BIOVALENTIA: BIOLOGICAL RESEARCH JOURNAL
***e*-ISSN: 2477-1392**
Vol. 6 No. 1, May 2020

PERFORMANCE OF SOMATIC EMBRYOGENESIS DEVELOPMENT UNDER DIFFERENT 2.4-D AND COCONUT WATER CONCENTRATION IN SUGARCANE VAR. BULULAWANG

Parawita Dewanti[*1,2], Safira Arikha Maryam[2], Laily Ilman Widuri[2], and Purnama Okviandari[1]

[1] *Center for Development of Advanced Science and Technology, University of Jember, East Java, Indonesia*
[2]*Faculty of Agriculture, University of Jember. Jalan Kalimantan 37, Kampus Tegal Boto, Jember 68121*
*Corresponding author

E-mail address: parawita@yahoo.co.id (Parawita Dewanti).
Peer review under responsibility of Biology Department Sriwijaya University

Abstract *:*
Mass propagation technology through somatic embryogenesis has become an alternative for producing sugarcane seedlings rapidly. Application of proper plant growth regulator and concentration contribute to support somatic embryogenesis development. This study applied the combination of liquid and solid culture during proliferation stage to promote cell dispersion of embryogenic callus, rapid embryo somatic production, and improve regeneration potency of somatic embryo. Application of 2.4-D and coconut water during proliferation may expected as proper combination for accelerating somatic embryo development and regeneration. Development of somatic embryogenesis in sugarcane var. Bululawang during proliferation were described in this study. Embryogenic callus from induction media were transferred to proliferation media containing MS Basal + vitamin supplemented with sucrose different level of 2.4-D (1 mgl^{-1}, 2 mgl^{-1}, 3 mgl^{-1}, 4 mgl^{-1}) and coconut water (0% and 5%) to enhance somatic embryo formation during proliferation. Result showed that low concentration of 2.4-D induced optimum somatic embryogenesis development in proliferation and regeneration. Concentration of single 2.4-D 1 mgl^{-1} without coconut water induced rapid development of scutelar and coleoptilar during proliferation and resulted in better shoot regeneration. In other way, 4 mgl^{-1} of 2.4-D concentration affected to inhibit scutelar and coloeptilar formed as the result of failure callus differentiation.

Keywords: Proliferation; somatic embryo, scutelar; coleoptilar, regeneration.

Received: September 12, 2019, Accepted: April 29, 2020

1. Introduction

Sugarcane (*Saccharum officinarum*. L) is the one of important sources for sugar production in the world. Indonesia is one of the Asian-sugar producing countries contribute to global sugar production. However, Indonesia including the third largest consumer at once a major importer of sugar in the world [1]. High demand of sugar consumption in the world require effort to sustain sugarcane productivity.

Bululawang (BL) is known as one of superior sugarcane variety in Indonesia [2][3]. Increasing Bululawang productivity require mass production of seedling to maintain production stability. Tissue culture is considered as propagation technique for producing sugarcane seedling in large scale. Seedling propagation through somatic embryogenesis in sugarcane has become a concern to be an alternative to increase seedling stock. Somatic embryogenesis is considered due to its potential to produce propagules in shorter period [4]. Somatic embryogenesis method already widely used for genetic transformation [5][6], producing virus free sugarcane [7], and developing synthetic seed [8][9].

Somatic embryogenesis produces bipolar structure of somatic embryo for accelerating seedling germination without gamet fusion process. There are particular stages during somatic embryogenesis development, such as: pro-embryonic mas (PEM) development, maturation of somatic embryo, and re-

generation [10]. Optimal induction of somatic embryogenesis is obtained form young leaf of sugarcane [11] that containing apical meristem region [12]. Spindle leaf is widely use for somatic embryogensis induction in sugarcane. High production of embryogenic callus form meristematic tissue is required for somatic embryogenesis development.

Seedling propagation via somatic embryogenesis pathway are commonly utilize solid media for induction, proliferation, and regeneration stage. Shifting solid media to liquid media could be used for regenerating somatic embryo [13]. This study applied the combination of liquid and solid culture during proliferation stage. Liquid culture enable to promote cell dispersion of embryogenic callus, rapid embryo somatic production, and improve regeneration potency of somatic embryo [14]. Composition of media also determine production of somatic embryo in sugarcane [15] and plant genotype [16]. Auksin is the most commonly hormone use for inducing callus during somatic embryogenesis, particularly 2.4-D [17][18]. Application of 2.4-D on induction media support cell divison and differentiation including callus embryogenic production [19]. The use of natural sitokinin such us coconut water (CW) also common applied in tissue culture. CW is effective for improving culture growth and somatic embryo induction [20]. CW also induce globular formation and increase embryogenic callus biomass [21].

Addition of 2.4-D and CW combination may resulted better development and proliferation of embryogenic callus in Bululawang sugarcane variety. Application of both plant growth regulator during proliferaton is limited. Application of 2.4-D and coconut water during proliferation may expected as proper combination for accelerating somatic embryo development and regeneration. The objective of this study was to describe somatic embryogenesis stage and regeneration of sugarcane var. Bululawang under different 2.4-D and coconut water concentration.

2. Materials and Methods

2.1 Callus Induction

The experiment was conducted at the *Center for Development of Advanced Sciences and Technology* (CDAST) Laboratory, University of Jember from January to June 2016. Spindle leaves from 4-6 months old sugarcane var. Bululawang collected from field were washed in tap water. Sterilization method was employed to avoid contamination by spraying explant using alcohol 70% and also burned in laminar air flow (LAF) during preparation. Sterilized spindle leaves were discarded until remained about 5 layers with 2-3 cm diameter and chopped in 2-3 mm thickness before transferring into induction media. Induction media used containing MS (Murashige dan Skoog) Basal + vitamin supplemented with 4 mgl^{-1} of 2.4-D, sucrose 30 gl^{-1}, agar 11 gl^{-1}, and casein hydrolisate 300 mgl^{-1}. Spindle leaf explants were incubated under dark condition and room temperature about 23-25°C for 6 weeks. Explants were subcultured after 3-4 weeks to induced embriogenic callus. Callus formation including Pro Embryo Mass (PEM) structure was observed weekly by macroscopic and microscopic observation using a stereo microscope (*Leica EZ4HD*).

2.2 Proliferation

Callus were transferred to proliferation media containing MS Basal + vitamin supplemented with sucrose 30 gl^{-1} , agar 11 gl^{-1}, casein hydrolisate 300 mgl^{-1} and prolin 500 mgl^{-1}. Different level of 2.4-D (1 mgl^{-1}, 2 mgl^{-1}, 3 mgl^{-1}, 4 mgl^{-1}) and coconut water (0% and 5%) were used to induce somatic embryogenesis. Combination of liquid and solid culture media were used during proliferation. Embryogenic callus were incubated on liquid media to disperse_callus by shaking on orbital shaker on 100 rpm under light condition. After 7 days callus were transferred to solid media to accelerate somatic embryo formation. Callus structure and somatic embryogenesis stages including globular, scutelar, and coleoptilar were observed by macroscopic and microscopic observation using a stereo microscope (*Leica EZ4HD*).

2.3 Regeneration of somatic embryo

Coleoptilar from proliferation stage were subcultured to regeneration media containing MS Basal + vitamin, 2 mgl^{-1} glysine, sucrose 30 gl^{-1}, and agar 11 gl^{-1}. Coleoptilar were growth on 16h light and 8h dark under temperature room 23-25°C until coleoptilar developed into shoot. Day of shoot initiation and shoot growth were recorded until after 4 week on regeneration media.

3. Results and Discussion

Spindle leaf explants were incubated in media containing MS + 2.4-D 4 mgl^{-1} + 300 mgl^{-1} casein hydrolysate under dark condition to initiate callus formation. Figure 1 showed the development process of callus induction during 6 weeks incubation. Before callus initiation, explants were performed swollen structure after 1-2 weeks incubation. This appearance indicated the occurrence of early cell division. Initiation of Pro Embryo Mass (PEM) structure was observed after two weeks incubation. Surface of spindle leaf displayed callus structure after 4

weeks and developed into embriogenic structure in 6 weeks (Figure 1).

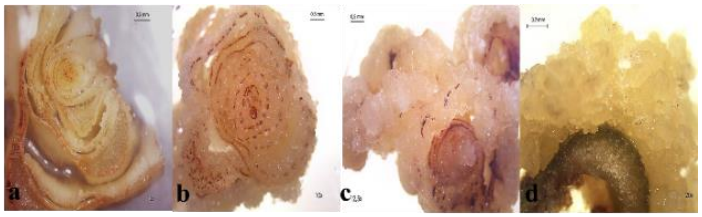

Figure 1. Callus induction from spindle leaf explant of sugarcane var. Bululawang at different age a) 1 week; b) 2 weeks; c) 4 weeks; d) 6 weeks.

Callus consisted of two characteristic, embryogenic and non embryogenic. Embryogenic callus performed whitish to yellow in colour, glossy, dry, and friable. This type of callus had high potential to regenerate into somatic embryo. Callus with brown in colour, rounded in shape, wet, and containing small nucleus, were eliminated as non embryogenic callus.These type of callus had less ability to promote somatic embryo development and differentiation. Previous study reported non embryogenic did not showed somatic embryo formation due to the involvement of metabolic activity such as phenolic compound. The presence of phenolic compound contributed to inhibit somatic embryo development [22][23].

Developmental pathway of somatic embryogenesis involved several process including cell dedifferentiation, activate cell division, and alteration of physiology, metabolism, and gene expression [24]. Somatic embryo development initiate from competent region of embryogenic callus derived from protodermal cells with high protein accumulation [25]. Embryogenic callus displayed small with a dense cytoplasm and obvious nucleus on outer layer and large cell with large vacuola in the inner layer of embryogenic callus [26]. These callus characteristics were selected for next proliferation stage.

Many studies reported the use of 3 mgl^{-1} [27][28] and 4 mgl^{-1} of 2.4-D [29][30] for optimum callus induction. High concentration of auxin 2.4-D triggered rapid cell division and differentiation to promote somatic cell production [31]. Proliferation is the important stage for multiplying and developing somatic embryo. In this research, embryogenic callus from induction media were transferred into liquid media to induce callus dispersion. Inducing callus dispersion by shaking on orbital shaker could split callus clumps into single cell. Incubation of embryonic cell in liquid culture could induce active division and growth of cells [32]. However, incubation of somatic embryo only applied for 7 days. The elongation of somatic embryo during proliferation required solid media to accelerate somatic embryo formation.

Proper composition of media for proliferation determine the quality and quantity of somatic embryo. In this study, different 2.4-D and coconut water concentration were applied for proliferation. Results showed that selection of low 2.4-D concentration was the key for proliferation stage, whereas coconut water did not affected to accelerate proliferation in sugarcane Bululawang variety.

Table 1. Time of initiation scutelar, coleoptilar, and shoot of sugarcane.

Time of initiation (day)				
2.4-D (mgl^{-1})	CW (%)	Scutelar	Coleoptilar	Shoot
1	0	13.5	28.5	7
	5	14	42	9
2	0	28	42	27.5
	5	28.5	42	15
3	0	25	44	17.5
	5	29	45	19
4	0	NA	NA	NA
	5	NA	NA	NA

Values are mean consisted of two replications. NA: Not available.

During early proliferation stage, the appearance of globular stage generally emerged after 7 days in all treatments. Scutelar and coleoptilar development was varied depended on 2.4-D and coconut water concentration. Data showed that concentration of 2.4-D 1 mgl^{-1} induced rapid development of suctelar and coleoptilar (Table 1). However, the application of coconut water combined with of 2.4-D 1 mgl^{-1} induced longer time for coleoptilar formation than single of 2.4-D. Higher 2.4-D concentration than 1 mgl^{-1} extended time initiation of scutelar, coleoptil, and resulted lower regeneration potential. Even, application of 4 mgl^{-1} 2.4-D did not perform somatic embryo development. This study revealed that 1 mgl^{-1} 2.4-D was the optimum concentration to induced better proliferation stage. The extend time of scutelar initiation by applying high 2.4-D concentration expected trigger continued cell division rather than cell elongation. Even, elevated level of 2.4-D induced the

development of non-embryogenic callus. During proliferation the growth of cell directed to differentiation phase. Nodular structure during globular stage contained bipolar structure with meristematic region. This meristematic region represented as competent area for scutelar formation. In the late of scutelum stage, this structure performed heart shape and green spot for early shoot initiation Lower auxin concentration applied during proliferation stage, promote somatic cell to produce important subtance for globular phase to develope into somatic embryogenesis stages such as scutelar and coleoptilar resulted in better plant regeneration [30]

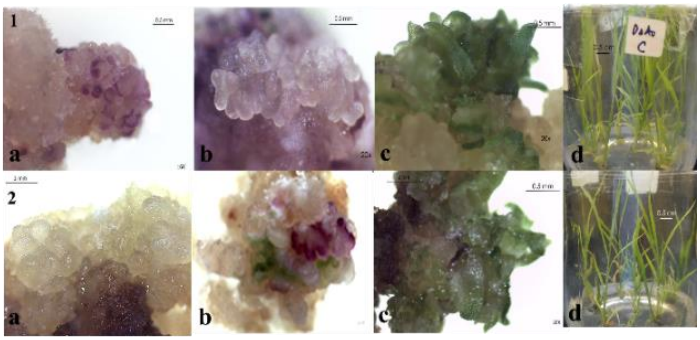

Figure 2. Somatic embryogenesis stages of sugarcane var. Bululawang on media containing 1) 2.4-D 1 mgl^{-1} + 0 % coconut water; 2) 2.4-D 1 mgl^{-1} + 5 % coconut water; a) Globular; b) Scutelar; c) Coleoptilar; d) planlet regeneration at 4 weeks after planting.

Development of scutelar under proper concetration required about two weeks followed by four weeks incubation for coleoptilar development. Coleoptilar clumps from preferable callus resulted in good shoot initiation during regeneration. Proper composition of media for proliferation determine the quality and quantity of somatic embryo. In this study, different 2.4-D and coconut water concentration were applied for proliferation.

Figure 2 was the performance of somatic embryo development of sugarcane under low 2.4-D concentration. Application of 2.4-D 1 mgl^{-1} without application of CW resulted better plant regeneration than combination with 5% CW. Callus clumps containing scutelar stage on media 2.4-D 1 mgl^{-1} + 5 % coconut water appeared green spot early than single 2.4-D 1 mgl^{-1}. However, coleoptilar and shoot growth during regeneration was better in single 2.4-D 1 mgl^{-1} .

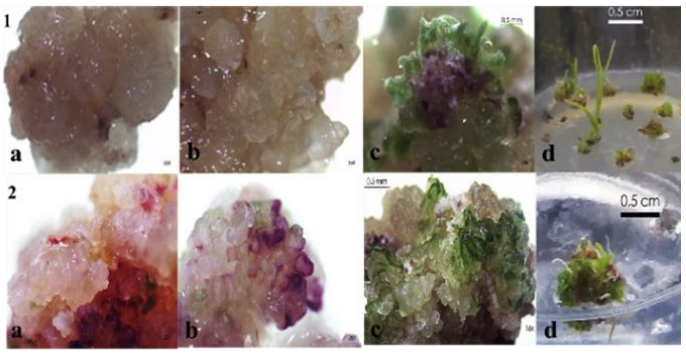

Figure 3. Somatic embryogenesis stages of sugarcane var. Bululawang on media containing 1) 2.4-D 2 mgl^{-1} + 0 % coconut water; 2) 2.4-D 2 mgl^{-1} + 5 % coconut water; a) Globular; b) Scutelar; c) Coleoptilar; d) planlet regeneration at 4 weeks after planting.

Different respon was displayed on callus growth under 2 mgl^{-1} of 2.4-D with and without CW application. Globular and scutelar performed wet structure, indicating delayed cell differentiation to develope into scutelar. Less scutelar development also performed (Figure 3). Scutellar formed from callus was not optimal and resulted in retarded shoot regeneration as well as 3 mgl^{-1} 2.4-D appplication (Figure 4). Shoot initiation perfomed better than 2 mgl^{-1} 2.4-D appplication, however shoot regeneration resulted in ununiform and albino explant.

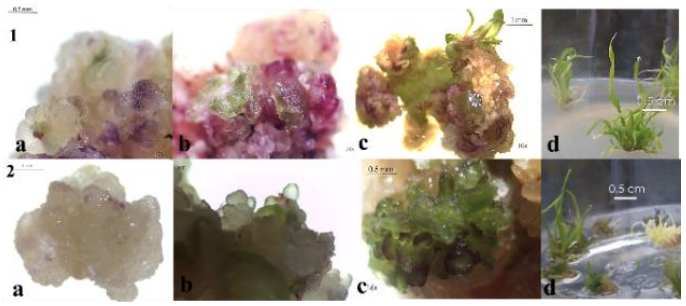

Figure 4. Somatic embryogenesis stages of sugarcane var. Bululawang on media containing 1) 2.4-D 3 mgl^{-1} + 0 % coconut water; 2) 2.4-D 3 mgl^{-1} + 5 % coconut water; a) Globular; b) Scutelar; c) Coleoptilar; d) planlet regeneration at 4 weeks after planting.

General purpose of 2.4-D application during induction are for cell division and differentiation. Higher 2.4-D concentration up to 1 mgl^{-1} during proliferation only promote continuous cell division and inhibit importance pathway to induce cell differentiation into somatic embryo stages (Figure 5). No number of scutelar and coloeptilar formed under 4 mgl^{-1} 2.4-D indicated as the result of failure callus differentiation. There was no green spot appeared in

callus surface. Green spot expected as competence spot for indicating shoot formation [33].

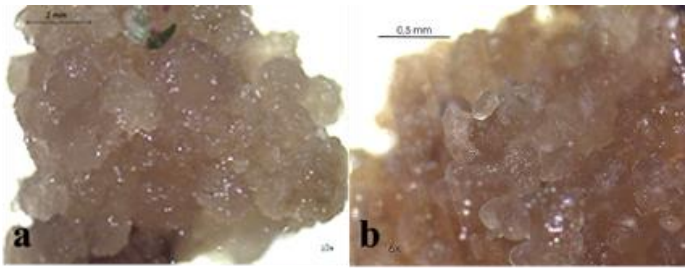

Figure 5. Media containing a) 2.4-D 4 mgl^{-1} + 0 % coconut water; b) 2.4-D 4 mgl^{-1} + 5 % coconut water did not induce somatic embryogenesis formation.

4. Conclusions

Development of somatic embryogenesis in proliferation required low concentration of 2.4-D to induce better shoot. Concentration of single 2.4-D 1 mgl^{-1} without coconut water induced rapid development of scutelar and coleoptilar during proliferation and resulted in better shoor regeneration. In other way, 4 mgl^{-1} of 2.4-D concentration affected to inhibit scutelar and coloeptilar formed as the result of failure callus differentiation.

5. Acknowledgement

This work was supported by grants from PTUPT Project 2019 number of contract 1767/UN25.3.1/LT/2019 and IDB (Islamic Development Bank) project 268/UN25.7/PIU-IDB/2019.

References

[1] Solomon, S and Y. R. Li. 2016. Editorial-The sugar industry of asian region. *Sugar Tech*, 18(6); 557–558. DOI: https://doi.org/10.1007/s12355-016-0500-8.

[2] Budi, S., E. S. Redjeki and A. E. Prihatiningrum. 2016. Effect variety and stratified plantlet nursery to the growth sugarcane (*Saccharum officinarum* L.) propagated in single bud. *Research Journal of Seed Science*, 9(2): 42-47 ISSN 1819-3552.

[3] Lestari, P., N. Hanani, and S. Syafrial. 2019. Technical efficiency analysis of sugar cane farming in Malang Regency, Indonesia. *Agricultural socio economic journal*, 19(1):1–8. P- ISSN: 1412-1425

[4] Raza, S., S.Qamarunisa, M. Hussain, I. Jamil, S. Anjum, A. Azhar, and J.A. Qureshi, 2012. Regeneration in sugarcane via somatic embryogenesis and genomic instability in regenerated plants. *Journal of Crop Science and Biotechnology*, 15(2):131-136.DOI:10.1007/s12892-011-0111-6

[5] Alcantara,G.B, R. Dibax, J. C. Bespalhok Filho, and E. Daros. 2014. Plant regeneration and histological study of the somatic embryogenesis of sugarcane (*Saccharum* spp.) cultivars RB855156 and RB72454 *Acta Scientiarum. Agronomy*. 36(1):63 . DOI:10.4025/actasciagron.v36i1.16342,

[6] Heringer, A.S, T. Barroso, A.F. Macedo, C. Santa-Catarina, G.H.M.F Souza, E.I.S. Floh, de G.A. Souza-Filho, and V.Silveira, 2015. Label-free quantitative proteomics of embryogenic and non-embryogenic callus during sugarcane somatic embryogenesis. *PLoS One*, 10(6):1-23. DOU: 10.1371/journal.pone.0127803.

[7] Dewanti,P. L. I. Widuri, C. Ainiyati, P. Okviandari, Maisaro, and B. Sugiharto. 2016. Elimination of SCMV (Sugarcane Mozaik Virus) and rapid propagation of virus-free sugarcane (*Saccharum officinarum* L.) using somatic embryogenesis. *Procedia Chemistry*, 18:. 96–102.

[8] Helal, N.A.S. 2011. The green revolution via synthetic (artificial) seeds: a review. *Research Journal of Agriculture and Biological Sciences.* 7(6)*:* 464-477, ISSN 1816-1561

[9] Ningtiyas, W.N, P. Dewanti, and B. Sugiharto. 2016. Preservation effect of PEG (Polyethylene Glycol) in sugarcane (*Saccharum officinarum*) NXI 1,3 Synthetic Seed, *Annales. Bogoriensis.* 20(2): 63–68.

[10] Fehér, A. 2015. Somatic embryogenesis - stress-induced remodeling of plant cell fate, *Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms*,1849(4): 385-402.

[11] Ali, A. M. Iqbal, A. Majid, N. H. Naveed, A. Rehman, and S. Afghan. 2013. In vitro conservation and production of vigorous and desiccate tolerant synthetic seed formation in sugarcane (*Saccharum officinarum* L .). *Annual Conference of Pakistan Society of Sugar Technologists, Rawalpindi Pakistan*. pp: 9-10.

[12] Yasmin,S. I. A. Khan, A. Khatri, N. Seema, M.

A. Siddiqui, and S. Bibi. 2011. Plant regeneration from irradiated embryogenic callus of sugarcane. *Pakistan Journal of Botany*. 43(5): 2423–2426.

[13] Pandey, S. P. Shukla, and P. Misra. 2018. Physical state of the culture medium triggers shift in morphogenetic pattern from shoot bud formation to somatic embryo in *Solanum khasianum*. *Physiology and molecular biology of plants*. 24(6):1295-1305.

[14] 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*). *International Journal of Agriultiral and Biol*ogy.21(4): 905–910. DOI: 10.17957/IJAB/15.0974

[15] Jahangir, G.Z. I. Ahmad Nasir, R. Ahmad Sial, M. Aslam Javid, and T. Husnain, 2010. Various hormonal supplementations activate sugarcane regeneration in-vitro. *Journal of Agricultural Science*, 2(4): 231. E-ISSN 1916-9760.

[16] Damayanti,F. S. Suharsono, A. Tjahjoleksono, and I. Mariska. 2018. Regeneration and histological study of somatic embryogenesis of sugarcane (*Saccharum officinarum* L.) cultivar PS 864. *Journal of Biological Researches*.24(1): 53–57. E-ISSN:2337-389X.

[17] Tarique,H.M, M. A. Mannan, M. S. R. Bhuiyan, and M. M. Rahaman, 2010. Micropropagation of sugarcane through leaf sheath culture. *International Journal of Sustainable. Crop Production*.5(2):13–15.

[18] Yadav, S and A. Ahmad. 2016. Standardisation of callus culture techniques for efficient sugarcane micropropagation.*Cibtech Journal of Bio-Protocols*. 2(2):29–32. ISSN: 2319–3840.

[19] Widuri, L.I, P. Dewanti, and B. Sugiharto. 2016. A simple protocol for somatic embryogenesis induction of in vitro sugarcane (Saccharum officinarum. L) by 2, 4-D and BAP. *BIOVALENTIA: Biological Research Journal*, *2*(1).: 1–9. E-ISSN: 2477-13.

[20] Al-KhayrI J.M. 2010. Somatic embryogenesis of date palm (*Phoenix dactylifera* L.) improved by coconut water. *Biotechnology*. 9(4): 477-484.

[21] Kierallah,H and N. Hussein. 2013. The role of coconut water and casein hydrolysat in somatic embryogenesis of date palm and genetic stability detection using RAPD markers. *Research in Biotechnology*. 4(3): 20–28. ISSN: 2229-791X .

[22] Silveira,V. A. M. de Vita, A. F. Macedo, M. F. R. Dias, E. I. S. Floh, and C. Santa-Catarina. 2013. Morphological and polyamine content changes in embryogenic and non-embryogenic callus of sugarcane. *Plant Cell, Tissue and Organ Culture (PCTOC)*. 114(3): 351-364.

[23] Ahmad, I. T. Hussain, I. Ashraf, M. Nafees, R. Maryam, and Muhammad Iqbal. 2013. Lethal effects of secondary metabolites on plant tissue culture. *American-Eurasian Journal of Agriculture Environment Science*. 13(4):539–47. https://doi.org/10.5829/idosi. aejaes.2013. 13.04.1975.

[24] Yang, X. and X. Zhang.2010. Regulation of somatic embryogenesis in higher plants, *Critical Reviews in Plant Science*. 29(1): 36-57.

[25] Oliveira, E.J, A.D. Koehler,D.I Rocha, D.I. Vieira, L.M., Pinheiro, M.V.M., de Matos, E.M., da Cruz, A.C.F., da Silva, T.C.R., Tanaka, F.A.O., F.T.S.Nogueira, and Otoni, W.C, 2017. Morpho-histological, histochemical, and molecular evidences related to cellular reprogramming during somatic embryogenesis of the model grass *Brachypodium distachyon*. *Protoplasma*, 254(5).

[26] Dhillon N.K. and S.S.2013. Histology of somatic embryos from maize embryo. 13(1):3571–3576.

[27] Dewanti,P. L. I. Widuri, F. N. Alfian, H. S. Addy, P. Okviandari, and B. Sugiharto, 2016. Rapid propagation of virus-free sugarcane (*Saccharum officinarum*) by somatic embryogenesis, *Agriculture and Agricultural Science Procedia*, 9:456-461.(9):

[28] Sardar, K. S. , T. Q. Sadaf, A. K. Imtiaz, and R. Saboohi, 2016.Establishment of in vitro callus in sugarcane (*Saccharum officinarum* L.) varieties influenced by different auxins," *African journalnya Biotechnology*. 29: 1541–1550.

[29] Sholeha, W. B. Sugiharto, D. Setyati, and P. Dewanti. 2015. Induksi embriogenesis somatik menggunakan 2,4-Dichlorophenoxyacetic Acid (2.4-D) dan kinetin pada eksplan gulungan daun muda tanaman tebu. Jurnal Ilmu

Dasar.16(1): 17–22, 2015.
[30] Kaur, R. and M. Kapoor. 2016. Plant regeneration through somatic embryogenesis in sugarcane. *Sugar Tech*. 18(1): 93–99.
[31] Ki Won, L. C. Ochirbat, J. Choi Gi, K.K. Yong, C. J. Hee, S. P. Hyung, K. W. Hoo, and L. S. Hoon. 2012. Factors influencing callus induction and plant regeneration of dahurian wildrye grass (*Elymus dahuricus* L.). *African Journal of Biotechnology*, 11 (4): 815–20. https://doi.org/10.5897/ajb11.1614.
[32] Muruganantham, M., S.Amutha, and A. Ganapathi. 2010. Somatic embryo productions by liquid shake culture of embryogenic calluses in *Vigna mungo* (L.) Hepper. *In Vitro Cellular & Developmental Biology-Plant*. 46(1):34-40.
[33] Melo E. F, R. S. Ramos, C. G. Melo, C. R. Rodrigues, M. S. Vieira, and M. H. P. Barbosa. 2014. The use of histological analysis for the detection of somatic embryos in sugarcane. *African Journal of Biotechnology*. 13(6) : 762–767.