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SHORT COMMUNICATION

Potential Embryogenic Callus Induction Protocol Through Cell Suspension Culture For High Frequency Plant Regeneration Of Maspine Pineapple (*Ananas comosus* L.)

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ABSTRACT

To explore the potential for embryogenic callus induction protocol through cell suspension culture for high frequency plant regeneration of Maspine pineapple (*Ananas comosus* L.), eight different culture media formulation were evaluated for their effects on the induction of somatic embryos from sucker explants. Explants were cultured on MS medium supplemented with various media concentration (NAA, Dicamba and BAP, Picloram, Kinetin and NAA, 2,4-D, TDZ, and TDZ and BAP). Embryogenic callus induction percentage, color and texture of the callus were assessed after five months of culture. The optimum medium for the proliferation of *in vitro* shoots from sucker explants was MS medium supplemented with 3 mg/L BAP. Meanwhile, the optimum medium for the induction of fastest and high percentage of embryogenic callus growth from *in vitro* leaf-based was MS medium supplemented with Picloram. Results of mean comparison showed that 3 mg/L Picloram were more effective on explants than 10 mg/L. Results of the double staining method proved that somatic embryogenesis occurred in MS supplemented with 3 mg/L Picloram. Under microscopic observations, the globular-stage of the embryos were revealed in callus cells which is relatively suitable for suspension cells inoculums, indicating that the tested PGR were significantly effective for somatic embryogenesis formation in this species. Most embryogenic callus from sucker explants was yellowish-mucilaginous-wet-friable. The developed protocol potentially leads to the production of embryogenic callus from sucker explants and plant regeneration through somatic embryogenesis.

Keywords: *Ananas comosus*, embryogenic callus, cell suspension, propagation, Maspine, Picloram

High demand of pineapple needs the cultivation done intensively by applying *in vitro* technique for regeneration. Tissue culture techniques has been successfully applied to produce millions of propagules per year, ranged from 40 (Dewald *et al*., 1988), 280 (Devi *et al*., 1997), 5,000 (Zepeda & Segawa 1981), 40,000 (Liu *et al*., 1989), 1,000,000 (Sripaoraya *et al*., 2003) from single explants. Conventionally, pineapple is vegetatively propagated, where the multiplication rate is low, and ranges from about 11 to 17 plants per five months (Lieu *et al*., 2004). However, many researchers such as Lakhsmi Sita *et al*. (1974), Mathews & Rangan (1979) and Zepeda & Sangawa (1981) reported direct regeneration, but hardly any report on regeneration from callus culture (Canals & Javier, 1994). The production can be scaled up manifold through the intervening of callus phase, compared to direct regeneration (Sha Yalli Khan *et al*., 2002) since it will produce larger number

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and uniform pineapple planting material in a relatively short period of time (Firoozabady & Gutterson, 2003) and also to improve plant performance (Ika & Ika, 2003). In the tissue culture technique, simple procedure for efficient plant regeneration by organogenesis from sucker and by somatic embryogenesis from leaf-based derived callus and cell suspension culture can be developed (Suneerat Sripaoraya *et al.,* 2002). Therefore, the objective of this study is to develop the protocol in cell suspension system from embryogenic cells for high frequency propagation of Maspine pineapple.

Explants (Figure 2A) were taken from suckers, length size range from 20 to 30 cm with approximately 20 leaves each. The outer leaves were excised from the suckers and washed under running tap water to remove dust and dirt. Subsequently, suckers were washed with Teepol™ (10 percent (%) potassium salt, KCl as the active ingredient), rinses under running tap water. After that, soaked for a few minutes in 0.025% mercury chloride ($HgCl_2$) and 70% Clorox™ (5.25% sodium hypochlorite, NaOCl the active ingredient) in laminar air flows. This is followed by soak for a while in an absolute ethanol (EtOH) before trimmed and culture. The cultures were maintained at ±25ºC, under 16 h photoperiod condition at 2,000 µmol m^{-2} s^{-1}.

Initially, two difference types of culture media formulation were used for early culture. The different formulation was used in order to observe the comparative effects of initial and subsequent shoot formation. The media formulation used was Murashige and Skoog (MS) containing 30 g/L sucrose, solidified with 8 g/L phytagel, supplemented with 3 mg/L 6-Benzylaminopurine (BAP) and 2 mg/L BAP respectively. After three to four months of shoots regeneration, the leaf-based were cut in size 5 x 5 mm. Then, placed them on eight different media formulation consisting of MS with various supplementation of 1 g/L Casein hydrolysate, 150 ml/L coconut water and 0.02 mg/L 1-Naphthylacetic Acid (NAA) (CI1); 2.5 mg/L dichloromethoxybenzoic acid (Dicamba) and 0.5 mg/L BAP (CI2); 3 mg/L trichloroaminopicolinic acid (Picloram) (CI3); 10 mg/L Picloram (CI4); 1.5 mg/L 1-NAA and 1 mg/L Kinetin (CI5); 2.5 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D) (CI6); 0.009 mg/L Thidiazuron (TDZ) (CI7); and 1 mg/L BAP and 0.5 mg/L TDZ (CI8) respectively in order to induce shoot-based embryogenic callus from *in vitro* shoot. The pH of all media was adjusted to 5.8 with 1.0 M NaOH or 1.0 M HCl prior to autoclaving. The cultures were placed in the dark at ±25ºC. These media formulation was chosen based on the results tested and optimised by Daquinta *et al*. (1996); Firoozabady & Moy (2004); He *et al*. (2012); Ika *et al*. (2012); Ika & Ika (2003); Sripaoraya *et al*. (2003); and Yapo *et al*. (2011) to initiate and induce somatic embryogenesis of pineapple cultivar Smooth Cayenne and Shenwan in their respective works before. Therefore, the selection of optimized medium was made to use in this project.

After two months, the desired embryogenic callus for cell suspension inoculums were initially selected and verified by double-staining technique. A sample of the cells to be examined was placed onto a slide and a few drops of 1% (v/v) acetone carmine were added. The samples were heated for a few seconds followed by rinsing with distilled water two to three times. A few drops of 0.1% (v/v) Evan's Blue was added to the sample and were incubated for two to three minutes and rinsed again with distilled water two to three times (Gupta & Ibaraki, 2006). The excess water was discarded and finally examined under a light microscope (Carl ZEISS, DSM940A).

Next, to facilitate the development and multiplication of embryogenic cells, 10 ml of liquid multiplication medium containing MS supplemented with 0.6 mg/L Picloram was initially dispensed into 100 ml Erlenmeyer flasks with about 500 mg embryogenic callus as the inoculums. The callus was homogenously dispersed when in contact with liquid medium. Subsequently, 10 ml of liquid multiplication medium was added to the cultures after two weeks of incubation. Later, the somatic embryos containing mostly the globular structures were sieved from the suspension cultures using a filter with 450 µm pore size. The subcultures processes were done every week until the desired volume of cells obtained. Cultures were maintained at ±25ºC, under 16 h photoperiod condition at 2,000 µmol m^{-2} s^{-1} under continuous agitation on a rotary shaker at 100 rpm.

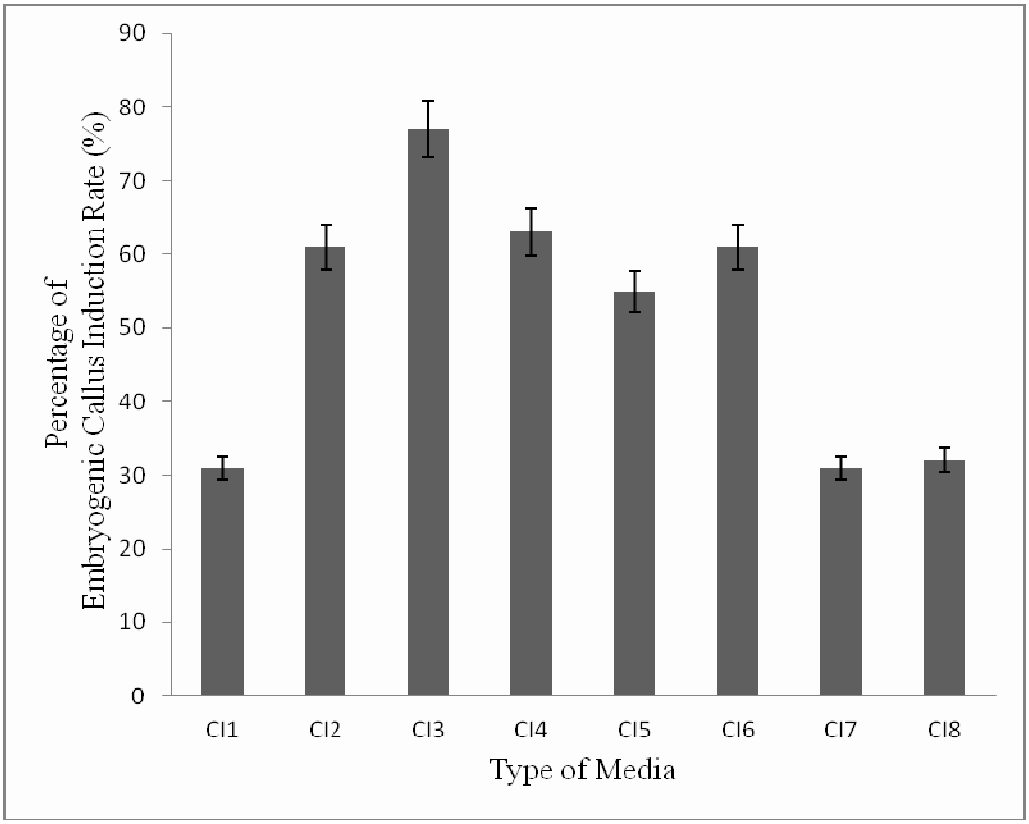

Figure 1. The percentage of embryogenic callus induction rate obtained from the explants cultured on MS medium supplemented with various plant growth regulators. Values are mean ± standard deviation for 30 replicates each treatment. MS supplemented with 1 g/L Casein hydrolysate, 150 ml/L coconut water and 0.02 mg/L 1-NAA (CI1); 2.5 mg/L Dicamba and 0.5 mg/L BAP (CI2); 3 mg/L Picloram (CI3); 10 mg/L Picloram (CI4); 1.5 g/L 1-NAA and 1 mg/L Kinetin (CI5); 2.5 mg/L 2,4-D (CI6); 0.009 mg/L TDZ (CI7); and 1 mg/L BAP and 0.5 mg/L TDZ (CI8).

After three to four months, the *in vitro* shoots were developed in MS medium supplemented with 3 mg/L BAP (Figure 2B). In facts, the observation also showed some abnormality of the shoots possibly due to excessive content of plant growth regulator (PGR) during the shoots induction. Therefore, lowering the concentration of plant growth hormone in subsequent subculturing process was done in order to avoid somaclonal variation or off-type on embryogenic callus structures development later. Furthermore, the prolong culture period was also believed contribute to the abnormality of the shoot formation.

The leaf-based of shoots was used as starter materials or explants to induce embryogenic callus (Figure 2C). In total, 30 replicates for each of medium composition (CI1, CI2, CI3, CI4, CI5, CI6, CI7 and CI8) were prepared with five explants in each replicate to obtain 150 samples. Nevertheless, observation proved that only MS medium supplemented with 3 mg/L Picloram as PGR exhibited the fastest growth rate (in two months culture period) and produced the highest percentage of embryogenic callus growth induction, 76.7% (Figure 1) at $P<0.5$. Out of 30 replicates in this medium composition, 23 replicates or 115 samples have had produced the potential embryogenic callus. These findings suggested that the Picloram at 3 mg/L was effective for embryogenic callus formation from leaf-based in the genus *Ananas* (Figure 2D). Miguel & Lirio (2010) also reported that Picloram in the medium was effective for embryogenic callus production in bromeliads. Therefore, Picloram might be effective plant growth regulators for embryogenic callus production in Bromeliaceae family. In addition, several cultures showed a formation of shoots structure developed from

the MS medium treated with cytokinin (CI2 and CI5) perhaps due to excessive content of BAP during the shoots induction. This structures such as clumps of shoot and whitish compact structures were transferred to developmental medium in order to regenerate the shoots over again.

Double-staining technique reveals the presence of embryogenic callus which stained blue (Figure 3B). Histological technique and fluorescein diacetate stain (FDA) were done to further confirm and validate the embryogenic callus formation and structure. This friable embryogenic callus was used in which highly totipotent embryogenic suspension cultures to be established (Figure 3A). The embryogenic cell suspensions were developed and multiplied in liquid medium containing MS supplemented with 0.6 mg/L Picloram (Figure 3C). However, the embryogenic callus-browning problem occurred during the third week of subculture in liquid induction medium. This oxidative browning/blackening problem was treated with the incorporation of polyvinylpyrrolidone (PVP), ethylenediaminetetraacetic acid (EDTA) and ascorbic acid into the liquid medium as well as putting in activated charcoal, citric acid and making half strength of MS during the early stage of suspension cultures development (Nurşen & Cüneyt, 2011).

In vitro technique of indirect plant regeneration is a rapid system to propagate and to produce large amounts of uniform pineapple plantlets. In this technique, the process begins with callus induction to which is formed by the vigorous division of plant cells prior to suspension culture treatment. Therefore, the cell suspension system served as a tool in mass propagation of Maspine pineapple. In this experiment, double-staining technique was used to detect the presence of embryogenic callus by the presence of blue color as an indicator of embryogenic cells. This validation test was done in order to ensure only fine somatic embryo to be cultivated, and later to regenerate it to produce clonal planting materials. This is because non-embryogenic cell is not competence to produce vigorous and large amounts of uniform pineapple plantlets since pineapple is a monocotyledonous crop, which is self-incompatible and highly heterozygous. Genetic engineering is important in pineapple improvement; therefore, plant regeneration is a prerequisite for genetic engineering of pineapple (Firoozabady & Moy, 2004). Evans *et al*. (1981) also mentioned the efficient protocols for plant regeneration needs to be implemented prior to application of cellular genetic techniques in crop improvement. This culture system for

Table 1. Effect of plant growth regulators on embryogenic callus formation from leaf-based *in vitro* culture of Maspine pineapple on eight different media.

Medium*	No. of sample	No. of sample producing embryogenic callus	Percentage of of sample producing embryogenic callus (%)
CI1	150	46 ± 73.5	31 ± 73.5
CI2	150	91 ± 41.7	61 ± 41.7
CI3	150	115 ± 24.7	77 ± 24.7
CI4	150	94 ± 39.6	63 ± 39.6
CI5	150	82 ± 48.1	55 ± 48.1
CI6	150	91 ± 41.7	61 ± 41.7
CI7	150	46 ± 73.5	31 ± 73.5
CI8	150	48 ± 72.1	32 ± 72.1

NOTE: * MS medium supplemented with various plant growth regulators containing 30 g/L sucrose and solidified with 8 g/L phytagel. Mean separation at $P<0.5$ as determined by *t*-test followed by the variability observed for quantitative characters as determined by ± standard deviation *(SD)*.

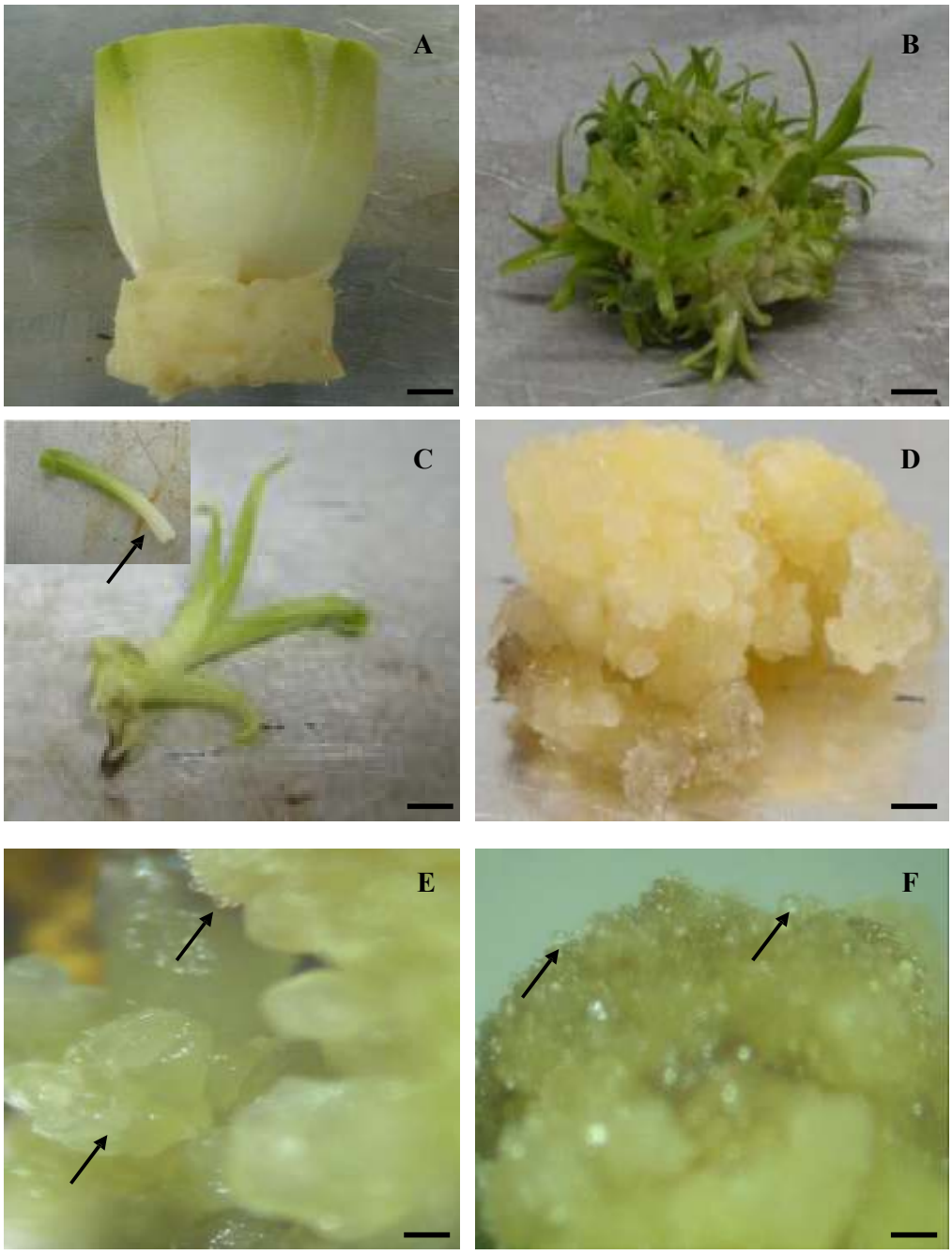

Figure 2. The steps of embryogenic callus induction of pineapple leaf-based explants treated by BAP and Picloram. (A) Slip explants, (B) Development of adventitious shoots in MS with 3 mg/L BAP after 3 months of culture, (C) *In vitro* shoot-based explants as indicated by the arrow, (D) Leaf-based derived yellowish-mucilaginous-wet-friable and large lump embryogenic callus induced on MS with 3 mg/L Picloram, and (E and F) Embryogenic cell clusters as indicated by the arrow developed after friable embryogenic tissue developed (Scale bar for A-F at 1 mm).

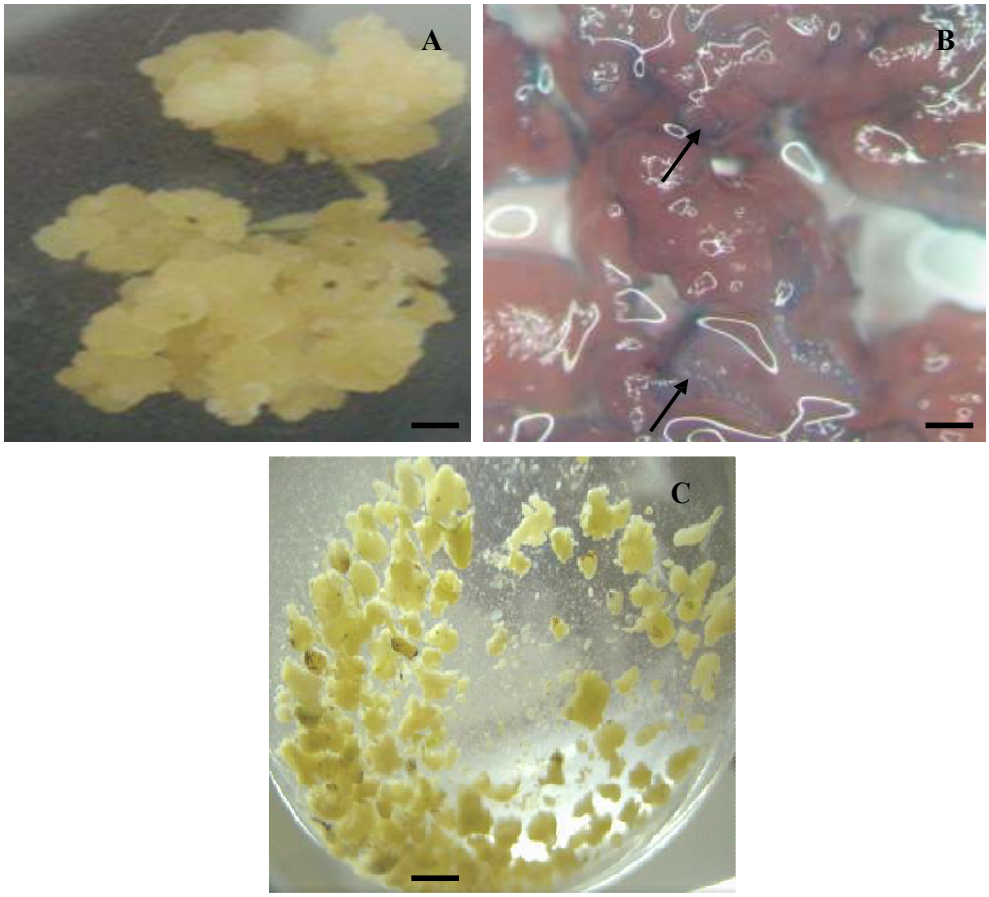

Figure 3. The events of embryogenic callus clusters in Maspine pineapple. (A) Image of rapid growing proembryogenic tissue cluster developed after two months of culture on MS with 3 mg/L Picloram (Scale bar at 1 cm) for suspension cultures inoculums, (B) Suspension cultures which stained blue as indicated by the arrow in double-staining technique (Scale bar at 0.5 cm), and (C) One-month-old suspension cells in MS with 3mg/L Picloram (Scale bar at 5 mm).

embryogenic callus formation may be also useful as materials for the genetic transformation studies in Maspine pineapple.

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REFERENCES

Canals, A.M. & Javier, F.B. (1994). Multiple shoot formation, callus induction and plant regeneration of pineapple (*Ananas comosus*). *Journal of Crop Science,* 19: 39-43.

Daquinta, M.A., Cisneros, A., Rodriguez, Y., Escalona, M., Pérez, M.C., Luna, I., & Borroto, C.G. (1996). Embryogenesis somatica en pina (*Ananas comosus*) and related species. *Acta Horticulturae*, 425: 243-246.

Devi, Y.S., Mujib, A., & Kundu, S.C. (1997). Efficient regenerative potential from long-term culture of pineapple. *Phytomorphology*, 47: 255-259.

DeWald, M.G. (1988). Production of pineapple plants *in vitro*. *Plant Cell Report*, 7: 535–537.

Firoozabady, E. & Gutterson, N. (2003). Cost-effective in vitro propagation methods for pineapple. *Plant Cell Report*, 21(9): 844-50.

Firoozabady, E. & Moy, Y. (2004). Regeneration of pineapple via somatic embryogenesis and organogenesis. *In Vitro Cellular & Developmental Biology Plant*, 40: 67-74.

Gupta, S.D. & Ibaraki, Y. (2006). Plant Tissue Culture Engineering. Dordrecht: Springer. Pp. 69-79.

He, Y.H., Fang, S.G., Hu, Z.Y., Ma, J., Luo, J., Wu, C.H., Cao, L., Lu, M., & Chen, C.J. (2012). Morphological and Anatomical Analysis of Pineapple Somatic Embryogenesis. *Acta Horticulturae Sinica*, 39(1): 57-63.

Ika R. & Ika, M. (2003). In vitro culture of pineapple by organogenesis and somatic embryogenesis: Its utilization and prospect. *Bulletin Agrobio*, 6(1): 34-40.

Ika, R, Ika, M., Nurul, K., & Gustaaf, A.W. (2012). Indirect Organogenesis and Somatic Embryogenesis of Pineapple Induced by Dichlorophenoxy Acetic Acid. *Jurnal AgroBiogen*, 8(1): 8-18.

Lakshmi Sita, G., Singh, R., & Layer, C.P.A. (1974). Plantlets through shoot tip culture in pineapple. *Current Science*, 43: 724.

Lieu, P.N., Tinh, N.N., Vui, P.V., Khai, T.P., & Teisson, C. (2004). Study of multiplication rate of conventional propagation in vivo of Cayenne (A. comosus L.) *In Proceeding of an Impact de 10 années de coopération française sur l'amélioration des productions fruitières au Vietnam* – SOFRI, May, Vietnam. Pp. 1-11.

Liu, L.J., Rosa-Marquez, E., & Lazardi, E. (1989). Smooth leaf (spineless) red spanish pineapple (Ananas comosus L. Merr) propagated in vitro. *Journal of Agriculture of the University of Puerto Rico*, 73: 301-311.

Mathews, V.H. & Rangan, T.S. (1979). Multiple plantlets in lateral bud and leaf explant in vitro cultures of pineapple. *Scientia Horticulture*, 11: 319–328.

Miguel, P.G. & Lirio, L.D.V. (2010). Strategies for the Micropropagation of Bromeliads. In S.M. Jain and S.J. Ochatt (Eds.), *Protocols for In vitro Propagation of Ornamental Plants*. New York: Humana Press. Pp. 47-66.

Nurşen, Ç. & Cüneyt, A. (2011). Inhibition of browning problem during micropropagation of *Sideritis trojana* bornm., an endemic medicinal herb of Turkey. *Romanian Biotechnological Letters,* 16(6): 6760-6765.

Sha Yalli Khan, P.S., Prakash, E., & Rao, K.R. (2002). Callus induction plantlet regeneration in *Bixa orellana* L., an annatto-yielding tree. *In Vitro Cellular & Developmental Biology Plant*, 38: 186-190.

Sripaoraya, S., Marchant, R., Power, J.B., & Davey, M.R. (2003). Plant regeneration by somatic embryogenesis and organogenesis in commercial pineapple (*Ananas comosus* L.). *In Vitro Cellular & Developmental Biology Plant*, 39: 450-454.

Yapo, E., Kouakou, T., Kone, M., Kouadio, J., Kouame, P., & Merillon, J.M. (2011). Regeneration of pineapple (*Ananas comosus* L.) plant through somatic embryogenesis. *Journal of Plant Biochemistry and Biotechnology*. 20(2): 196-204.

Zepeda, C. & Sagawa, Y. (1981). *In vitro* propagation of pineapple. *HortScience*, 16: 495.