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ESTABLISHMENT OF CELL SUSPENSION CULTURES OF FOUR ABACA CULTIVARS, 'ABUAB', 'INOSA', 'PARANG', AND 'TANGONGON'

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ABSTRACT

Established sterile cultures of four abaca cultivars previously inoculated onto MC + 22.19 µM 6-benzyladenine (BA) were induced to form meristematic buds (scalps) on MS + 1 µM indoleacetic acid (IAA) supplemented with either 50 µM BA or 1 µM thidiazuron (TDZ). Explants of all cultivars inoculated onto TDZ containing medium produced higher number of shoots and scalps than BA supplemented culture medium. 'Parang' cultivar produced the highest number of scalps followed by 'Tangongon', 'Abuab' and 'Inosa'. Globule structure formation was observed in modified liquid medium consisted of ½ MS macronutrients and Fe-EDTA with 5 µM 2,4-D and 1 µM zeatin. Fifty to sixty globules from each cultivar were collected and maintained for three weeks in fresh culture medium for the establishment of embryogenic cell suspension cultures. The highest number of cells released from globules was observed at five days after incubation except for 'Parang' cultivar which attained its peak after four days of incubation. Cell viability ranging from 59 to 87.1% was observed at four to five days of incubation in all abaca cultivars. Mature embryogenic cells of the four abaca cultivars were induced to undergo somatic embryogenesis in MS + 10 µM BA, MS + 10 µM BA + 7.6 µM Abscissic acid (ABA) and MS + 10 µM BA + 7.6 µM ABA + 0.01% w/v malt extract, however no further growth was observed in the three media formulations after 3-4 weeks of incubation. On the other hand, highest number of cells released from globules was observed at five days after incubation except for 'Parang' cultivar which attained its peak after four days of incubation. Cell viability ranging from 59 to 87.1% was observed at four to five days of incubation in all abaca cultivars. However, in MS + 9.1 µM zeatin medium, mature embryogenic cells of 'Tangongon' and 'Abuab' cultivars could successfully develop into somatic embryos. The fully developed somatic embryos obtained from 'Tangongon' cells continued to proliferate but no germination occurred. Moreover, only root formation was observed in 'Abuab' cultivar, indicating the absence of embryo bipolarity.

Key words: thidiazuron, somatic embryo, globule formation, meristematic buds, cell viability

INTRODUCTION

Abaca (*Musa textilis* Nee) known worldwide as Manila hemp is one of the country's major agricultural export and is indigenous to the Philippines. This crop is an essential source of income to many Filipinos, mostly in the regions of Bicol, Eastern Visayas and some part of Mindanao. The demand for the abaca raw fiber as a biodegradable material continuously increased in the recent past and has shown great importance locally and internationally encouraging local producers to produce more and expand abaca production areas. In spite of the increasing demand for abaca fiber, its production is limited by the outbreak of fungal and viral diseases. Efforts to control these diseases

that continue to threaten the abaca industry and the country's share in the world market should therefore be the focus of research in abaca.

Tissue culture is a very useful technique in mass propagation of disease-free and true-to-type planting materials of important crops like abaca. This technique has long been adopted by other research laboratories that are engaged in mass propagation of disease-free abaca cultivars. However, the disease-free plants can be infected with various diseases (Tegen and Mohammed, 2016). At present, genetic engineering is considered one of the effective tools in producing resistant important plant species to specific diseases that include the banana cultivars. Therefore, as a relative of banana this technique may also hold true for abaca to produce disease resistant cultivars. However, the success of this technique is largely dependent on regenerating whole plants through tissue culture. Embryogenic cells obtained from established cell suspension cultures are potential materials for genetic engineering, since these cell cultures which will be induced to undergo somatic embryogenesis produce non-chimeric regenerants or plantlets.

This research sought to optimize culture conditions for induction of somatic embryogenesis and plantlet regeneration in four abaca cultivars, 'Abuab', 'Inosa', 'Parang' and 'Tangongon', which will then be adopted in future research for crop improvement through genetic engineering.

MATERIALS AND METHODS

Enhancement of meristematic bud (scalps) formation. The in vitro plantlets of four abaca cultivars, 'Abuab', 'Inosa', 'Parang', and 'Tangongon' cultured on MS + 22.198 µM 6-benzyladenine (BA) medium were used as sources of explants. The basal regions of the shoots were used as explants for meristematic bud (scalps) formation. Explants were cultured on MS + 1 µM indole acetic acid (IAA) supplemented with either 1 µM thidiazuron (TDZ) or 50 µM BA. Sucrose was added at 4% w/v into the culture medium and solidified with 0.6% w/v agar. The number of meristematic buds (scalps) formed and number of shoots proliferated were recorded at 3 weeks after inoculation on both culture media formulations.

Establishment of embryogenic cell suspension cultures. Meristematic buds (scalps) developed from the basal region of *in vitro* cultures of four abaca cultivars were excised and transferred into modified liquid maintenance culture medium for the induction of globule structure formation. The components of liquid medium was ½ MS macronutrients and Fe-EDTA, full strength MS micronutrients and modified vitamins with 1.19 µM thiamine HCl and 3% sucrose plus 5 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 1 µM zeatin. To reduce browning of scalps, ascorbic acid at 56.278 µM was added into the culture medium and the scalps were transferred to fresh medium every other day for the first two weeks. Succeeding transfer of scalps in fresh medium was done monthly until globules were formed.

Fifty to sixty globules were placed into each flask containing 10 mL liquid medium. The density of cells released from globules into the suspension was determined every 3 weeks through direct cell counting from a 20 µL/ml sample of each abaca cultivar using a hemocytometer under light microscope. Cells were transferred to the same culture medium but devoid of 2,4-D and zeatin and were incubated for one to three weeks to induce cell maturation. Another set of cell suspension cultures was established to determine cell density and viability in four abaca cultivars at 3, 4, 5 and 7 days of incubation. All cultures were incubated in dark condition and were continuously agitated at 80 – 100 rpm on a gyratory shaker. Cell viability was determined by staining the cells with Evan's Blue (Fernandez-Da Silva and Menéndez-Yuffá, 2006).

Induction of somatic embryogenesis. Embryogenic cells obtained from three weeks of continuous incubation in maintenance medium were transferred to liquid MS medium devoid of 2,4-D and zeatin

for the induction of cell maturation. Mature cells of four abaca cultivars obtained from 3 weeks of incubation were transferred into three liquid media formulations: A) MS + 10 µM BA; B) MS + 10 µM BA + 7.6 µM abscissic acid (ABA); and C) MS + 10 µM BA + 7.6 µM ABA + 0.01% w/v malt extract for induction of somatic embryogenesis. Cells were incubated for 3 weeks. Cell growth was monitored under the light microscope. Embryogenic cells of the four abaca cultivars were plated onto semi-solid medium of similar composition as for the induction of somatic embryogenesis and incubated for 3-4 weeks.

A separate experiment using globular masses from embryogenic cell suspensions of 'Tangongon' and 'Abuab' cultivars obtained from the new set of cell suspension cultures incubated for 5 days in liquid maintenance medium were collected and directly plated onto semi-solid MS medium + 9.1 µM zeatin, without passing through a culture medium devoid of plant growth regulator. Embryo germination was monitored on the plated cells.

Experimental design and data analysis. All experiments were laid out in a Completely Randomized Design with three replications each. Data on scalp formation were compared using t-test at 5% significance level while the experiments on establishment of cell suspensions, induction of somatic embryogenesis and plantlet regeneration were reported using descriptive statistics.

RESULTS AND DISCUSSION

Enhancement of meristematic bud (scalps) formation

The effect of BA and TDZ on meristematic buds (scalps) and shoot formation was investigated on 'Abuab', 'Inosa', 'Parang' and 'Tangongon' cultivars previously established onto MS + 22.20 µM BA (Fig.1). The number of meristematic buds (scalps) and shoots formed on the four abaca cultivars are presented in Table 1. It was noted that at 3 weeks of incubation, explants of the four abaca cultivars inoculated onto medium containing 1 µM TDZ resulted to higher number of meristematic buds (scalps) than explants inoculated on medium containing 50 µM BA. The highest number of scalps was produced by 'Parang', followed by 'Tangongon', 'Abuab' and lastly, 'Inosa'. Although, it was observed that TDZ supplementation enhanced meristematic bud formation in all the abaca cultivars investigated, only 'Abuab' cultivar did not show significant difference between the two media formulations.

Table 1. Average number of meristematic buds (scalps) and shoots formed on *in vitro* derived explants of abaca cultivars inoculated onto two media formulations after 3 weeks.

Cultivars	**Number of meristematic buds (scalps) formed**		**Number of shoot formed**	
	6-Benzyladenine (**BA**)	Thidiazuron **(TDZ)**	6-Benzyladenine (**BA**)	Thidiazuron **(TDZ)**
'Abuab'	8.7a	12.8a	3.7a	4.8b
'Inosa'	4.1a	12.5b	6.5a	6.7a
'Parang'	11.1a	16.8b	4.1a	6.4b
'Tangongon'	9.7a	15.6b	4.2a	3.8a

Values per variable followed by a different letter in a row are significant at 5% level using t-test

Similar to scalp formation, higher number of shoots formed was observed in TDZ than in BA supplemented culture media. Different abaca cultivars have varying responses to a given plant growth regulator. The highest number of shoots formed in TDZ supplemented medium was obtained from 'Inosa', followed by 'Parang', 'Abuab' and lastly, 'Tangongon'. 'Abuab' and 'Parang' showed significant differences between the two treatments while 'Inosa' cultivar had the least number of meristematic buds than shoots formed. The meristematic buds (scalps) formed on explants inoculated

onto culture medium supplemented with TDZ are smaller in size compared with those on BA containing medium. These had to be transferred onto BA containing medium for further growth and enlargement. These results were consistent with the findings of Shirani et al. (2010) that demonstrated induction and good quality scalps in banana could be obtained with medium containing high concentration of TDZ (7.5 µM). Greatest percentage of axillary bud proliferation in banana and shoot proliferation in some banana cultivars increased with increasing TDZ concentration (Shen et al. 2010).

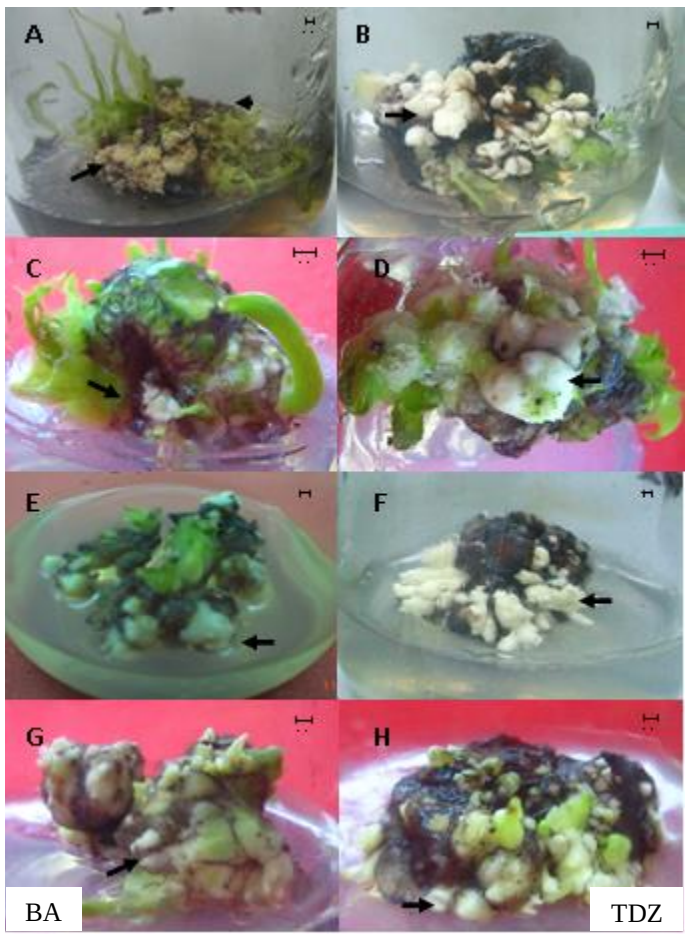

Fig. 1. Meristematic buds (scalps, arrows) and shoot formation in Abuab (A & B), Parang (C & D), Tangongon (E & F) and Inosa (G & H) cultured in MS + 1 µM IAA + 50 µM BA (BA) and MS + 1 µM IAA + 1 µM TDZ (TDZ).

Establishment of embryogenic cell suspension cultures

Continuous incubation for two to three weeks resulted in browning of tissues and culture medium, hence, transfer interval to freshly prepared culture medium was shortened to every other day for the first 2 weeks of culture. The browning of tissues and culture medium was due to the oxidation of phenolic compounds that exude from the explants, however, could be overcome by frequent transfer of tissues to fresh medium and with the addition of ascorbic acid to the culture medium (Banerjee and Langhe, 1985; Srangsam and Kanchanapoom, 2003). The formation of globular

structures on the surface of the scalps was observed 4-5 weeks after inoculation irrespective of explants source (Table 2 and Fig. 2A, arrow). The 'Abuab' cultivar showed the earliest days to globule formation which took 26.1 days after incubation from the initial inoculation of the scalps, followed by 'Inosa' at 28.9 days, 'Parang' at 31.0 days and lastly, 'Tangongon' at 32.7 days after incubation (Table 2). It was also observed that 'Abuab' was the earliest to release globules at an average of 39.7 days from the first sighting of meristematic globule formation.These results also indicated that the formation and release of glossy and yellowish globules (Fig 2C) varied with different abaca cultivars. These globular structures were released into the culture medium together with loose, few single cells (Fig. 2B).

Table 2. Average number of days to globule formation and release of globules from scalps inoculated into modified ½ MS liquid medium + 5 µM 2,4-D + 1 µM zeatin in four abaca cultivars.

Cultivars	Days to globule formation	Days to globule release from 1st sighting
'Abuab'	26.1	39.7
'Inosa'	28.9	55.3
'Parang'	31.0	70.8
'Tangongon'	32.7	61.5

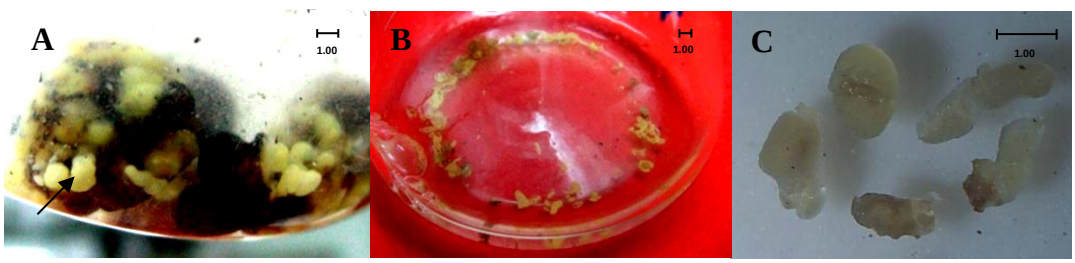

Fig. 2. Formation of meristematic globules on scalps inoculated in liquid MS + 5 µM 2,4-D + 1 µM zeatin (A); meristematic globules released (B); and glossy and yellowish globules observed under a stereomicroscope (10x; Meiji Technology) (C).

The highest number of cells released into the culture medium after 3 weeks of incubation was observed in 'Inosa' (5.30 x 10^6 cells/10 mL suspension), followed by 'Parang' (3.29 x 10^6), 'Tangongon' (2.92 x 10^6) and lastly, 'Abuab' (1.17 x 10^6). However, it was observed that % cell viability was relatively low after 3 weeks of incubation. Although dividing cells were observed, these were very few in number. Hence, a new set of cell cultures were established. As shown in Fig. 3, the number of cells released into the liquid culture medium increased with time, i.e. from 3 days with the highest number of cells observed at 5 days and declined at 7 days after incubation into ½ MS macronutrients and Fe-EDTA supplemented with 5 µM 2,4-D and 1 µM zeatin, except for 'Parang' cultivar which attained its peak after 4 days of incubation. Although, cell count of 'Parang' cultivar declined at 5 days after incubation, nevertheless, it still had the highest cell count followed by 'Inosa', 'Tangongon' and lastly, 'Abuab'. In contrast to cell count, viability of cells of the four abaca cultivars relatively decreased with time (Table 3). The rate of decrease in cell viability from 3 days to 7 days of incubation varied with abaca cultivars. It should be noted that 'Tangongon' cultivar showed a decline in % cell viability after 4 days and recovered after 5 and 7 days of incubation. This was probably because cells of 'Tangongon' cultivar exude less phenolic compounds or toxic products which are known to inhibit cell division (Kenneth and Torres, 1989 as cited by Sadik, 2007).

These results indicate that suspension cultures should be refreshed or transferred into fresh medium, at a regular interval depending on the abaca cultivars. Transfer of cells to newly prepared culture medium be done in their exponential or linear phase to maintain actively growing and dividing cells and high cell viability. At 3 days of incubation, the cells were still very young or have not yet stabilized in the new culture medium that may have contributed to low number of cells released into the culture medium. However, cells obtained after 5 days were relatively mature and with prominent nucleus (Fig. 4, arrow), an indication of a morphogenetic competence of the cells (Dhed'a et al., 1991). Further microscopic investigation revealed cell division which form discrete culture masses are shown in Fig. 5. It also shows the general appearance of cells of the four abaca cultivar at 5 days after inoculation. Generally, small and dense spherical embryogenic cells with prominent nucleus were obtained in the four abaca cultivars as the examples in Fig. 5A. These embryogenic cells were capable of dividing, and eventually gave rise to "daughter" cells (Fig. 5C-B). In the case of a large cells internal division occurred resulting to a compact mass of cells (Fig. 5C). In some instances, repeated cell division in the "daughter" cell occurred, which later form multi-cellular mass leading to formation of cell aggregates (Fig. 5C).

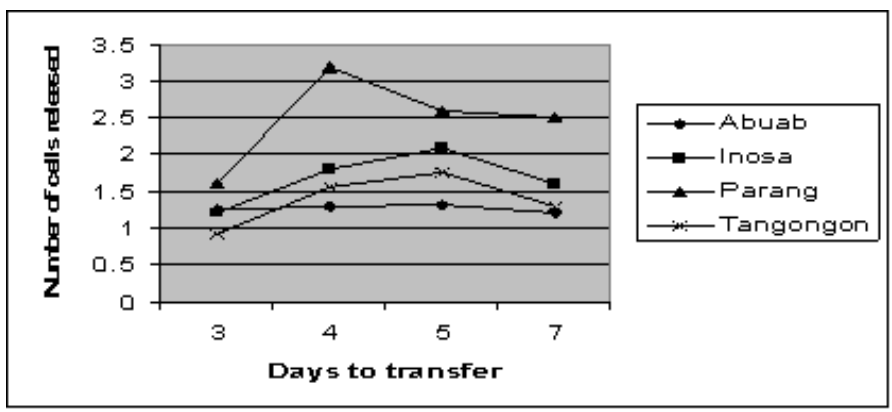

Fig 3. Average number of cells released from globules obtained on four abaca cultivars at different days of incubation into modified ½ MS liquid medium + 5 µM 2,4-D + 1 µM zeatin.

Table 3. Percent viability of cells released from globules obtained on four abaca cultivars at different days of incubation into modified ½ MS liquid medium + 5 µM 2,4-D + 1 µM zeatin.

Cultivars	**Cell viability (%)**			
	3 days	4 days	5 days	7 days
'Abuab'	91.1	87.1	84.7	74.4
'Inosa'	64.1	64.7	62.5	56.2
'Parang'	67.0	66.0	59.0	55.0
'Tangongon'	84.2	64.9	76.6	77.6

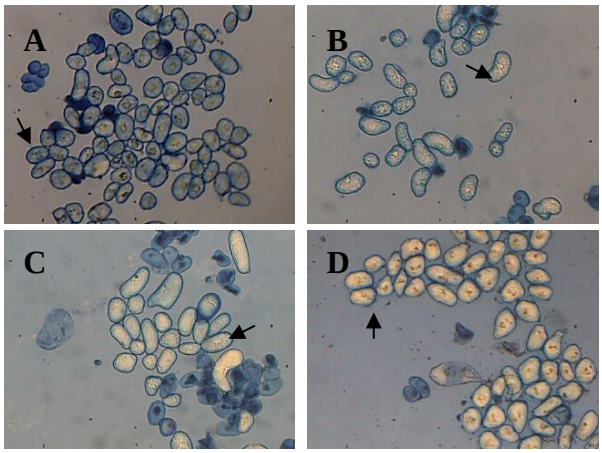

Fig 4. Cells with prominent nucleus (arrow) that were released from globules of abaca cultivars after 5 days of incubation: Abuab (A), Inosa (B), Parang (C) and Tangongon (D) (100x; Spencer compound microscope). Cells viability was evaluated by Evan's Blue staining and the dead cells were stained blue.

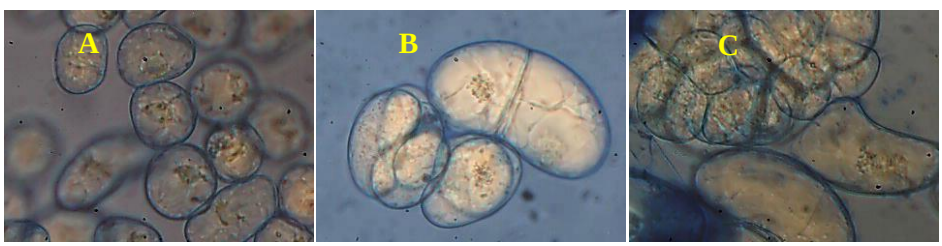

Fig. 5. Embryogenic cells of abaca (**A**) competent of cell division (**B**) that will further form into cluster of viable cells (**C**) (400x; Spencer compound microscope).

In the study of Ozawa and Komamine (1989), high frequency of regeneration was obtained from cell suspension cultures of *Oryza sativa* L. when cells were subcultured at 3-days interval rather than 7 days interval. Megia et al. (1993) reported that suspension cultures of cooking banana of cv. Bluggoe (Musa spp., ABB group) tended to lose cell totipotency as they aged which have caused the decline in cell number with continuous incubation of the four abaca cultivars. Similarly, Panis and Swennen (1993) reported that cell suspensions of Musa sp. older than two weeks manifest a low survival rate because cells were highly vacuolated. These phenomena were also observed in the four abaca cultivars that were continuously incubated in the same culture medium. The highly vacuolated cells are non-embryogenic and are therefore not suitable for transformation. Swennen et al. (1998) reported that a 4-day-old cell suspension culture of Cavendish banana was used for bombardment and regeneration of transgenic banana was successfully achieved. The morphohistological study on different banana embryogenic cell suspensions conducted by Georg et et al. (2000) revealed that the age of the cell suspension influenced the development of cell aggregates. These findings suggest that the age of cell suspension cultures and interval between subculture are important considerations to obtain a high rate of cell growth and eventually plantlet regeneration.

Induction of somatic embryogenesis

Cells that were inoculated in these various culture media differed in terms of cell organization (Fig. 6). Individual or single cells were commonly seen in medium with BA alone (Fig. 6A), whereas cell clusters were mostly observed in culture media containing BA in combination with ABA (Fig. 6B) and in culture medium supplemented with BA, ABA and malt extract (Fig. 6C). However, mature cells of the four abaca cultivars did not show further growth in the three media formulations after 3-4 weeks in culture. It was noted that in cell suspension cultures of 'Inosa' and 'Parang' contamination almost always manifest at the later stage of every newly established cell suspension cultures, which may be caused by endophytic bacteria present in the explants. Contaminants such as endogenous bacteria that escaped initial disinfection and microorganisms introduced during the tissue culture process may survive in the plant material for several subculture cycles without expressing symptoms in the tissue or visible signs in the culture medium (Strosse et al. 2004).

Induction of somatic embryos and plantlet regeneration in Tangongon and Abuab cultivars

Embryogenic cells of the four abaca cultivars inoculated in the three liquid media formulations failed to induce somatic embryogenesis. However, from the new set of experiment done, somatic embryos were formed in 'Tangongon' cultivar at various days after plating as shown in Fig. 7. Embryogenic cells (Fig. 7A) released from globules of 'Tangongon' into the maintenance medium were collected and plated onto the germination medium. After 17 days of incubation, embryogenic cells appeared opaque, yellowish and glossy structures (Fig. 7B), while at 36th day these structures increased in size (Fig. 7C) which continuously progressed as observed at 70th day of incubation.

Fig. 6. Embryogenic cells of Parang taken 21 days after inoculation in **A**) MS + 10 µM BA, **B**) MS + 10 µM BA + 7.6 µM ABA, and **C**) MS + 10 µM BA + 7.6 µM ABA + 0.01% w/v malt extract (100x). Photos taken using LW Scientific, MiniVid USB Camera and magnified x100.

Fig. 8 shows the fully developed somatic embryos from cells of 'Tangongon' cultivar after 70 days from plating. However, somatic embryos failed to germinate. A modification of the germination medium has to be considered to initiate embryo germination.

Fig. 9 illustrates a similar response of 'Abuab' cultivar to the germination medium as observed in 'Tangongon' cultivar during the early stages of somatic embryogenesis. It was noted that cells (Fig. 9A) after 8 days of incubation became swollen (Fig. 9B). Subsequent transfer onto solid medium of similar composition showed further cell enlargement and development of somatic embryos (Fig. 9C) that eventually lead to the formation of root (Fig. 9D). The root continued to elongate but shoot development did not proceed. The findings in 'Abuab' cultivar indicated the absence of embryo bipolarity which may be caused by poor individualization of the shoot and root meristems. These findings were also observed in 'Cau man', a banana cultivar in Vietnam (Viet and Huong, 2004). Furthermore, Litz and Gray (1992) elucidated that somatic embryogenesis is triggered by subculturing explants onto culture medium without auxin however subsequent development of cotyledons and bipolarity is suppressed. It should be noted that the cell suspension cultures of the 'Abuab' cultivar

that were plated onto the germination medium were directly obtained from auxin containing medium, which may have caused the absence of bipolarity in the developing embryos.

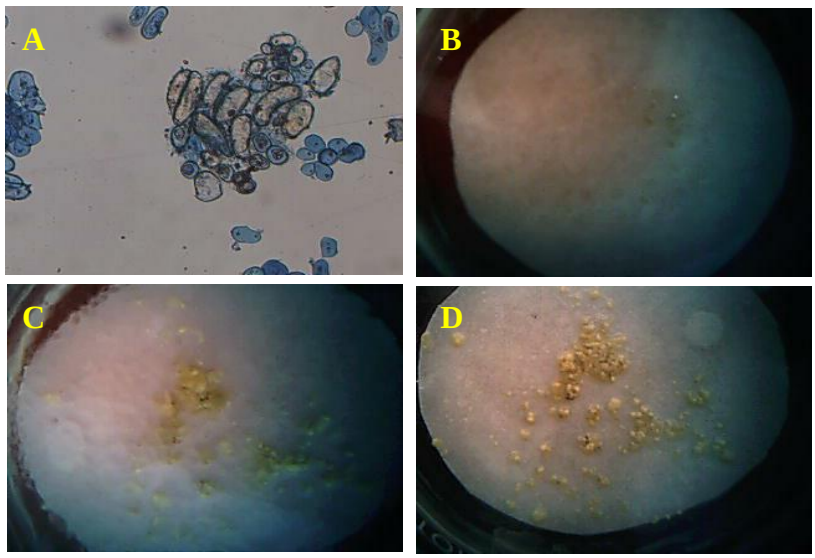

Fig. 7. Somatic embryo formation of abaca cv. 'Tangongon': Mature embryogenic cell cultures before plating onto MS + 9.1µM zeatin (**A**), plated cells at 17th day (**B**), 36th day (**C**) and 70th day (**D**) after plating.

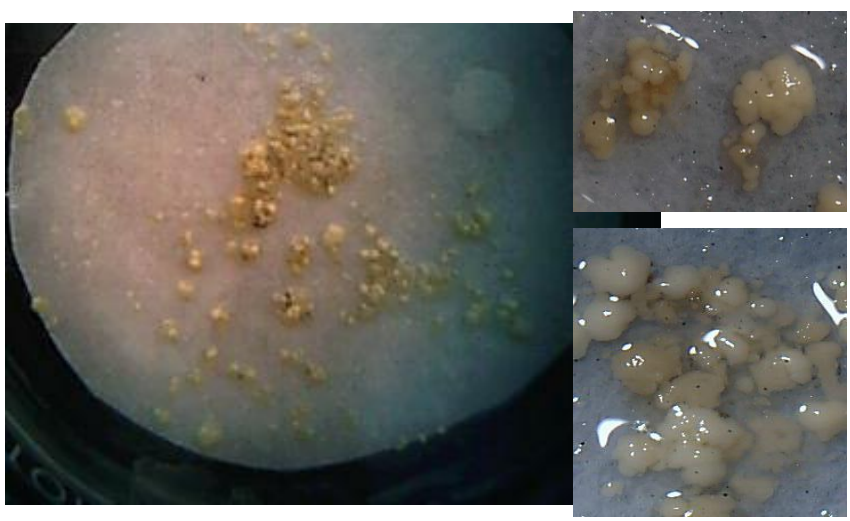

Fig. 8. Somatic embryos formed from embryogenic cells of 'Tangongon' after 70 days from plating on MS medium + 9.1 µM zeatin.

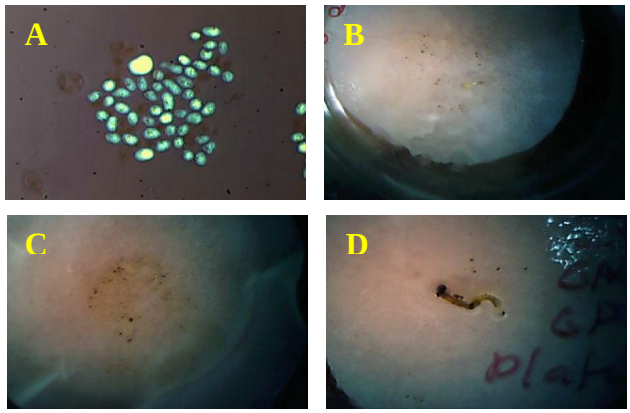

Fig. 9. Embryogenic cell cultures of 'Abuab' cultivar before plating onto MS medium + 9.1µM zeatin (**A**), cells after 8th (**B**) and 15th (**C**) day, after plating and germinated somatic embryos after 28th day of plating (**D**).

CONCLUSION

Different abaca cultivars had varying responses to a given culture medium formulation. TDZ supplemented culture medium favored meristematic bud and shoot formation in four abaca cultivars but scalps were small. Mature, embryogenic cells of the four abaca cultivars obtained from three weeks of continuous incubation were induced to undergo somatic embryogenesis but failed to develop into somatic embryos. Hence, an independent experiment was conducted in 'Tangongon' and 'Abuab' cultivars using another medium formulation which consisted of MS + 9.1µM zeatin to allow the induction of somatic embryos without passing through a culture medium devoid of PGR.

Somatic embryos that developed from cells of 'Abuab' cultivar are unipolar in nature generating roots only upon germination. While fully developed plants were not produced from cells of the four cultivars, it was demonstrated that germination is possible at least for 'Abuab'. These findings reflect the need for further improvement in *in-vitro* culture conditions to induce development of bipolar embryos that would generate both roots and shoots

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