Short communication

Enhancement of regeneration efficiency in banana (Musa acuminata cv. Berangan) by using proline and glutamine

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A B S T R A C T

Regeneration of Musa acuminata cv. Berangan (AAA) from embryogenic cell suspension was enhanced by using proline and glutamine. Sixteen embryo development media consisting of different concentrations of proline and glutamine were tested. Glutamine was preferred as the source of organic nitrogen supplement compared to proline. The embryos developed in liquid media were shown to produce 10.6-fold higher number of plantlets in lesser duration (1.5 to 2.5 months) as compared to solid media. Optimum somatic embryo development was observed in liquid media supplemented with 400 mg/l glutamine (M4GL) with an average regeneration of 33,844 ± 402 plantlets per ml of settled cell volume (SCV). Although both amino acids have proven to promote the embryo development, concentrations of proline between 200 to 400 mg/l in liquid media caused abnormal embryo differentiation.

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1. Introduction

Embryogenic cell suspensions (ECSs) are comprised of rapidly proliferating totipotent single cells. These totipotent single cells make the embryogenic cells amenable for genetic manipulations as well as an ideal system for mass propagation. Many published reports have described plant regeneration from somatic embryogenesis in banana through callus intermediary derived from leaf bases and rhizome fragments (Kulkarni et al., 2006), highly proliferating scalps (Sipen et al., 2008), immature male flowers (Sidha et al., 2007, Wong et al., 2006 and Jalil et al., 2003,2008) and immature female flowers (Grapin et al., 2000), and bract (Divakaran and Nair, 2011).

Based on the literature, to date, immature male flowers have been extensively used as the source of explants and mostly cultured on media established by Côte et al. (2006) to induce embryogenic callus. However, the current protocols for banana somatic embryogenesis are constrained by low embryo germination concomitantly leading to low plant regeneration rates and long cultural period.

Amino acids are source of organic nitrogen supplement during culture. Previous studies in several species such as alfalfa, maize, sorghum, pineapple, rice and other monocots have shown that amino acid supplements could enhance somatic embryogenesis and in vitro plant regeneration (Claparols et al., 1993; Rao et al., 1995; Hasamaki et al., 2005; Grewel et al., 2006). It was due to the association of the organic nitrogen compared to inorganic sources with the ease of transportation of the former into the plant cells (Kim and Moon, 2007).

Although both amino acids have been widely used in many plant species to improve somatic embryo maturation, study of the inclusion of amino acids into the culture media of banana for embryo development and concomitant improvement in the regeneration was lacking. Therefore, this work was done to study the effect of L-proline and L-glutamine on somatic embryo development of banana (cv. Berangan, AAA) and subsequently determine the optimum concentration to improve the existing protocol and develop the most efficient banana regeneration system.

2. Materials and Methods

2.1. Initiation and maintenance of ECS

Immature male flower clusters of Musa acuminata cv. Berangan were cultured for six months to induce embryogenic calli, which were then used to initiate cell suspensions according to the method described by Jalil et al. (2003). ECSs were maintained in M2 propagation medium consisting of Murashige and Skoog (MS) (Murashige

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Abbreviations: 2,4-D, 2,4-Dichlorophenoxyacetic acid; BAP, benzylaminopurine; cv, cultivar. 
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Table 1
Composition of M3 development media tested. L denotes liquid M3 media and S denotes solidified M3 media.

<table>
<thead>
<tr>
<th>Media</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSOL</td>
<td>MS basal medium, 20 g/l sucrose</td>
</tr>
<tr>
<td>M1GL</td>
<td>MS basal medium, 100 mg/L glutamine, 20 g/l sucrose</td>
</tr>
<tr>
<td>M2GL</td>
<td>MS basal medium, 200 mg/L glutamine, 20 g/l sucrose</td>
</tr>
<tr>
<td>M3GL</td>
<td>MS basal medium, 300 mg/L glutamine, 20 g/l sucrose</td>
</tr>
<tr>
<td>M4GL</td>
<td>MS basal medium, 400 mg/L glutamine, 20 g/l sucrose</td>
</tr>
<tr>
<td>M1PL</td>
<td>MS basal medium, 100 mg/L proline, 20 g/l sucrose</td>
</tr>
<tr>
<td>M2PL</td>
<td>MS basal medium, 200 mg/L proline, 20 g/l sucrose</td>
</tr>
<tr>
<td>M3PL</td>
<td>MS basal medium, 300 mg/L proline, 20 g/l sucrose</td>
</tr>
<tr>
<td>M4PL</td>
<td>MS basal medium, 400 mg/L proline, 20 g/l sucrose</td>
</tr>
<tr>
<td>MSOS</td>
<td>MS basal medium, 30 g/l sucrose, 2 g/l PhytagelTM</td>
</tr>
<tr>
<td>M1GS</td>
<td>MS basal medium, 100 mg/L glutamine, 30 g/l sucrose, 2 g/l PhytagelTM</td>
</tr>
<tr>
<td>M2GS</td>
<td>MS basal medium, 200 mg/L glutamine, 30 g/l sucrose, 2 g/l PhytagelTM</td>
</tr>
<tr>
<td>M3GS</td>
<td>MS basal medium, 300 mg/L glutamine, 30 g/l sucrose, 2 g/l PhytagelTM</td>
</tr>
<tr>
<td>M4GS</td>
<td>MS basal medium, 400 mg/L glutamine, 30 g/l sucrose, 2 g/l PhytagelTM</td>
</tr>
<tr>
<td>M1PS</td>
<td>MS basal medium, 100 mg/L proline, 30 g/l sucrose, 2 g/l PhytagelTM</td>
</tr>
<tr>
<td>M2PS</td>
<td>MS basal medium, 200 mg/L proline, 30 g/l sucrose, 2 g/l PhytagelTM</td>
</tr>
<tr>
<td>M3PS</td>
<td>MS basal medium, 300 mg/L proline, 30 g/l sucrose, 2 g/l PhytagelTM</td>
</tr>
<tr>
<td>M4PS</td>
<td>MS basal medium, 400 mg/L proline, 30 g/l sucrose, 2 g/l PhytagelTM</td>
</tr>
</tbody>
</table>

and Skoog, 1962) basal medium supplemented with 1.1 mg/l 2,4-D, 0.25 mg/l zeatin, 10 mg/l ascorbic acid and 20 g/l sucrose with pH 5.7 prior to autoclaving. Observation on somatic embryo initiation was done for about 3–6 months culture. ECs were subcultured every 14 days and sieved through 425 μm mesh sieve every third subculture to maintain fine cells. Approximately 2 ml of settled cell volume (SCV) was inoculated into 250 ml conical flask containing 50 ml of M2 media. The cultures were maintained at 25 ± 1 °C on orbital shaker at 80 rpm with a photoperiod of 16 h/day under 31.4 μmol m−2 s−1 light intensity provided by fluorescent lamps. Experiments were repeated twice with 3 replications done for each treatment (3 flasks per treatment).

2.2. Optimization of embryo development media (M3)

In this study, the influence of varying concentrations of L-proline and L-glutamine in solid and liquid M3 embryo development media was compared. In liquid media, the solidifying agent was removed (Table 1). M3 media consists of MS basal supplemented with 30 g/l sucrose solidified in 2 g/l phytagel ( Duchefa, Netherlands) and 20 g/l sucrose in liquid media with varying concentrations of glutamine or proline. Glutamine or proline of concentrations at 0, 100, 200, 300 and 400 mg/l were tested. All media were adjusted to pH 5.7 prior to autoclaving.

ECs in M2 media were sieved with 425 μm mesh sieve and allowed to settle in 50 ml Falcon tube. The SCV to M2 media was adjusted to a ratio of 1:4. The cells were then suspended and 200 μl was inoculated either in 20 ml of liquid M3 media or on 70 mm-diameter Whatman No. 1 filter paper placed on solid M3 media. Culture conditions for cells placed in M3 liquid medium were the same as the initiation stage, whereas cells in M3 solid media were maintained in dark conditions until formation of somatic embryos was observed. Experiments were repeated twice with 3 replications for each treatment (3 petri dishes containing cells per treatment).

2.3. Shoot regeneration of somatic embryos

The mature embryos obtained from M3 media were cultured in M4 regeneration media containing MS basal, 6 mg/l BAP, 30 mg/l sucrose and 2 g/l phytagel. After 30 days, the plantlets were subcultured onto M4 media with 3 mg/l BAP. Cultures were incubated in a photoperiod of 16 h/day under 31.4 μmol m−2 s−1 light intensity provided by fluorescent lamps for about 2 months. Experiments were repeated twice with three replications done for each treatment (3 petri dishes containing somatic embryos per treatment).

2.4. Rooting of shoots and plantlet acclimatization

Plantlets about 3 cm in height were cultured in M5 rooting media consisting of MS basal, 30 g/l sucrose, 1% (w/v) activated charcoal and 2.2 mg/l phytagel adjusted to pH 5.7. Cultures were incubated for 16 h/day under 31.4 μmol m−2 s−1 light intensity provided by fluorescent lamps. Plants were ready to be acclimatized after reaching about 8 cm minimum height. Acclimatization was done by transferring the plants into polybags containing soil mixture and placed in the netted nursery under natural condition (80% humidity, 28–30 °C, 2000–5000lx, 12 h/day) for 1.5–2 months before transferring to the field.

2.5. Preparation for histological sections

Specimens selected for histological studies were fixed for 24 h in formaldehyde-absolute ethanol-acetic acid (FAA) solution consisting of 5% (v/v) formaldehyde, 45% (v/v) absolute ethanol and 5% (v/v) glacial acetic acid. The samples were then dehydrated in an ethanol series; 30% for 30 min, 50% for 45 min, 70% for 45 min, 80% for 60 min and 100% for 120 min. Specimen were then embedded in basic resin (Technovit 7100) and sectioned at 3.5 μm. Sections were double-stained with naphthol blue black (Sigma, USA) 1 g naphthol blue black in 100 ml 7% (v/v) acetic acid (Fisher, 1968).

2.6. Statistical analysis

The data collected were analysed by ANOVA and the mean values were tested to evaluate the significance at 1% confidence level using Tukey’s HSD test.

3. Results and Discussion

Development of Musa acuminata cv. Berangan somatic embryos was affected by both media composition and phase (liquid and solid). The embryos developed in liquid media were shown to produce more plantlets in a shorter time compared to solid media (Table 2). The maximum number of plantlets obtained in liquid culture medium (M4GL) was 10.6-fold higher than on solid medium (M4GS). This result was in agreement with previous study by Wong et al. (2006) on Musa acuminata cv. Mas (AA). This could be due to ease of nutrient uptake into the cells where media and cells were in close proximity.

Different amino acid supplements resulted in different effects on development and regeneration of Berangan somatic embryos. Glutamine was the preferred source of organic nitrogen compared to
Table 2
Effects of liquid and solid development media with different concentrations of L-glutamine or L-proline on the production and culture duration of banana cv. Berangan plantlets.

<table>
<thead>
<tr>
<th>Media phase</th>
<th>Embryo development media</th>
<th>M3 Plantlets per ml SCV</th>
<th>Culture duration (months)</th>
<th>Abnormality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liquid</td>
<td>MSOL</td>
<td>9,902 ± 356b</td>
<td>2.5–3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>M1GL</td>
<td>17,341 ± 442a</td>
<td>1.5–2.5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>M2GL</td>
<td>20,961 ± 325a</td>
<td>1.5–2.5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>M3GL</td>
<td>25,602 ± 721b</td>
<td>1.5–2.5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>M4GL</td>
<td>33,844 ± 402a</td>
<td>1.5–2.5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>M1PL</td>
<td>15,430 ± 360a</td>
<td>2–2.5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>M2PL</td>
<td>16,336 ± 494a</td>
<td>2–2.5</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>M3PL</td>
<td>17,775 ± 452d</td>
<td>2–2.5</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>M4PL</td>
<td>16,400 ± 1871e</td>
<td>2–2.5</td>
<td>5.7</td>
</tr>
<tr>
<td>Solid</td>
<td>MSOS</td>
<td>186 ± 17d</td>
<td>3.5–5.5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>M1GS</td>
<td>1,352 ± 883h</td>
<td>2.5–3.5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>M2GS</td>
<td>1,803 ± 735h</td>
<td>2.5–3.5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>M3GS</td>
<td>2,917 ± 1922h</td>
<td>2.5–3.5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>M4GS</td>
<td>3,181 ± 199h</td>
<td>2.5–3.5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>M1PS</td>
<td>539 ± 38j</td>
<td>2.5–4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>M2PS</td>
<td>1,098 ± 533i</td>
<td>2.5–4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>M3PS</td>
<td>1,850 ± 676hi</td>
<td>2.5–4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>M4PS</td>
<td>2,797 ± 1211h</td>
<td>2.5–4</td>
<td>0</td>
</tr>
</tbody>
</table>

*Values are the means ± SE of 3 replicates; different letters indicate significant differences between media at P < 0.01.

Proline. The highest plantlet number was obtained in liquid development medium supplemented with 400 mg/l glutamine, yielding an average 33,844 of regenerated plantlets per ml SCV. The presence of both glutamine and proline promoted embryo development in Berangan. Media without amino acid supplementation (MSO solid and liquid) not only produced lower plantlet numbers (186 and 9,902 plantlets) per ml SCV respectively, but also required a longer time to achieve maturity than those cultured in the presence of amino acids. However, the embryos developed in glutamine based media turned opaque white within 1.5–2 months compared to those on medium with proline (2–2.5 months). Embryos in media supplemented with glutamine produced normal plantlets. This indicated that exogenous amino acid serves as nitrogen source for the synthesis of proteins which is important to embryo differentiation and maturation. Similar result was obtained in chestnut (Robichaud et al., 2004), eastern white pine (Carin et al., 2000), white spruce (Ashihara et al., 2001) and hybrid tea rose (Marchant et al., 1996).

The beneficial role of glutamine in the histodifferentiation of embryos has been observed in many species such as date palm (Zouine and El-Hadrami, 2007), bitter melon (Thiruvengadam et al., 2006) and horse gram (Varisai et al., 2004). It was reported that

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Figure 1. Comparison on embryo development on different M3 media. (A–C): Embryos after 3 months in culture on M3 solid media. A) Translucent embryos in MSOS media were still at earlier stage of development. B) Some of the embryos in M4PS matured into solid white structure. (C) Most of the embryos in M4GS had turned solid white. (D–F): Embryos 1.5 months after being cultured in liquid M3 media; (D) MSOL, (E) M4PL and (F) M4GL, a: embryo at early stage of development, b: solid white embryos at maturity. Bar (A–F) = 1 mm.
the frequency of somatic embryos increased with the incorporation of glutamine in the media (Thiruvengadam et al., 2006 and Varisai et al., 2004). Exogenous supply of glutamine was also reported to increase soluble storage protein (Zouine and El-Hadrami, et al., 2007), which enhanced protein synthesis and allowed rapid division of embryogenic cells (Stasolla et al., 2001).

From this study, glutamine was shown to be superior to proline as a supplement in the development media of Berangan somatic embryos in both solid and liquid media. Proline liquid based media also showed negative effects in the embryos, wherein it caused abnormalities such as fused root-like structure, extensive browning cells and retardation (Figure 2). The percentage of abnormalities increased with the increasing proline concentration (Table 2). All abnormalities appeared after 1–2 months of culture in development media. Abnormal embryos with pseudo-radicle structure and/or browning became necrotic after 1–3 weeks, whereas retarded embryos maintained its structure for as long as 6 months before becoming necrotic.

According to Caligo et al. (1985), proline has an inhibitory effect if added during the embryo development stage of carrot, in which it could produce embryos either with no polarity, or if they do, they were lacking root meristem. Trovato et al. (2008) stated that plant cells were capable of accumulating and quickly degrading excess proline when needed. Proline usually accumulates or is synthesized at high levels during osmotic stress condition (Delauney and Verma, 1993; Hu et al., 1992 and Kiyosue et al., 1996), and eventually, the proline concentration will drop when stress is relieved (Kiyosue et al., 1996; Peng et al., 1996 and Nakashima et al., 1998).

According to Hellmann et al. (2000), this adaptation is important as excess amount of proline is toxic to the cell, due to its conversion product PSC that causes the production of reactive oxygen species (ROS) which induces apoptosis and programmed cell death (PCD). In this study, the high concentration of proline may cause toxicity to cells which later causes abnormalities and necrosis of the developing embryos. The toxicity level might also become more severe in liquid culture as proline was supplemented every 14 days during subculture.

Embryo during the early stage of development in both media appeared as translucent globular structure, which turned solid white as it matured (Figure 1). Histological sections of the embryos showed normal embryogenesis structure as described by Jalil et al. (2008). The embryos were later regenerated into morphologically normal plants (Figure 2) and are currently being acclimatized to be tested in the field for clonal fidelity study.

4. Conclusion
From this study, it can be concluded that 400 mg/l glutamine in solid and liquid development media was superior to all the media tested, in terms of duration and number of regenerated plantlets obtained. Proline supplementation to the media was inferior to glutamine in enhancing development in banana somatic embryos and also induced abnormality to the developing embryos at high concentration in liquid media.

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References


