INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
18–1438/2019/21–4–905–910
DOI: 10.17957/IJAB/15.0974
http://www.fspublishers.org

Full Length Article

Liquid Culture of Somatic Embryogenesis Cell Proliferation of Sugarcane (*Saccharum officinarum*)

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Abstract

Somatic embryogenesis has turned out to be a major breakthrough in sugarcane biotechnology research. The embryogenic cell line is capable of being stored for a long time and it will not undergo a characteristic change. The aim of this study was to obtain such a medium, which may promote an excellent growth of embryogenic callus of sugarcane. Embryogenic cell was able to proliferate in a liquid medium of MS containing 2,4-D, L-proline and casein hydrolisate with the addition of arginine. However, the lack of 2,4-D as an auxin in medium causing cell death in the culture. This study showed that 2,4-D as an auxin in proliferation medium has an important role to proliferate embryogenic cell in a liquid medium and the resultant embryogenic cell did not lose its capability to regenerate as a new whole plant. © 2019 Friends Science Publishers

Keywords: Sugarcane; Somatic embryogenesis; Somatic embryo; Liquid medium; Proliferation

Introduction

Sugarcane (*Saccharum officinarum* L.) is one of the commercial crops in Indonesia. Breeding of Sugarcane is not easy owing to its high level of heterozygosity, complex cytogenetics and the sterility of its male and female parts. Propagating sugarcane vegetatively using stem cutting is easier and more popular to do. However, it has a high range of microbes which favor systemic disease transmission even if it is subjected to chemical therapy or heating before planting (Maretzki *et al*., 1969; Jabeen *et al*., 2009; Arjun and Rao, 2015). Nowadays, the most effective way to propagate sugarcane is tissue culture (Dewanti *et al*., 2015; Wekesa *et al.*, 2015). The development of somatic embryogenesis is a major breakthrough in biotechnology research on sugarcane.

Somatic embryogenesis is a biotechnological approach mainly used for the clonal propagation of different plants worldwide (Heringer *et al.*, 2018). It is the process of developing somatic cells into new plants by forming an embryo without fertilization (Kaur and Kapoor, 2015). Somatic embryogenesis is subdivided into indirect and direct based on the process involved. The difference between them is that there is a callus phase in indirect somatic embryogenesis and no callus phase in direct somatic embryogenesis. Only a few somatic embryos could be formed via the direct process of somatic embryogenesis whereas in indirect somatic embryogenesis there is a high yield of somatic embryos (Williams and Maheswaran, 1986; Percy *et al*., 2000; Suprasanna *et al*., 2005). Development of somatic embryogenesis involves two big steps: The somatic cells differentiate and become embryogenic competent cells, then proliferate as embryogenic cells, and those embryogenic cells show an embryogenic capability and become differentiated to form somatic embryos (Jimenez, 2011).

Efficient and reliable plant regeneration can be obtained from embryogenic tissue culture in most species of Poaceae such as sugarcane. The fact that when embryogenic cell lines are stored for long periods of time there is no genetic change that may make them reliable totipotent cells not only for genetic transformation but also for efficient germplasm conservation. Agar, being a gelling agent for solid tissue culture media, is a general way to activate sugarcane embryogenic cell. The solid media have proven to give a great result in embryogenic cells which could be regenerated more easily. But compared with solid media, liquid media are more suitable and adaptable for reducing cost and labor (Suarez, 2013). Currently, a liquid culture of callus derived from somatic embryogenesis has a great potential for propagation at a rapid rate (Thorat *et al.*, 2017). But establishing and maintaining embryogenic cell liquid culture have however, proven to be a Herculean task. This research describes a definite medium, which promotes an excellent growth of embryogenic callus from sugarcane.

To cite this paper: Alfian, F.N., N.N. Afdhoria, P. Dewanti, D.P. Restanto and B. Sugiharto, 2019. Liquid culture of somatic embryogenesis cell proliferation of sugarcane (*Saccharum officinarum*). *Intl. J. Agric. Biol*., 21: 905–910

Materials and Methods

Plant Material and Preparations

Experiments were performed in Molecular Biology and Biotechnology Division, Center of Development and Advanced Science and Technology (CDAST), University of Jember, East Java, Indonesia during January–October of the years 2017. A spindle leaf isolated from 6 months old commercial sugarcane var. NXI 1-3 was chosen as plant material for inducing embryogenic callus. This plant material was cultivated in the trial field of the University of Jember in Jubung, Jember, East Java. Explants were sterilized using 96% alcohol and sliced in the transverse section for about 2 mm in thickness and 5 mm in diameter width aseptically in the laminar air flow from Biobase.

Induction of Embryogenic Callus

The transverse section of spindle leaf was cultured on MS media based on previous experiment (Alfian, 2015) containing 3 mg/L 2,4-D + 300 mg/L casein hydrolisate + 30 g/L sucrose +12 g/L agar, which has been optimized for embryogenic callus induction in accordance with prior experiment. The pH was djusted to 6.2 prior to autoclave at 121°C and 103 kPa for 15 min. Microscopic observation was carried out using Leica EZ4HD setero microscope to achieve a better morphological analysis. This culture was put in a totally dark condition for 6 weeks with 3 weeks interval subculture to activate spindle leaf to produce embryogenic callus. The percentage success of embryogenic callus induction (ECI) was measured with the equation (Tahir *et al*., 2011):

$$\text{ECI} = \frac{\text{Number of callus induced}}{\text{Number of total explant inoculated}} \times 100\%$$

Initiation of Somatic Embryo from Embryogenic Callus

Embryogenic callus from induction media was moved into a new media in ordr to induce the somatic embryo stage using MS media containing 1 mg/L 2,4-D + 300 mg/L casein hydrolisate + 560 mg/L L-proline + 30 g/L sucrose + 12 g/L agar. The pH was adjusted to 6.2 prior to autoclaving at 121°C and 103 kPa for 15 min. This culture was on for 3 weeks under dark condition. Development stage of somatic embryo maturation was observed every week with a stereo microscope from Leica EZ4HD. The formation of globular, scutellar and coleoptile structure is the first stage of specific characterization of embryogenic callus development.

Proliferation of Soamtic Embryo in Liquid Medium and Regeneration

The somatic embryo has three major developmental stages in monocots such as sugarcane. These are globular, scutellar, as well as coleoptile stage. However, only the scutellar structure could continue proliferating in a liquid medium. We put the selected friable embryogenic callus, which reached the somatic embryo stage of scutellar in a liquid proliferation medium. The homogeneity of the liquid cell culture was maintained to obtain the fine cell culture. Cell culture was produced from 9 week-old embryogenic callus (0.25 g fresh weight) in Erlenmeyer flask, which contained liquid MS medium (25 mL) supplemented with varying combination. The pH was adjusted to 6.2 prior to autoclaving. The flask was put on a rotary shaker at a regular speed of 100 rpm, in the dark at 27±28°C. After 7 days, the medium was replaced with a fresh medium and maintained on a rotary shaker at 80 rpm for the next eight weeks. The cell culture was sub-cultured every 7 days which precipitated the cell from the medium and the medium was poured into a new vessel, and replaced with a new fresh medium from the old vessel with the same volume (Mustafa *et al*., 2011). Several combinations of media were used to discover the best combination to produce either embryogenic callus or fine cell culture (Table 1). The aggregates from nine week-old liquid medium proliferation were filtered with sterile filter paper in order to separate the liquid medium and the aggregates. The aggregates were dried inside Laminar Air Flow (LAF) cabinet and planted again in solid MS media fortified with 1 mg/L 2,4-D + 560 mg/L L-proline + 500 mg/L casein hydrolisate + 50 mg/L L-arginine + 30 g/L sucrose + 11 g/L agar for three weeks. The shoot was transferred to MS medium which contain 100 mg/L L-glutamine + 30 g/L Sucrose + 11 g/L agar to perform shoot regeneration for four weeks. Plantlets were acclimatized to sterile sand medium for 2 weeks in growth chamber before transferring into the soil in the pots.

Results

Somatic Embryo Development

Embryogenic callus: Embryogenic callus was activated in embryogenic callus induction media for six weeks. The percentage of successfully induced embryogenic callus is shown in Fig. 1. The spindle leaf of sugarcane began to swell after one week of culture and 6% of total explants inoculated started to form pre-embryo mass stage of embryogenic callus. Over 50% of embryogenic callus was formed in three weeks after inoculation and reached 92% at the end of activation time. The morphology of embryogenic callus and non-embryogenic callus is shown in Fig. 2. The microscopic observation showed that callus formed on the surface or the cut end of sugarcane spindle leaf. Callus began to induced at three weeks of culture and become more pronounced at six weeks of culture. Three week-old has been indicated as embryogenic callus characterization with a clear surface, yellow to translucent in color and a friable structure as shown in Fig. 2a. At this time, some part of spindle leaf still failed to develop into a callus. Embryogenic callus had a rapid growth and development until six weeks old after culture. Some part of callus developed into the globular

Table 1: Medium combination for Liquid Media Proliferation

Combination	Concentration		
	I	II	III
MS (from Phytotech Lab)	4.43 g/L	4.43 g/L	4.43 g/L
L-Proline	560 mg/L	560 mg/L	560 mg/L
Casein Hidrolysate	500 mg/L	500 mg/L	500 mg/L
2,4-D	-	1 mg/L	1 mg/L
L-Arginine	-	-	50 mg/L

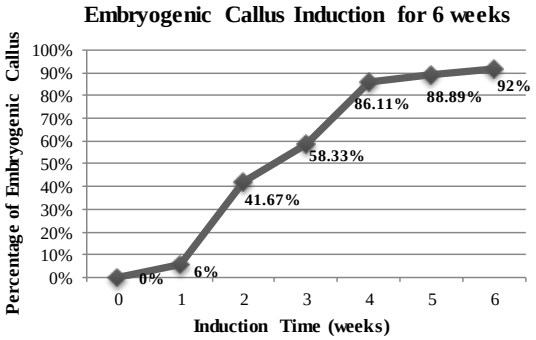

Fig. 1: Percentage of Embryogenic Callus that formed for 6 weeks in total induction time

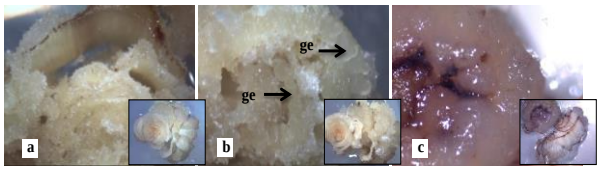

Fig. 2: Morphology of Embryogenic and Non Embryogenic Callus. a) Callus formed in the surface cut end of sugarcane spindle leaf in 3 weeks culture. b) Callus formed in the surface cut end of sugarcane spindle leaf in 6 weeks culture (some globular structure has been formed, showed in arrow). c) Non embryogenic callus. (ge: globular embryo)

structure indicated with an arrow in Fig. 2b. Six weeks old embryogenic callus was massive in size of callus which was formed from spindle leaf. Non-embryogenic callus also appeared in this experiment and was characterized by the watery structure of callus, brown to dark in color and is not capable of developing into a somatic embryo as shown in Fig. 2c.

Somatic embryo development: The embryogenic callus chosen for the next stage was pre-embryo mass structure (Fig. 3a). Pre-embryo mass structure was the first stage of somatic embryo formation which had a transparent, glossy, and friable structure. After two weeks, pre-embryo mass was grown in the new culture medium, and the structure developed into a globular structure that was more compact than pre-embryo mass (Fig. 3c). Globular structure sprang up from the surface part of callus. It allowed observation to be done visually because it was easier to observe the structure. Somatic embryo grew rapidly until three weeks after being transferred to the new culture medium, that contained a lower level of auxin and a scutellar structure was clearly observed (Fig. 3e). This scutellar structure, that was more adaptable to environmental changes, was used as a material for liquid media proliferation. The histological analysis of pre-embryo mass and globular structure of somatic embryo revealed that embryogenic cells were small, richly cytoplasmic and actively dividing with a big nucleus and conspicuous starch grains. These cells were generally present in small, compact aggregates and indicated competence for embryogenesis (Fig. 3b, d and f). The non-embryogenic cells were large, vacuolated and often elongated with sparse cytoplasm and few starch grains and such cells did not indicate morphogenetic competence (Figueroa *et al*., 2006).

Proliferation of Somatic Embryo in Liquid Medium and Regeneration

Three different combinations of liquid proliferation medium were investigated to determine the best composition for the development of fine embryogenic cell culture. Main difference of this medium combination was the presence or absence of auxin (2,4-D) and amino acid (L-arginine) in the culture medium. The culture was rotated at 100 rpm in the first week of culture in the liquid medium to release the small aggregates from embryogenic clumps that had reached scutellar stage (Fig. 4a) and maintained to rotate at 80 rpm from the second week until last six weeks observation with five days interval for subculture. First medium gave no response to cell growth in the liquid proliferation medium (Fig. 4b). Depletion of auxin triggered browning of cells in liquid medium, which hindered cells proliferation. The addition of 1 mg/L 2,4-D in the second medium gave a significant result compared with that obtained in the first medium, where the cells proliferated (Fig. 4c). However, the third medium produced the best result among all combination media with the addition of 1 mg/L 2,4-D and 50 mg/L L-arginine (Fig. 4d).

Regeneration was undertaken to prove that proliferated cells through a liquid medium did not lose their embryogenic ability. The cells aggregates proliferated for six weeks in liquid medium and filtered were returned to solid regeneration medium (Fig. 4e). The green spot appeared after three weeks culture in the solid medium (Fig. 4f). It was proven that somatic embryo that has undergone proliferation in liquid media did not lose its capability to regenerate as it developed into coleoptile structure of somatic embryo stage after five weeks culture (Fig. 4g). This coleoptile structure of somatic embryo could grow easily and become a shoot and after being released from callus clumps, the roots also appeared without transfer to rooting medium (Fig. 4h). Shoot grew into a whole plantlet in the culture jar after 8 weeks of culture (Fig. 4i). The plantlet with a strong shoot and long root could be acclimatized in a sterile sand medium. The acclimatization only took place for two weeks in a growth chamber (Fig. 4j). Acclimatized plants with a good condition were moved to the greenhouse (Fig. 4k).

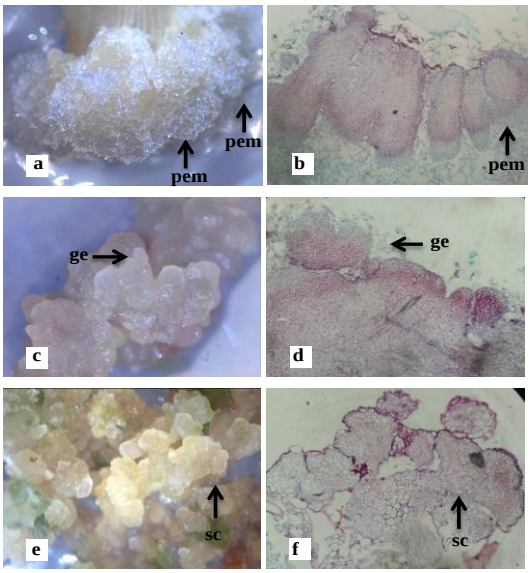

Fig. 3: Development of somatic embryo stage from time to time, a) pre-embryo mass structure of somatic embryo in 1 week after culture in the new medium. b) histologycal observation from pre-embryo mass structure. c) globular structure of somatic embryo in 2 weeks after culture. d) histologycal observation from globular structure. e) scutellar structure of somatic embryo in 2 weeks after culture. f) histologycal observation from scutellar structure. (pem: pre-embyo; ge: globular embryo; sc: scutellar)

Discussion

Spindle leaf is the most commonly explant performed for callus culture in sugarcane somatic embryogenesis research (Mahmud *et al*., 2014; Sandhu *et al*., 2016). Callus was first observed at the cut end of the spindle leaf as an explant after one week of culture. The callus was semi-translucent and consisted of loose and elongated cells. The callus started to look clear 11–15 days after planting. Callus induction began at the age of eight days after planting. However, a clearer callus did not begin to form and begin to differentiate to become embryogenic (embryoids) until 14±21 days after planting. An embryonic callus is a callus that is 30–55 days old after planting. Both embryogenic callus and non-embryogenic callus could be present in the same clump. This phenomenon could be caused by characteristics of each cell or tissue and internal factors. Several studies have confirmed that embryogenic competent and incompetent callus appear on the same explant, indicating that although they are genetically similar, responses to a certain stimulus may be different, with a small percentage of the cells becoming responsive (Ho and Vasil, 1983).

The somatic embryo was an indicator that a series of somatic embryogenesis processes were successfully reached (Sholeha *et al*., 2015; Widuri *et al*., 2016). A special characteristic of the somatic embryo is that two meristems are developed at the end of the stage, shoot and root meristems. The regeneration process becomes easier to do with no root induction in rooting medium. Transfer to the new culture medium may be needed for the formed callus at least six weeks after planting. If the callus is younger than six weeks, there would be little or no callus that can regenerate into plants. Callus age is an important factor to be considered in inducing somatic embryos (Ho and Vasil, 1983). Only callus that is 6–10 weeks after planting can induce somatic embryo (Roy *et al*., 2011; Haq and Memon, 2012). The new culture medium must consist of a lower level auxin from callus induction medium. We used synthetic auxin (2,4-D at 3 mg/L level) in embryogenic callus activation media and transferred embryogenic callus in somatic embryo activation media with a lower level of auxin, that was only 1 mg/L. High level of auxin given at the start of culture triggered somatic cell to induce a specific gene, which produces a certain substance to complete a full somatic embryogenic process (Purnamaningsih, 2002). Auxin has a real effect on somatic embryo formation after successful induction of embryogenic callus (Almeida *et al*., 2018). A significant fall in the auxin level in culture medium caused the embryogenic cells to elongate and form somatic embryo with the addition of L-proline into the culture medium (Moghaddam *et al*., 2000; Gill *et al*., 2004; Takahashi and Takamizo, 2013).

Somatic embryo phases could be seen on the surface of embryogenic callus. These stages occur within a short time and their ability reduces with the duration of incubation in the media. The first somatic embryo to be initiated had small, yellowish white color and friable, that can be noticed on the surface of the callus (Khalil, 2002). The most suitable parts for inducing callus were leaf sheath and midrib. The deepest leaf rolls (1–2 rolls) produced a finer texture callus compared with the callus produced from 4–5 leaf rolls that have a friable texture and were more suitable for inducing embryogenic callus. Leaf rolls that exceed the 6th roll cannot produce embryogenic callus but roots (Alfian, 2015). The density and number of embryogenic callus produced depend on the age of the leaves and the distance of the leaves used as explant from the basal section. Explant distance from the basal area about 1–2 cm forms almost 100% embryogenic callus (Ho and Vasil, 1983). Morphological observation became one of the important factors in this research. The embryogenic callus has certain features such as clear white to yellowish, glossy, and friable structures while the non-embryogenic callus is characterized by its milky to brownish-yellow color, more wet and soft, and has a compact callus structure that is not easy to differentiate (Alcantara *et al*., 2014). Embryogenic callus characteristic was presented as a rounded shape with prominant nuclei, a high nucleus with small vacuoles. On the other side, non embryogenic callus was descripted as elongated, dispersed and vacuolated cells with a low nucleus. The non embryogenic callus was not be able to perform the regeneration process even upon the expposure of regeneration stimulus (Silveira *et al*., 2013).

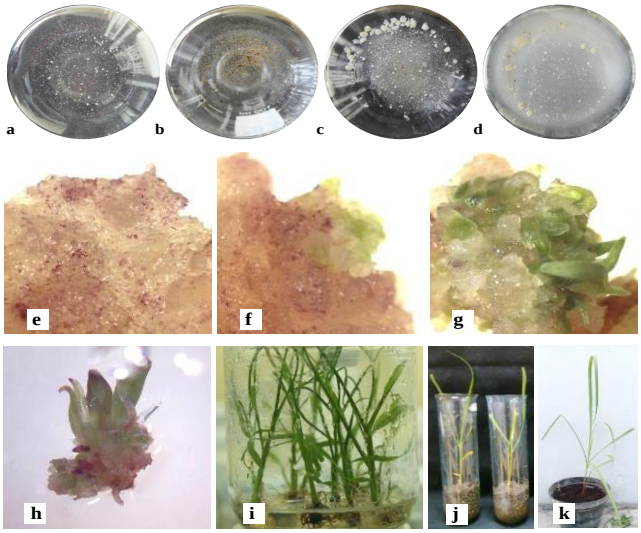

Fig. 4: The development from cell culture in the different combination liquid media proliferation to a whole new plantlet after regeneration. a) at 1 week of culture in all combination medium. And at the end time (6 weeks) of culture, which are: b) medium I (auxin -, arginine -). c) medium II (auxin √, arginine -). d) medium III (auxin √, arginine √). e) development of cell from liquid culture to regeneration in solid culture. f) green spot appeared after 3 weeks culture in solid media. g) shoot has been ready to develop become a plantlet. h) shoot that has been released from callus part. i) plantlet growth in culture jar. j) plantlet after 2 weeks acclimatization in the growth chamber. k) plant growth in greenhouse for 2 months

Callus multiplies as an unorganized mass of cells which makes it more difficult to observe its cellular phenomenon. A chemically defined liquid medium is expected to be an overcome the limitations of callus culture in solid medium. A friable callus was transferred to an agitated liquid medium and it readily dispersed after break up. The movement of the cell in relation to absorption of nutrient medium improves gaseous exchange and eliminates the nutrient gradient within the medium and at the surface of the cells. The first proliferation liquid medium which is auxin and amino acid-free medium gave no response to cell growth in the liquid proliferation medium. Depletion of auxin triggered browning phenomenon to cells in a liquid medium which hindered cells from proliferating. The presence of browning would hinder the absorption of nutrients by explants; it slows down the process of smoothening. When the plants are injured, such as during the process of cutting the leaf, the phenolic substances that are mainly present in the vacuoles, will be mixed with the contents of the plastids and other organelles and a dark color appears (Gill *et al*., 2004; Yadav *et al*., 2012; Ahmad *et al*., 2013). These events will prevent enzyme activity and can cause death, both in explants and in the media.

A liquid media provides a better homogenization to te culture, which both oxygen transport and aeration in the aerial and root zone as compared to solid media (Sajid and Pervaiz, 2008). The addition of low level auxin to the liquid culture in the second medium gave a significant result compared with what obtained in the first medium, where the cells could proliferate well (Purnamaningsih, 2002). A low level of auxin is needed to give the best effort and maintain the torque of cell division. However, the third medium produced the best result among all combination media with the addition of auxin 1 mg/L 2,4-D and amino acid 50 mg/L L-Arginine. Several studies also support the results that amino acid has been used as organic nitrogen source in *in vitro* cultures of monocots to enhance somatic embryogenesis (Komor *et al*., 1981; Loukanina and Thorpe, 2008). Sugarcane as one of most productive monocots plants also take up amino acids including L-arginine that could significantly increase the cell growth rate. A high uptake of L-arginine is due to its low *Km* value of uptake system and low susceptibility to general trans inhibitory effect of the other amino acids. It is reported that extracellular arginine uptake for suspension cell growth was four times greater than lysine in the sugarcane cell (Sachdeva *et al*., 2005; Asad *et al*., 2009).

Conclusion

Liquid media for sugarcane somatic embryo proliferation could be used with the addition of 2,4-D and L-arginine to get a high number of embryogenic cells that could elongate and develop. Somatic embryo proliferated from liquid media could be regenerated normally with only the addition of L-glutamine because of its ability to form shoot and root meristems as it grows to become coleoptile.

Acknowledgements

This work was supported by grants from PTUPT Research Project 2017 number of contract 0515/UN25.3.1/LT/2017

for Dr. Ir. Parawita Dewanti, M.P. in the cooperation of Ministry of Research Technology and Higher Education and the University of Jember, and Prof. Dr. Ir. Bambang Sugiharto, M.Agr.Sc the Chief Director of Center for Development of Advanced Science and Technology (CDAST) of University of Jember.

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(Received 12 September 2018; Accepted 24 November 2018)