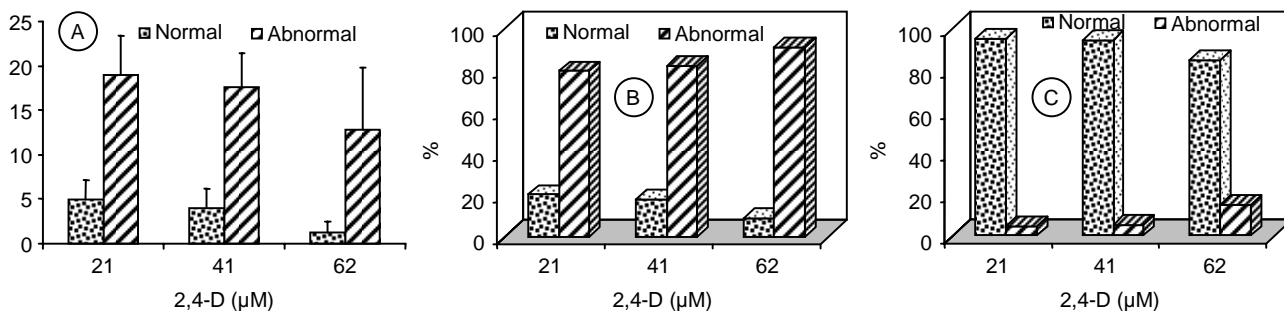

Figure 6. The effect of the level of 2,4-D to the regeneration of pineapple calli: the number of normal and abnormal shoots (A), the percentage of normal and abnormal shoots (B), and the conversion of abnormal shoot into normal shoot after 3.5 months additional incubation period (C).

formed in groups. The similar structure was observed by Firoozabady and Moy (2004). This structure was called as friable embryogenic tissue (FET) since individual globular embryos were group in a matrix.

Histological and microscopical examination showed a weakening of the cell-cell interaction of the N-organic compounds and AdS treated cells (Figure 8A). The similar fenomenon was also observed by Cunha and Fernandez-Ferreira (2012). This weakening indicated the starting stage of the somatic embryogenesis initiation. This assumption was strengthened with the existence of the isodiametric and meristematic cells which showing intense red staining of haematoxylin-eosin due to a densely rich cytoplasm with a high nucleoplasmic ratio, and reduced vacuole size. These meristematic cells were grouped into an aggregate called proembryogenic mass (PEM). A high magnification (400 times) of the embryogenic cells revealed the granular cells (Figure 8B) and asymmetrical cell division (Figure 8C). These granular cells might be contain of starch grains as a mark of embryogenic cell. Furthermore they developed into globular embryos (Figure 8D). It was suggested that Figure 8E shown the early stage of scutellar embryo which had a jutty structure on the
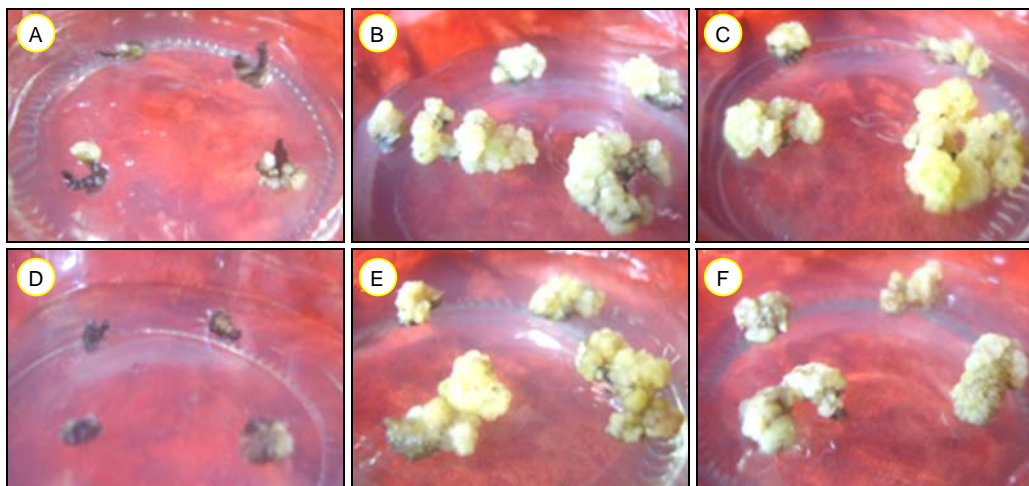
Figure 7. The performance of pineapple embryogenic calli on different kinds of media: MS medium + N-organic compounds (A), MS medium + N-organic compounds + 0.05 µM AdS (B), MS medium + N-organic compounds + 0.1 µM AdS (C), Bac medium + N-organic compounds (D), Bac medium + N-organic compounds + 0.05 mM AdS (E), and Bac medium + N-organic compounds + 0.1 µM AdS (F).
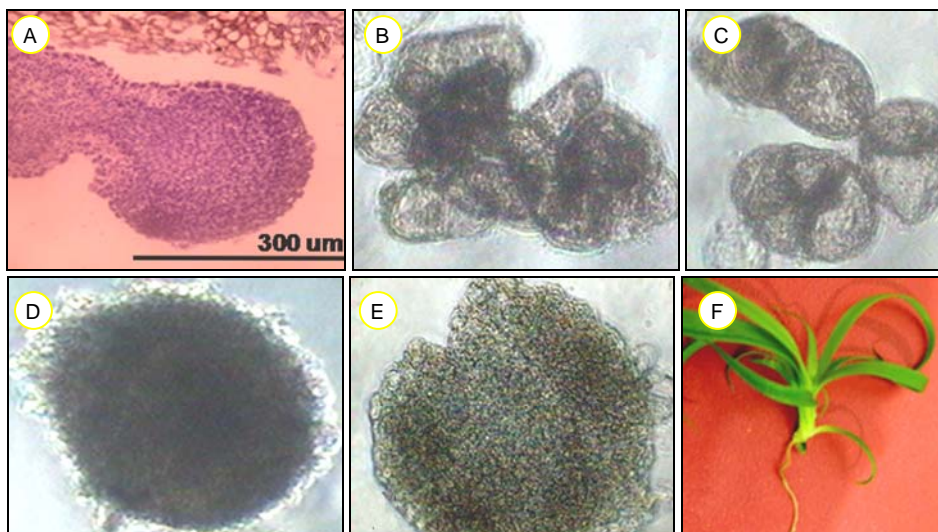
Figure 8. The events of pineapple somatic embryogenesis on MS medium with addition of N-organic compounds and 0.05 µM AdS: PEM (A), the separated embryogenic cells (B), a polarized cell and asymmetrical division of the 2-4-celled proembryos (C), globular stage (D), early scutellar stage (E), and germinated somatic embryos (F).

top. Unfortunately, the coleoptilar stage could not be observed in this study. Finally, the mature embryos developed into germinated embryos or plantlets (Figure 8F) which clearly showed bipolarity (shoot and root). After 5 months, the embryogenic cells turned to dark brown and mixed with the non embryogenic cells. It indicated that the embryogenic competent lost accompanied with long period of the culture and they need to be subcultured immediately before they turn to brown color completely.

Generally, all of embryogenic callus induction media yielded globular and semi dispersible calli. However, the browning calli were observed on media without addition of AdS (Table 1). Moreover, there was no interaction between basal media and the rate of AdS and there was no significant difference of all treatment to the growth of embryogenic calli (Table 2). It indicated that the role of N-organic compounds were more important than the role of AdS to proliferate embryogenic calli.

As reported by many researchers, nitrogen (N) is also fundamental element for plant cell and tissue culture, being essential for the synthesis of DNA, RNA, and protein. When MS medium compared to Bac medium, the total N in MS medium is higher (60 mM) than that in Bac medium (35 mM). However, the ratio between N-oxidized (NO_3^-) and N-reduced (NH_4^+) is higher in Bac medium (2.5 : 1) than that in MS medium (2 : 1). It means that the ratio of N-oxidized and N-reduced in both media is rather not different. Therefore, there was not difference response resulted from all of media to the growth of calli (Table 2). Cunha and Fernandez-Ferreira (2012) mentioned that the balance between both ionic forms (NO_3^- and NH_4^+) played a dramatic role on the induction of somatic embryogenesis of flax hypocotyls explants. Nitrate is important for calli differentiation and growth.

On the regeneration step, there was no interaction between the embryogenic callus induction medium with the regeneration medium to the level of

Table 1. The performance of pineapple calli on embryogenic callus formation media.

Treatment	Performance of the calli
MS+	Globular, semi dispersible, some of them were browning
MSAdS0.05	Globular, semi dispersible, whitish
MSAdS0.1	Globular, semi dispersible, whitish and greenish
Bac+	Globular, semi dispersible, some of them were browning
BacAdS0.05	Globular, semi dispersible, yellowish
BacAdS0.1	Globular, semi dispersible, yellowish

MS+ = MS medium + N-organic compounds, MSAdS0.05 = MS medium + N-organic compounds + 0.05 µM AdS, MSAdS0.1 = MS medium + N-organic compounds + 0.1 µM AdS, Bac+ = Bac medium + N-organic compounds, BacAdS0.05 = Bac medium + N-organic compounds + 0.05 µM AdS, BacAdS0.1 = Bac medium + N-organic compounds + 0.1 µM AdS.
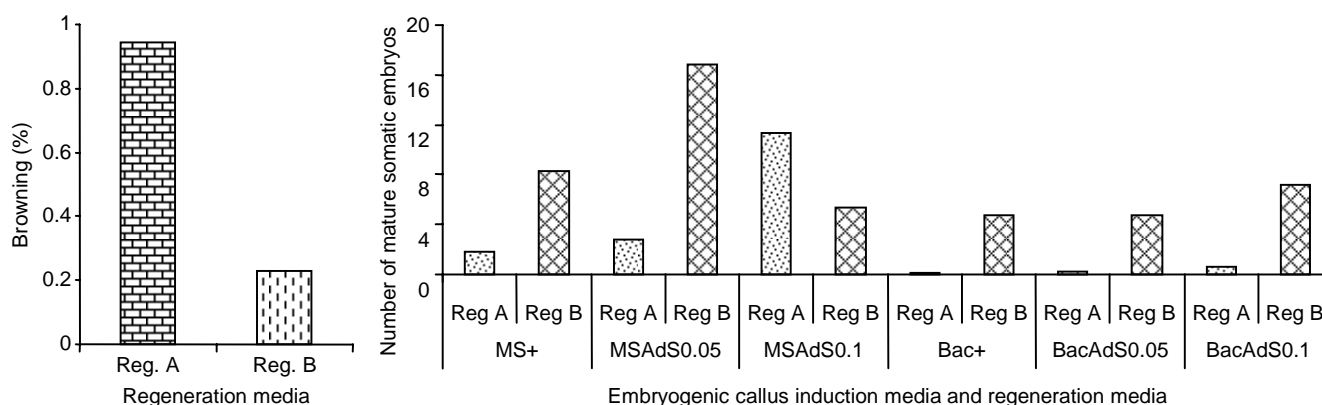
Reg. A = modified MS medium without nicotinic acid and pyridoxine HCl with addition of 0.9 µM (0.18 mg l^{-1}) IBA, 1.1 µM (0.25 mg l^{-1}) BA, 0.09 µM (0.03 mg l^{-1}) GA$_3$, Reg. B = MS medium with addition of 0.018 mM (4 mg l^{-1}) BA, MS+ = MS medium + N-organic compounds, MSAdS0.05 = MS medium + N-organic compounds + 0.05 µM AdS, MSAdS0.1 = MS medium + N-organic compounds + 0.1 µM AdS, Bac+ = Bac medium + N-organic compounds, BacAdS0.05 = Bac medium + N-organic compounds + 0.05 µM AdS, BacAdS0.1 = Bac medium + N-organic compounds + 0.1 µM AdS.

Figure 9. The effect of regeneration medium to the level of browning and the interaction between the embryogenic callus induction medium with the regeneration medium to the number of mature somatic embryos, 6.5 months after subculture.

Table 2. The effect of basal media and adenine sulphate to the growth of pineapple *FETs* cultivar Smooth Cayenne.

Basal media	Adenine sulphate (µM)	The longest diameter of calli (mm)	The shortest diameter of calli (mm)	The average of calli diameter (mm)
MS+	0	6.8	5.6	6.2
	0.05	10.3	5.1	7.7
	0.1	8.9	6.0	7.5
Bac+	0	8.2	5.1	6.7
	0.05	7.3	4.3	5.8
	0.1	8.4	5.4	6.9

MS+ = MS + N-organic compounds, Bac+ = Bac + N-organic compounds.
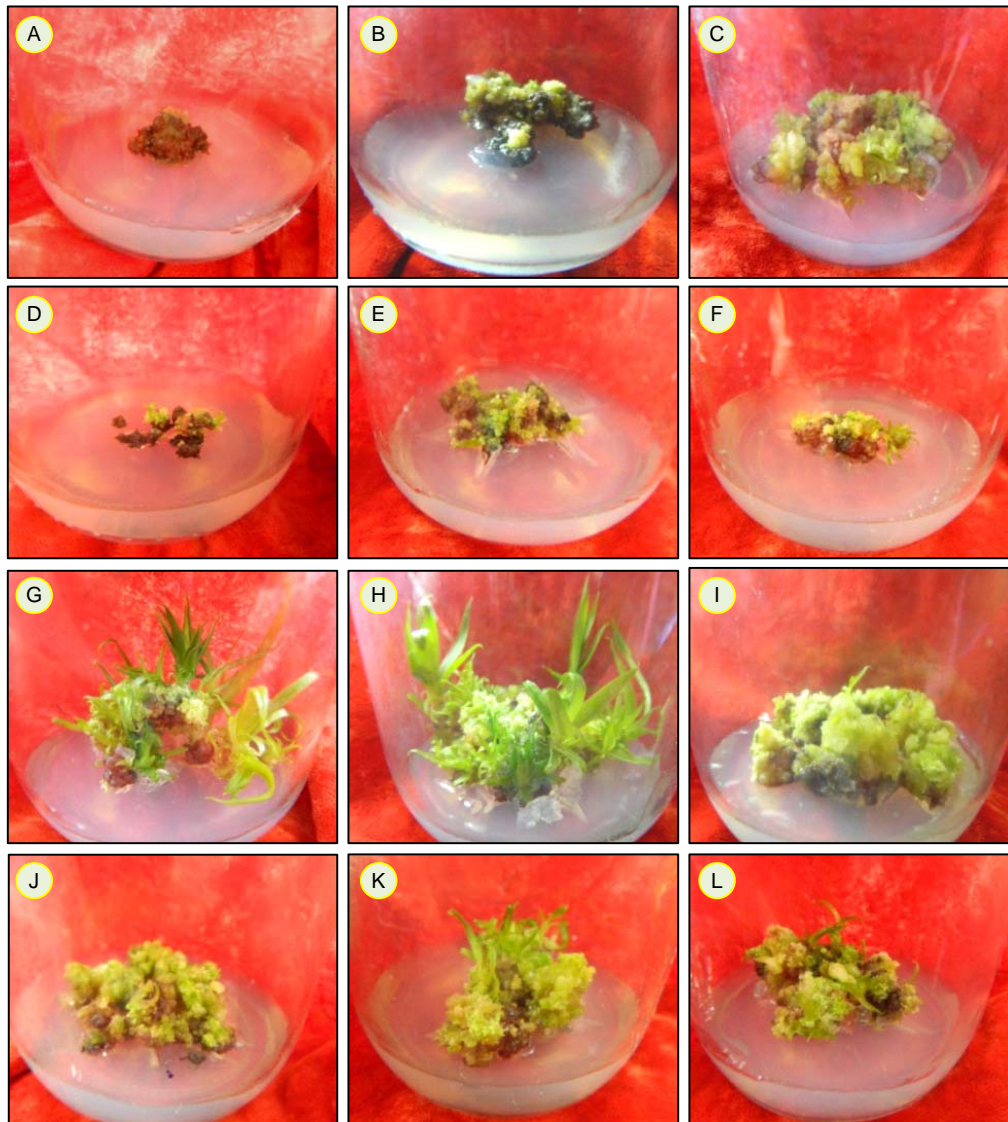
Figure 10. The performance of pineapple somatic embryos (8 months) on six different kinds of embryogenic callus induction media (A and G = MS medium + N-organic compounds, B and H = MS medium + N-organic compounds + 0.05 µM AdS, C and I = MS medium + N-organic compounds + 0.1 µM AdS, D and J = Bac medium + N-organic compounds, E and K = Bac medium + N-organic compounds + 0.05 µM AdS, and F and L = Bac medium + N-organic compounds + 0.1 µM AdS) and two different kinds of regeneration media (the upper rows = MS + 0.9 µM IBA + 1.1 µM BA + 0.09 µM GA_3 and the lower rows = MS basal medium supplemented with 0.018 mM BA).

browning but the single factor of regeneration medium affected that variable. The MS medium containing of 0.018 mM BA medium could reduce the level of browning (Figure 9A).

As mentioned above, that AdS did not affect the proliferation of embryogenic calli. Interestingly, there was interaction between the embryogenic callus induction medium with the regeneration medium to the number of mature somatic embryos (Figure 9 and 10). On this medium, the number of mature somatic embryos reached about 17 embryos/explant. Structurally, AdS is similar with cytokinin compounds since it has an adenine ring which has a rich of nitrogen compound. The AdS may act as nitrogen source in the synthesis of RNA, DNA, and protein for cell division. Therefore it may play important role in the embryo development.

CONCLUSION

The single auxin of 2,4-D was not enough to induce embryogenic cells. Therefore a part non embryogenic calli were regenerated through indirect organogenesis. The best treatment for organogenesis was 21 μM 2,4-D because it provided high level of callus formation (80%), the highest fresh weight (0.2 g/explant), and the highest number of shoot (about 25 shoots/explant). The friable embryogenic callus formation could be induced by N-organic compounds with or without addition of AdS. There was interaction between the embryogenic callus induction medium with the regeneration medium to the number of mature somatic embryos. The combination treatment of MS medium enriched with N-organic compounds and 0.05 μM AdS as embryogenic callus induction medium and MS medium with addition of BA 0.018 mM BA as regeneration medium was the best treatment which yielded about 17 embryos/explant.

ACKNOWLEDGEMENTS

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