Robust in vitro propagation and regeneration of *ubikuning* high beta carotene cassava genotype through somatic embryogenesis

SUPATMI1♥, HANI FITRIANI1♥♥, NURHAMIDARR RAHMAN1, N. SRI HARTATI1, ENNY SUDARMONOWATI1

Research Centre for Biotechnology, Indonesian Institutes of Sciences. Cibinong Science Centre, Jl. Raya Bogor KM 46, Cibinong 16911, West Java, Indonesia. Tel.: +62 218754587, Fax: +62 218754588, *email: patmi_bio@yahoo.com, **hfitriani76@yahoo.com


Abstract. Supatmi, Fitriani H, Rahman N, Hartati NS, Sudarmonowati E. 2017. Robust in vitro propagation and regeneration of *ubikuning* high beta carotene cassava genotype through somatic embryogenesis. *Nusantara Bioscience* 9: 352-360. *Ubi kuning* is a local genotype of cassava with high beta carotene content but the development of this genotype is still low because of plant disease susceptibility. Objectives of this study were to robust induce and regenerate somatic embryos of *ubikuning* in vitro as well as to define a protocol of cyclic somatic embryogenesis of *ubikuning*. Different size of leaf lobes, various concentration of picloram and different light conditions were tested to produce an effective and efficient somatic embryos (SEs). The best response of the induction of embryogenic callus was observed in leaf lobes explant with range size of 1-3 mm and >5mm cultured on induction medium (MS + 4% sucrose + 4 µM CuSO4 + 0.1 mM Glutamine + 0.8% Microagar) supplemented with either 10 or 18 mg/L picloram grown under dark light for 4 weeks. Retransferring embryogenic callus to the same medium supplemented with 16 mg/L picloram gave the advanced development of primary somatic embryos (PSEs) after 70 d grown under both dark and light condition treatments. A positive correlation between globular and cotyledon stages was obtained in all treatments (P≤ 0.01). The highest shoot and root growth (30% and 25%) was achieved in the regeneration of cotyledonary like-tissues cultured on callus embryogenic media (CEM) (MS basal+ 2.5 µM CuSO4 + 3% sucrose + 2.75 g/L phytagel) supplemented with 1.6 mg/L of BAP (6-Benzylaminopurine).

Keywords: Light conditions, picloram, somatic embryogenesis, *ubikuning*

INTRODUCTION

Cassava (*Manihott esculenta* Crantz.), known as one of important crop in the lowland tropics, is a major source of calories to nearly 500 million people worldwide (Montagnac et al. 2009). Cassava is also the targeted crops in the biofortification programme for increasing its nutritional value because it becomes a staple food in most of Sub-saharan Africa (Thakkar et al. 2009). In Indonesia, the total production of cassava roots reach more than 5 million tons per year (Hermiati et al. 2012). However, most of used-cassava varieties still have poor nutritional quality such as low contents of provitamin A (proVA) carotenoids, iron, zinc and protein and also toxic cianogens (Njoku et al. 2005; Atehnkeng et al. 2006; and Feitosa et al. 2007), reported (Ihemere 2003; Hankoua et al. 2005; Saelim et al. 2007). The potential to increase cassava productivity, nutritional value and for breeding purposes, is the potentiality of using cassava as a raw material for many technologies and purposes. According to Von Arnold et al. (2002) somatic embryogenesis is the development process of plant bipolar embryos, known as somatic embryos, which it is derived from a nonzygotic cell without vascular connections with the original tissue. Besides somatic embryogenesis is used for clonal mass propagation, it is also a valuable tool for genetic improvement of many plant species because of its single cell origins (Stasolla and Yeung 2003). Somatic embryos play a key role in genetic transformation methods, cell selection, somatic hybrid and polyploid plant production (Aboshama 2011; Kamle et al. 2011). Two critical events are involved in the early programme of somatic embryogenesis process i.e., (i) the induction of cytodifferentiation of pre-embryonic cells, and, (ii) the unfolding development of these pro-embryonic cells forms the advanced embryonic stages (primary somatic embryos) (Kumar et al. 2008). In vitro plant propagation of cassava through somatic embryogenesis has been successfully reported (Ihamere 2003; Hankoua et al. 2005; Saelim et al. 2005; Atehnkeng et al. 2006; and Feitosa et al. 2007), although the successful induction of those somatic embryos is depended on many factors including genotypes, explant source, the concentration and combination type of plant.
growth regulators as well as the environmental conditions (Saelim et al. 2006; Sudarmonowati et al. 2009). Ihemere (2003) has reported that cassava cultivars originated from Africa gave a different response in culture media during the process of somatic embryos induction compared with South American varieties. Therefore, objectives of this study were to induce and regenerate somatic embryos of ubi kuning in vitro as well as to define a protocol of cyclic somatic embryogenesis by employing different size of leaf lobes as explant source, various concentrations of picloram in culture media and different treatments of light conditions.

MATERIALS AND METHODS

Plant materials
The axenic culture of ubi kuning cassava was prepared by harvesting shoot stems from Field Station of Research Center for Biotechnology, Indonesian Institute of Sciences which were then surface sterilized using Sunlight (commercial detergent) under tap water running for 45 min. Afterward, they were sterilized by 4% Dethane (antifungicide) for 60 min followed by 70% ethanol (5% sodium hypochlorite solution) for 5 min and 70% ethanol for 1 min. After washing three times with sterilized reverse osmosis water, shoot apex and axillary buds were excised and cultured on Murashige and Skoog (MS medium) (Murashige and Skoog 1962) supplemented with 4% sucrose and solidified with 0.7% Swallow agar. The pH was adjusted to 5.8 prior to autoclaving. The proliferated shoots were repeatedly transferred in the same medium at 30 days intervals. These culture were grown under white fluorescent light for 16 h/day photoperiod and 25 ± 2°C until they produced young leaf lobes.

Embryogenic callus induction
Young leaf lobes (immature leaf lobes from shoot tips) taken from shoots and axillary bud in vitro were isolated and used for induction of somatic embryos. They were excised by using scalpel and then cultured on induction medium (MS + 4% sucrose + 4 μM CuSO4 + 0.1 mM Glutamine + 0.8% Microagar) at 25-28°C. Different size type of leaf lobes (1-3 mm and >5mm-long) and various concentrations of phytohormone picloram (10, 16, 18 mg/L) were tested for their efficiencies in the production of embryogenic callus by employing different size type of leaf lobes and used for induction of somatic embryos. They were excised by using scalpel and then cultured on induction medium (MS + 4% sucrose + 4 μM CuSO4 + 0.1 mM Glutamine + 0.8% Microagar) at 25-28°C. Different size type of leaf lobes (1-3 mm and >5mm-long) and various concentrations of phytohormone picloram (10, 16, 18 mg/L) were tested for their efficiency in somatic embryogenesis medium and cultured in dark condition at the same condition for 3-4 weeks. Embryogenic callus formation was observed using stereo microscope with 8x magnification LEICA E24HD. The frequency of embryogenic callus formation [ (explants with embryogenic callus/ total of explants)*100] was then evaluated after 3 weeks of culture.

Induction of cyclic somatic embryogenesis
After 4 weeks of culture on callus induction medium, embryogenic callus segments were then cut into small clump pieces and transferred to induction medium with various concentration of picloram (10, 16, 18 mg/L). They were then placed into a different light conditions (dark and light conditions) to produce cyclic somatic embryogenesis with the first cycle was known as primary somatic embryos (PSE). Each treatment consisted of three replication with each replication comprised of 10 explants. Proembrionic stage developed from embryogenic callus segments was recorded after 70 days of culture on treatment medium and light conditions. Macroscopic features of pre-embryonic stages i.e globular and cotyledonary-like tissues shape of somatic embryos were observed using a stereo microscope LEICA E24HD with 8x magnification.

Regeneration of somatic embryo
The mature primary somatic embryos (PSEs) which could be seen from the formation of cotyledonary like-tissue was then cultured in CEM (callus embryogenic media) comprised of MS basal+ 2.5 μM CuSO4 + 3% sucrose + 2.75 g/L phytagel supplemented with various concentrations of BAP (0; 0.8; 1.2; 1.6 mg/L). Each treatment consisted of 4 replication plate with each plate comprised of 5 explants. The observation was conducted after three weeks of culture including the number of survival rate, shoot and root formation, and the cotyledonary like tissues using a stereo microscope LEICA E24HD with 8x magnification.

Statistical analysis
Number of globular, cotyledonary stages, shoot and root growth resulted from treatment media were analysed by a mixed-model ANOVA and means by the Duncan test. Data resulted from a different light conditions (dark and light condition) treatment was also analyzed by analysis of variance T test. Correlation coefficients were analysed by Bivariate test continued with Pearson test. All of results were analysed using SPSS software (SPSS for windows 16.0 version).

RESULTS AND DISCUSSIONS

Induction of embryogenic callus
In order to induce callus formation, preliminary experiment using various explants (i.e nodes, leaves and immature leaf lobes) of ubi kuning cassava cultured on MS supplemented with various plant growth regulators such as NAA (1-Naphthaleneacetic acid) and Kinetin has been conducted (data not shown). Among these explants, callus was only obtained using leaf lobes despite these callus did not initially form embryogenic callus. Afterward, leaf lobes were primary used as explant material in the induction of embryogenic callus by employing different size type of leaf lobes and various concentration of picloram. Different size type of leaf lobes (1-3 mm and >5mm-long) have been tested for their efficiencies in the production of embryogenic callus by culturing on to (MS + 4% sucrose + 4 μM CuSO4 + 0.1 mM Glutamine + 0.8% Microagar) supplemented with various concentrations of picloram (10, 16, 18 mg/L) in dark light conditions at 25-28°C. Most of explants treated with various concentration of picloram could develop into callus formation after they were incubated for 3 weeks in dark light condition. The highest
callus formation (85 and 100%) was obtained after leaf lobes were cultured on to induction medium supplemented with 16 mg/L picloram (PG16) (Table 1). Meanwhile, the lowest frequency of callus formation (63 and 44%) was achieved in those explants cultured on to induction medium supplemented with 10 mg/L picloram (PG10) (Table 1).

Of all size type of leaf lobes which were treated, both size of 1-3 and >5 mm showed a high response of callus formation. However, among the developed-callus formation, not all of them were embryogenic. The obtained embryogenic calluses were characterized by their yellowish colour and friability (Figure 1). Results showed that no embryogenic callus was obtained when explants of size >5mm were cultured on induction medium supplemented with 16 mg/L picloram (PG16) (Table 1). On the other hand, leaf lobes with size of >5mm cultured on medium induction supplemented with 10 mg/L picloram (PG10) or 18 mg/L picloram (PG18) resulted in 100% embryogenic callus response (Figure 1b&1e). The best response of embryogenic callus formation was also obtained from leaf lobes with range size of 1-3 mm cultured on both PG10 and PG18 media with the percentage of 60 and 64, respectively (Table 1, Figures 1a&P1d). This is in accordance with Saelim et al. (2006) who reported that leaf lobes is the best material for the induction of somatic embryos in cassava Asian cultivars and with Ravindran et al. (2015) in African and Indian cassava cultivars. Furthermore, leaf lobes had a high response in the production of somatic embryos in most of culture media supplemented with various plant growth regulators although the response of somatic embryo development is depended on physiological state of plants for providing leaf lobes (Atehnkeng et al. 2006, Sudarmonowati et al. 2009).

Somatic embryos was initially formed by the development of glowsy embryogenic callus after three weeks cultured on induction medium supplemented with picloram under dark light condition at 25-28°C (Figure 1). Moreover, observations using stereo microscope showed that the early pro-embryonic stage, known as noduler shape of somatic embryos, was formed on the callus surface (Figure 1). Of all well-developed somatic embryos, the fastest and the largest number of noduler shape was apparently obtained in PG10 and PG18 media (Figure 1a-1e). According to Zavattieri et al. (2010), auxin (i.e picloram) is considered to be the most important plant growth regulators in the regulation of somatic embryogenesis. Both the endogenous auxins and the application of exogenous auxins determines the success process of somatic embryo induction and further PSEs stage development to become plantlets (Jimenez. 2001).

These results were similar to other published papers, which employed media supplemented with various concentrations of picloram in the induction of somatic embryos in many cassava genotypes such as KU 50 and Hanatee (Saelim et al. 2006), cassava genotypes from Northeast Brazil (Feitosa et al. 2007), Indonesian local genotype (i.e Iding, Gebang, Apuy, Roti) (Sudarmonowati et al. 2009 and Fitriani et al. 2012), although the source of explants, the composition of culture media, and the concentration of plant growth regulators were generally different.

**Maturation and Germination of Primary Somatic Embryos (PSEs)**

The maturation process of somatic embryogenesis is an important stage because the degree of maturation can significantly affect the germination capability of somatic embryos (SEs) (Malabadi & Van Staden 2005; Hankoua et al. 2006). During the maturation stage, the somatic embryos will accumulate reserves and achieve desiccation tolerance (Hankoua et al. 2006). This study showed that somatic embryos stop developing the nodular stage in induction media containing picloram grown under dark light conditions for 4 week. For this reason, those nodular stages were re-transferred onto the same media. To evaluate the development of those somatic embryos; therefore, they were grown under different light condition (dark and light condition) treatments. Interestingly, those nodular stages of somatic embryos were subsequently developed into pre-embryonic stages i.e globular stage and immature cotyledon stage both under dark and light conditions. The development of pre-embryonic stages in terms of light white and yellow globular, heart, torpedo and green immature cotyledon stages was observed from 7 to 70 days (Table 2 and Figure 2).

**Table 1.** The response of *ubi kuning* callus formation derived from a different leaf lobes size type cultured on a different picloram concentration medium after grown under dark condition for 3 weeks

<table>
<thead>
<tr>
<th>Callus development</th>
<th>Leaf lobes</th>
<th>Induction media</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type size</td>
<td>PG10</td>
</tr>
<tr>
<td>Callus formation (%)</td>
<td>1-3 mm</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>&gt;5 mm</td>
<td>44</td>
</tr>
<tr>
<td>Embryogenic callus formation (%)</td>
<td>1-3 mm</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>&gt;5 mm</td>
<td>100</td>
</tr>
</tbody>
</table>

Note: Induction media (MS + 4% sukrosa + 4 µM CuSO4 + 0.1 mM glutamine + 0.8% microagar) supplemented with 10 mg/L picloram (PG10), 16 mg/L picloram (PG16), 18 mg/L picloram (PG18)

**Table 2.** The differences of *ubi kuning* primary somatic embryos stages 70 d after treatment with various concentrations of picloram under dark and light conditions

<table>
<thead>
<tr>
<th>Media</th>
<th>Globular stage</th>
<th>Immature cotyledon stages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dark condition</td>
<td>Light condition</td>
</tr>
<tr>
<td></td>
<td>ns</td>
<td>*</td>
</tr>
<tr>
<td>PG10</td>
<td>2.00 ± 0.87  ns</td>
<td>5.00 ± 1.74  b</td>
</tr>
<tr>
<td>PG16</td>
<td>3.00 ± 1.02  ns</td>
<td>17.00 ± 5.71  a</td>
</tr>
<tr>
<td>PG18</td>
<td>3.19 ± 2.45  ns</td>
<td>3.19 ± 1.55  b</td>
</tr>
</tbody>
</table>

Note: Means ± standard error within a column followed by the different letters in each column are significantly different at P≤0.05 by ANOVA. ns: not significance, *: significantly different
In this study, the observation of PSEs maturation and germination was focused on the observation of globular and immature cotyledon stages due to the fastest cycle of other somatic embryo stages i.e heart and torpedo stage which could not be observed effectively. Those PSEs cycle was observed microscopically after 7 days cultured in both different picloram concentration and light conditions (Table 2 and Figure 2).

Generally, the differentiation of PSEs beyond the globular stage and its subsequent maturation requires the removal of growth regulators from the medium or reduction of their concentrations (Kordestani & Karami 2008). However, in this study, retransferring to the same media supplemented with picloram concentration grown either under dark or light condition showed the advanced development of *ubi kuning* somatic embryos. Interestingly, 16mg/L picloram-containing medium gave the best response for PSEs development compared to other picloram concentrations (Figure 2c).

From correlation analysis between globular and immature cotyledon stages grown under both dark and light conditions, results showed that the significant correlation between different SEs stage and light condition has been obtained (Table 3 and Figure 3). A positive correlation ($P \leq 0.05$) was observed at globular and immature cotyledon stage grown under dark conditions ($R^2 = 0.339$). Furthermore, a positive correlation ($P \leq 0.01$) was also observed at globular and immature cotyledon grown under light conditions ($R^2 = 0.424$). It means that the more globular formation would likely form the more immature cotyledon when they were grown both under dark and light conditions. Therefore, it is predicted that the germination to be shoots will be highly obtained. This result was in accordance to Saelim et al. (2007) who reported that the maturation of primary and secondary somatic embryos could happen when the cyclic somatic embryogenesis including the formation of globular, heart and torpedo stages can form distinct bipolar structures with expanded cotyledons (Saelim et al. 2007). Jimenez (2001) also reported that the formation of pre-embryonic mass is associated with the increase of DNA demethylation leading to the gene product synthesis; therefore, pre-embryonic mass should complete the globular stage of somatic embryogenesis prior to generating a new plant variety.
Regeneration of cotyledonary like-tissues of *ubi kuning* somatic embryos

One of the important aspect for in vitro clonal propagation through somatic embryogenesis is the ability of plant to regenerate and to self-multiply from cell cultures and tissues to be plantlets. In the process of plant propagation in vitro, the need of plant growth regulators is very important in order to trigger the shoot growth for massive somatic embryo regeneration (Atehnkeng et al. 2006). One of the plant growth regulators often used is BAP. In this study, the varied concentration of BAP (0; 0,8; 1,2; 1,6 mg/L) in the CEM (Callus embryogenic media) which comprised of MS basal + 2.5 μM CuSO4 + 3% sucrose + 2.75 g/L phytagel could initiate the formation of shoot and root of *ubi kuning* PSEs in the late-pre-embryonic stages namely cotyledonary like-tissues after 7 DAP (days after planting). The emerging shoot could be seen from the elongation of shoot-up and the formation of the initial leave candidates, while the root formation could be seen from the white long spot in the basal of explants (Table 4 and Figure 4).

---

**Figure 2.** The differences of somatic embryos development at globular and immature cotyledon stages after 7 days of culture on induction medium supplemented with various picloram concentrations under both dark and light condition.

---

Dark condition | Light condition
---|---

PG10

PG16

PG18
Results from the first day after planting (1 DAP) to the third DAP showed that not all observed explants could develop the shoot or root formation. In order to know the differences amongst the treatments, a further analysis using ANOVA test followed by Duncan test ($\alpha=5\%$) was conducted after three weeks of observation (Table 4). Results showed that the cotyledonary-like tissues of *ubi kuning* derived from PSEs showed a positive response after they were treated in CEM media supplemented with various concentrations of BAP. Those responses could be seen in the observed parameters including the percentage of survival rate, the shoot growth, the root growth and the development of further cotyledonary-like tissues. However, the further analysis using Duncan test showed that those observed parameters did not show a significant difference in each treatment media. This results indicated that the treatment of various concentration of BAP did not show a different response in all observed explants because three weeks is not sufficient to get optimal response. According to Saelim et al. (2006), the regeneration process or shoot growth of callus is very complex depended on many factors including genotype, type of explants, the balance concentration of plant growth regulators and the physiological conditions of callus. Based on three-weeks of observation, all the explants were in good conditions. Generally, the survival rate of explants was 100% during the observation. This might correlate with the appropriate concentration of CEM media and plant growth regulators which did not cause the tissue damage so that the explant death could be hindered effectively. Another shoot growth parameters showed that explants treated in CEM media supplemented with 1.6 mg/L of BAP had a higher percentage of shoot growth (30%) (Table 4 and Figure 4). The increase number of shoot formation obtained in the high concentration of BAP apparently happen due to the ability of cytokinin to increase the protein synthesis (Mohd et al. 2011). According to Kurakawa et al. (2007), cytokinin is an affective plant growth regulators which is...
consistently triggered the double shoot formation in meristem culture, apex shoot culture and shoot culture. However, the need of cytokinin in each plants is different in both type and concentration, but most of plants give the positive response in the addition of synthetic cytokinin such as BAP.

Based on the root formation of the explants, result showed that the root growth of cotyledonary like-tissues derived from PSEs of *ubi kuning* was obtained from 15% to 25%. The highest root growth was achieved in explants cultured on treatment media supplemented with 1.6 mg/L BAP. Meanwhile, the supplementation of cytokinin (BAP) in the treatment media could not inhibit the root growth development and also trigger the shoot formation. This could be seen in most of explants cultured in all CEM media supplemented with various concentration of BAP showing the root development although the root growth across the treatment media did not significantly different based on data analysis using ANOVA followed by Duncan test (Table 4 and Figures 4).

Of all cyclic somatic embryogenesis and regeneration of *ubi kuning*, most of regenerated somatic embryos could form plantlet (Figure 5). However, the efficiency of multiplication rate and the best media for plantlet growth as well as the efficiency of plantlet survival rate in the field are still further observed.

As conclusion, somatic embryogenesis of *ubi kuning* is significantly depended on the size of leaf lobe explants, the concentration of picloram in the culture medium and also the light condition. The cyclic somatic embryogenesis protocol and the efficiency of somatic embryos development of *ubi kuning* has been established, allows for the application of *ubi kuning* somatic embryos for clonal propagation and other genetic engineering purposes. The regeneration of somatic embryos to be plantlets had been established although the efficiency of plantlet survival rate in the field should be further observed.

---

**Table 4.** The morphological performances of cotyledonary like tissues derived from PSEs of *ubi kuning* after culturing in treatment media for three weeks. The left row showed the treatment media consisted with various concentration of BAP, while the above column showed the growth parameters including shoot, root, shoot and root formation and the cotyledonary like tissues formation.
Figure 5. Cyclic somatic embryogenesis and regeneration of ubi kuning derived from leaf lobes explants. A. Leaf lobe explant with size of 1-3 mm; B. Embryogenic callus; C. Preembrionic stages of somatic embryos; D. Immature cotyledon stage of somatic embryos; E. Cotyledonary like-tissues; F. Shoot growth; G. Shoot and root growth; H. Plantlet with young full-leaves and root; I. Mature plantlets ready to be acclimatized in the field.

ACKNOWLEDGEMENTS

This research was part of the “DIPA Unggulan” Research Grant of Indonesian Institute of Sciences (LIPI) Project 2017. The authors would like to thanks to Mr. Nawawi for providing explants from the fields and Mr. Tomey Indrianto for regenerating explants to be plantlets.

REFERENCES


Hartati NS, Fitriani H, Supatmi, Sudarmonowati E. 2012. The tuber root characterization and nutrition from seven cassava genotypes (*Manihott esculenta*). Agricola 2 (2): 101-110. [Indonesian]


Ihemere UE. 2003. Somatic Embryogenesis and Transformation of Cassava for Enhanced Starch Production [Dissertation]. Ohio State University, USA.


