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Development of a Cell Suspension Protocol for Abaca (*Musa textilis* Nee 'Inosa')

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A protocol for the establishment of embryogenic cell suspension in abaca was developed based on the International Network for the Improvement of Banana and Plantain (INIBAP) procedure for *Musa*. Meristematic buds from shoot cultures were excised and cultured on P5 medium for several cycles until 'scalps' formed. These scalps were then used to produce embryogenic complexes (ECs) in 2,4-D-containing media. Discrete primary somatic embryos (SEs) were observed on ECs 3–4 wk after initial culture. After 6 mo, 6 % of the 150 inoculated scalps formed into ECs that had at least 10 SEs. These were then used to commence cell suspensions. Nine liquid media formulations were tested, and only M2 medium produced cell lines that had a characteristic bright yellow suspension indicative of embryogenic potential. However, doubling time during the initiation phase with M2 medium was lower than the doubling time in the rest of the media tested. In several months, cell lines in M2 medium stabilized with an average doubling time of about 4 wk. The old medium was replenished with fresh medium every 4–7 d, replacing at least 80% of the old medium. Maintenance of fine homogenous suspension was done at monthly intervals by transferring 2 mL aliquots with a settled cell volume (SCV) of 0.2–0.3 mL from a 1-mo-old suspension culture to an 8 mL fresh medium in 125-mL Erlenmeyer flasks. As for embryo development, regeneration media trials showed that Murashige and Skoog (MS) and M2 media promoted the formation of yellow, nodular calli, or pro-embryogenic masses (PEMs). MS and M2 medium supplemented with 1 ppm AgNO3 (with or without ascorbic acid) could enhance PEM formation, especially if the cells were pre-induced to form PEMs by gradual reduction of 2,4-D while at the cell suspension stage. Sucrose or glutamine does not seem to have any promotive effect on PEM induction during the regeneration phase. These treatments, however, were not favorable for the conversion of PEMs into mature somatic embryos. Likewise, M3 regeneration media promoted PEM formation but failed to induce somatic embryogenesis in a separate experiment. PEMs derived from the 2,4-D reduction experiment were proliferated in M2 medium and transferred in P5 for embryo development. PEMs bearing mature somatic embryos in P5 medium were transferred to abaca shoot proliferation medium where fully developed shoots were obtained in 6 mo. Although the protocol requires validation, our results clearly demonstrate that abaca could undergo *in vitro* somatic embryogenesis (SE) and offer a transformation platform for modern genetic improvement work.

Key Words: abaca, embryogenic cell suspension, 'Inosa', regeneration, somatic embryogenesis

Abbreviations: ABTD – abaca bunchy top disease, EC – embryogenic complex, INIBAP – International Network for the Improvement of Banana and Plantain, PEM – pro-embryogenic mass, PGR – plant growth regulator, PhilFIDA – Philippine Fiber Development Authority, SCV – settled cell volume, SE – somatic embryogenesis

INTRODUCTION

Abaca (*Musa textilis* Nee) or Manila hemp is indigenous to the Philippines. As one of the country's major agricultural exports, it is an essential source of income to many Filipinos, in the Bicol, Eastern Visayas and Davao Regions (PSA 2019; Lacuna-Richman 2002). The demand for the abaca fiber is projected to increase by 12% per annum, driven by renewed interest in biodegradable resources encouraging producers and processors to expand abaca production areas to produce more fiber (PhilFIDA 2018).

Despite the increasing popularity of abaca fiber and its products, the outbreak of diseases has significantly affected the productivity of abaca as an industrial crop (Bajet and Magnaye 2002). Abaca bunchy top disease (ABTD) is the most economically significant viral disease of abaca. ABTD can cause widespread devastation, often requiring the destruction of abaca plantations (Raymundo et al. 2001, 2002). Mass propagation of disease-free abaca planting materials through shoot-tip culture (Tolentino 1984; Aspuria 2003) is a way to repopulate old abaca stands. Unfortunately, the technique cannot overcome the problems caused by fungal and viral diseases because inoculum in previously planted abaca plantations could

re-infect newly established plants.

The relatively long breeding cycle of abaca hinders the development of improved varieties resistant to diseases through conventional methods (Lalusin and Villavicencio 2015). Disease-resistant abaca cultivars through the integration of genetic engineering into the breeding programs may offer a powerful tool to overcome these limitations. Still, the success of genetic engineering is dependent mainly on regenerating whole plants through tissue culture.

In banana and plantains, genetic improvement employs the somatic embryogenesis (SE) technique. The procedure involves initiating cell suspension cultures from callus or embryogenic tissue. Cells obtained from these suspensions develop into bipolar embryos and regenerate into whole plants (Strosse et al. 2003). Embryogenic cells from suspension cultures are preferred material for genetic transformation because regenerants are non-chimeric, which minimizes problems in the selection and characterization of transformed plants.

Somatic embryogenesis in *Musa* spp. is known to be dependent on genotype, explant type, the age of the plant material, and the culture medium (Youssef et al. 2010). In bananas and plantains, explants such as male flowers (Escalant et al. 1993; Côte et al. 1996; Navarro et al. 1997; Ganapathi et al. 1999; Khalil et al. 2002), rhizome tissues and leaf sheaths (Jamaluddin and Novak 1992), zygotic embryos (Navarro et al. 1997) and meristematic buds/ scalps (Dhed'a et al. 1991; Megia et al. 1993; Panis et al. 1993; Schoofs et al. 1998) are useful in generating cell suspensions. Although different types of explants can be used for establishing embryogenic cell suspensions in *Musa*, the scalp (meristematic buds) methodology which relies on highly proliferating shoot tips is much preferred to initiate embryogenic cell suspension cultures in banana (Dhed'a et al. 1991; Schoofs et al. 1998; Strosse et al. 2004) because it can be made available year-round. The 'Saba' banana cell suspension protocol also uses scalps (Aspuria and de Juras 2007).

In abaca, embryogenic cell suspensions could be successfully initiated using regenerated shoots and scalp (Aspuria 1997; Aspuria et al. 2003; Aspuria and Garcia 2018) largely based on the Dhed'a protocol, but embryo development and maturation and the subsequent germination of (unipolar) embryos remain major bottlenecks in the protocol as well as lethal browning and the presence of endophytes (Bernardo et al. 2014; Aspuria and Garcia 2018). Further, SE requires the acquisition of embryogenic competence and involves an induction phase to transition from somatic to embryogenic cells (Dodeman et al. 1997). Organic supplements like glutamine (Husin et al. 2014) and sucrose (Tremblay and Tremblay 1995) or growth regulators such as 2,4-D (Jimenez 2005) or other substituents such as AgNO3 and ascorbic acid can control browning and facilitate the conversion of cells into somatic embryos, especially if the cells exhibit some form of recalcitrance or premature necrosis.

Abaca is generally perceived as a recalcitrant species to the cell suspension procedure. The absence of published and replicable cell suspension procedures for abaca in local literature reflects the difficulty of adapting the cell suspension technique to abaca, as opposed to the optimized cell suspension protocol achieved in other *Musa* species (Strosse et al. 2006). In this study, we have developed a cell suspension protocol for abaca based on the INIBAP protocol for *Musa*, clearly demonstrating that abaca is amenable to the somatic embryogenesis technique. We also report on the induction of embryogenic complexes (ECs) using scalps and detail the steps and considerations in commencing abaca cells suspension from these ECs. We examined a combination of factors that are known to improve SE including reduction of 2,4-D in the culture medium as a stimulus to express SE, the addition of organic additives, i.e. sucrose and glutamine to enhance SE and the use of silver nitrate (AgNO3) as an ethylene inhibitor as well as control for lethal browning. And, finally, we show the formation of pro-embryogenic masses (PEMs) from embryogenic cells and their conversion into mature somatic embryos and fully developed shoots.

MATERIALS AND METHODS

The study describes a protocol for the regeneration of 'Inosa' abaca that is a close parallel of the INIBAP method (Strosse et al. 2003).

Multiplication and Maintenance of Shoot Cultures

Proliferating *in vitro* cultures of 'Inosa' abaca obtained in 2012 from PhilFIDA-Tacloban Tissue Culture Laboratory were used throughout the study. Shoot cultures were sub-cultured every 4–6 wk in freshly prepared abaca shoot multiplication medium, MS + 5 ppm 6-benzylaminopurine (BAP) + 40 g/L sucrose (Tolentino 1984). The cultures were kept under continuous light supplied by white fluorescent lamps with photosynthetic photon flux density (PPFD) of 25–40 μmol m^{-2} s^{-1}. The culture room was maintained at 25 ± 2°C (standard condition).

Production of White Meristematic Buds and Scalps from Shoot Cultures

White meristematic buds interspersed with leaves could

be induced to form in 'Inosa' after repeated subcultures by de-topping the leaves and cutting close to the base (Fig. 1A; Bernardo et al. 2013). To produce "scalps" (Fig. 1B), sectors of white meristematic buds (3–5 mm^3) were placed on INIBAP P5 medium (MS, 10 mg/L ascorbic acid, 0.175 mg/L IAA, 2.273 mg/L BAP, 30 g/L sucrose, 3 g/L Gelrite, pH 5.8; Strosse et al. 2003) and subcultured monthly. In each subculture, the meristems appeared "cauliflower-like" (Schoofs et al. 1998) from proliferative growth and could be subdivided into two to three sectors each of 5 mm size and then cultured separately. Blackened, dead tissues and sectors showing shoot growth were removed at each subculture. The meristematic bud/scalp cultures were kept in total darkness for 6–8 mo at a temperature of 27 ± 2°C. Glass vessels of 50 mL capacity were used which contained 20 mL of the P5 medium.

Induction of Embryogenic Complex from Scalps

Individual scalps (Fig. 1B) derived from P5 medium were inoculated onto INIBAP ZZss medium (Strosse et al. 2003) in small glass vessels (ca. 50 cm^3) covered with aluminum foil and parafilm. ZZss medium is composed of ½ MS macro-elements, full-strength MS micro-elements, MS vitamins, 10 mg/L ascorbic acid, 1 mg/L 2,4-D, 0.219 mg/L zeatin, 30 g/L sucrose solidified with 3 g/L Gelrite® and adjusted to pH 5.8 before autoclaving. The cultures were maintained in the dark at 27 ± 2°C for 6–7 mo without subculturing. The formation of nodular structures and the development of embryogenic complexes were observed monthly.

Establishment of Cell Suspension Cultures from Embryogenic Complex Induced from Scalps

Six- to seven-month-old embryogenic complex bearing at least 10 discrete SEs (Fig. 1C) induced in INIBAP semi-solid medium was used. Two hundred to two-hundred-fifty milligrams (200–250 mg) of the embryogenic complex were inoculated to nine different media formulations (Table 1) in 50 mL Erlenmeyer flasks tightly covered with foil and sealed with parafilm (Fig. 1D). The ECs in liquid media were incubated in total darkness at 27 ± 2°C in an orbital shaker using 100 rpm. Heterogenous suspension formed in a week and portions of the dissociated callus were present in the liquid medium. Ten drops of freshly prepared medium were replenished every 4 d for 1 mo and then every 10–14 d in the succeeding months until a homogenous suspension mostly consisting of individual and group of cells was achieved (~3–6 mo). Fine embryogenic suspensions (Fig. 1E) were maintained by using a small pipette tip or by occasional sieving when cell aggregates have become larger than 2 mm^3.

Once a fine suspension is obtained (Fig. 1F), total cell count and viable cells were measured using a hemocytometer every 4 d over 16 d to ensure that the cells are actively proliferating in the respective medium. Viable and dead cells were set apart by Evan's blue staining (1%, w/v). Non-stained cells were considered viable. The experiment with the different formulations was designed as a single factor experiment in CRD.

The capacity to undergo somatic embryogenesis was routinely tested by plating 1 mL cell suspension on 25 mL basal MS medium in disposable Petri plates (Fig. 1F).

Effect of the Gradual Withdrawal of 2,4-D from M2 Liquid Medium on Viable Cell Count and PEM Formation in M3 Solid Regeneration Medium

Established cell suspensions in M2 medium were used. A two mL aliquot with ca. 0.1 SCV mL^{-1} was resuspended in 8 mL of standard M2 medium to make a total volume of 10 mL in a 125 mL Erlenmeyer flask and sealed with aluminum foil. Treatments were T1: no refreshment, T2: with refreshment of standard M2 medium, and T3: with refreshment with M2 medium devoid of 2,4-D. The suspensions were refreshed at 4-d intervals. Cell count, SCV, and cell morphology were recorded on the 15^{th} and 30^{th} days of incubation. There were a total of 9 flasks for each treatment.

After a month, 0.2 mL SCV from each treatment was plated in M3 regeneration medium and incubated in the dark for 3 mo. M3 regeneration medium contains Schenk and Hildebrandt (1972) basal salts, amino acids, four types of cytokinin and two carbohydrate sources (Côte et al. 1996). Four plates for each of the nine flask replicates per treatment were used to set up the experiment. Hence, there were a total of 36 plates per treatment (4 plates x 9 cell suspensions = 36 plated cells). Development and the proportion of somatic embryos were observed every 2 wk for 3 consecutive months.

Both the cell suspension and induction of somatic embryogenesis stages were designed as a single-factor experiment in CRD. The data were analyzed using repeated measures design ANOVA, and the time x treatment interaction was tested. If there were no significant interactions detected, the main effect of time and the main effect of treatments were further tested. This premise is attributed to the natural multiplication process of cells as a biological system.

Effect of Increased Glutamine Levels on Viable Cell Count and PEM Formation

Similar to the experiment on the gradual reduction of 2,4-D, established cell suspensions in M2 medium were used.

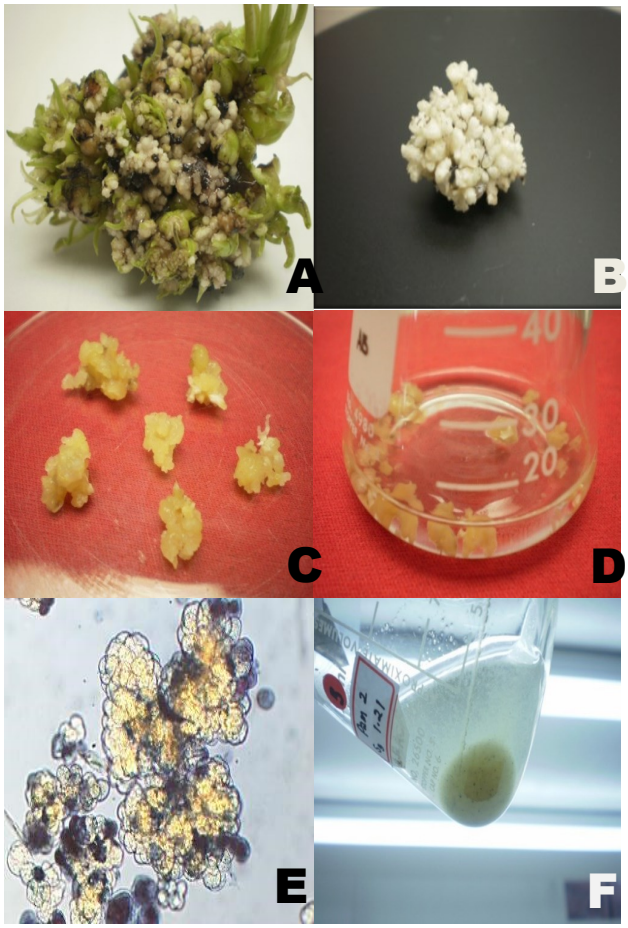

Fig. 1. Steps in the generation of cell suspensions and induction of somatic embryogenesis in abaca. A. Multiplying shoot cultures of abaca 'Inosa' were de-topped every 4 wk above the growing point and cultured successively on abaca multiplication medium. When repeated for several subculture cycles, white meristematic buds are produced, which can be separated from the mass of tissue for further culture in P5 medium. B. Scalp from repeated culture of white meristematic buds in P5 medium in the dark was used to initiate embryogenic complexes (ECs). C. 6-mo-old ECs bearing discrete somatic embryos. D. Ca. 200–250 mg ECs in 10 mL liquid medium. E. Heterogenous suspension formed in a week and portions of the dissociated callus were present in the liquid medium. F. Ten drops of freshly prepared medium were replenished every 4 d for 1 mo and then every 10–14 d in the succeeding months until a fine suspension was achieved (~3–6 mo).

A two mL aliquot with ca. 0.1 SCV mL^{-1} was resuspended in 8 mL of standard M2 medium to make a total volume of 10 mL in a 125 mL Erlenmeyer flask and sealed with aluminum foil. Treatments were T1: 100 ppm glutamine and T2: 400 ppm glutamine. The suspensions were refreshed at 4-d intervals. Cell count, SCV, and cell morphology were recorded on the 15th and 30th days of incubation. There were a total of 9–10 flasks for each treatment.

After 1 mo, 0.2 mL SCV from each treatment was plated in ZZss medium and incubated in the dark for 3 mo. Four plates for each of the 10 flask replicates per treatment were used to set up the experiment. Hence, there were a total of 40 plates per treatment (4 plates x 10 cell suspensions = 40 plated cells). Development of PEMs and the proportion of somatic embryos were observed every 2 wk for 3 consecutive months.

Both the cell suspension and induction of somatic embryogenesis stages were designed as a single-factor experiment in CRD. The data were analyzed using repeated measures design ANOVA. The time x treatment interaction was tested. If there were no significant interactions detected, the main effect of time and the main effect of treatments were further tested.

Effect of Sucrose, $AgNO_3$, and Glutamine on the Formation of PEMs in M3 Medium

M3 regeneration medium (Côte et al. 1996) with various combinations of $AgNO_3$ (0, 1, 2, 3 ppm), sucrose (3.5 g L^{-1} and 4.5 g L^{-1}), and glutamine (100, 200, 300 and 400 ppm glutamine) were tested. A total of 33 treatments were generated, including a control without plant growth regulators. One mL of cell suspension from a 1-mo-old suspension culture with 0.1 mL SCV was plated onto a disposable Petri dish (90 x 150 mm) filled with 25 mL of semi-solid medium and sealed with parafilm. The cultures were kept in the dark for 3 mo at a room temperature of 25 ± 2 °C.

Formation of PEMs and the efficiency of somatic embryogenesis were measured in terms of the number of somatic embryos per mL of cell suspension. Each treatment was replicated three times in CRD.

IAA, Ascorbic Acid and $AgNO_3$ on the Formation of PEMs

One mL of cell suspension (0.1 mL SCV) from 1-mo-old cultures were plated onto semi-solid MS medium with IAA (0.5 to 2.5 ppm) and $AgNO_3$ (1,2,3 ppm), with or without 10 ppm ascorbic acid, yielding a total of 32 treatments, including a control without Plant Growth Regulators (PGRs) and additives. Twenty mL of each media combination was contained in a 90 x 150 mm disposable plastic Petri dish and then sealed with parafilm. The cultures were kept in the dark for 3 mo at a room temperature of 25 ± 2°C.

Formation of PEMs and the efficiency of somatic embryogenesis were recorded in terms of the number of somatic embryos per mL of cell suspension. Each treatment was replicated three times in CRD.

Proliferation of PEMs and Induction of Shoot Formation from PEMs

PEMs which developed from plated cell cultures from treatments with gradual reduction of 2,4-D were transferred onto M2 solid medium and incubated in the dark for 2 mo under 25 ± 2°C to allow for further proliferation. PEMs that showed intense yellow color, with firm, nodular structures after 2 mo were then transferred in P5 medium and allowed to continue development under standard culture conditions.

Shoots that developed from pro-embryo masses were transferred to abaca shoot multiplication medium then cultured under standard conditions for further shoot development.

RESULTS AND DISCUSSION

Production of White Meristematic Buds and Scalps from Multiplying Shoot Cultures and the Induction of Embryogenic Complex (EC) from Scalps

Multiplying shoot cultures of abaca 'Inosa' could be induced to produce numerous white meristematic buds by de-topping just above the growing point and leaving a small portion of the base and most of the corm (Bernardo et al. 2013). After several cycles on abaca multiplication medium, meristematic buds interspersed with few discrete shoot/leaves would form in the mass of tissue (Fig. 1A). This seems to be unique to 'Inosa' as other varieties do not develop small and numerous bud clumps, but instead form glossy and bulbous structures (Aspuria and Garcia 2018). On the other hand, the change in external morphology may have originated from the long history of culture cycles in multiplication medium. Meristematic bud sectors in P5 medium in the dark result in a "cauliflower-like" appearance (Fig. 1B) after 1 mo. Successive cycles in P5 medium would yield a compact structure, henceforth, "scalps" (Fig. 1C). These are suitable materials for embryogenic complex induction (Fig. 1D; Strosse et al. 2003) used in generating cell suspension cultures (Fig. 1E) and from these suspensions, pro-embryogenic masses (Fig. 1F).

Five types of embryogenic complex (or callus) may be induced from scalps depending on the pre-culture step in a specified medium and the kind of growth regulators used in the induction medium. Only type V calli induced with 2,4-D produce cells that are competent for somatic embryogenesis (Huang et al. 2000). Type V calli comprise numerous white and translucent pro-embryos on the surface (similarly described by Strosse et al. (2003) as the "ideal callus" for cell suspension). ZZss medium contains 1 mg L^{-1} 2,4-D to induce embryogenesis similar to most cell suspension protocols developed for banana. In abaca, 1 ppm 2,4-D induced somatic embryogenesis from scalp explants (Aspuria and Garcia 2018).

The ECs that developed from abaca scalps consisted of individual embryos similar to the pattern observed in immature male flowers of banana (Sutherland 2006; Youssef et al. 2010) rather than the "ideal callus" described by Strosse et al. (2003) as having many translucent pro-embryos and non-organized embryogenic cell clusters. Primary somatic embryos were observed 3–4 wk after the initial culture of the scalps and in the succeeding 6 mo (Fig. 2 and 3A–6D). Strosse et al. (2003) noted that "the appearance of individual embryos is a promising indication of the embryogenic capacity of the starting material." Of the 150 abaca scalps inoculated, 6% of the ECs had at least 10 recognizable SEs of various sizes and stages of development on the 6–7th month which is a modest number in comparison with other embryogenesis systems attempted (Strosse et al. 2004). The formation of somatic embryos also dismisses the seemingly recalcitrant nature of abaca, at least in 'Inosa'. Even the more widely used male flower (banana) technology had lower embryogenic response (Strosse et al. 2004).

The frequency of EC induction also varies depending on explant type, developmental stage, and genotype (Okole and Schulz 1996), among other factors. It is, therefore, not uncommon to get non-embryogenic callus (Strosse et al. 2003) in some cases, even when following the more successful procedures (Schoofs et al. 1998).

Establishment and Maintenance of Cell Suspensions

The influence of sucrose concentration and basal salts concentration (coded as GFM1 to GFM5) were tested in terms of total cell count and cell morphology. In addition, the effect of IAA + BA (coded as EAG1, EAG2, and EAG3) in cell proliferation was evaluated in comparison with

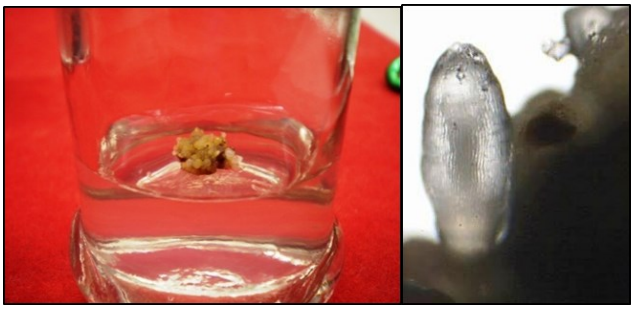

Fig. 2. Abaca 'Inosa' embryogenic complex in ZZss medium 1 mo after inoculation (left). Somatic embryoids form in discrete sectors of the embryogenic complex (right).

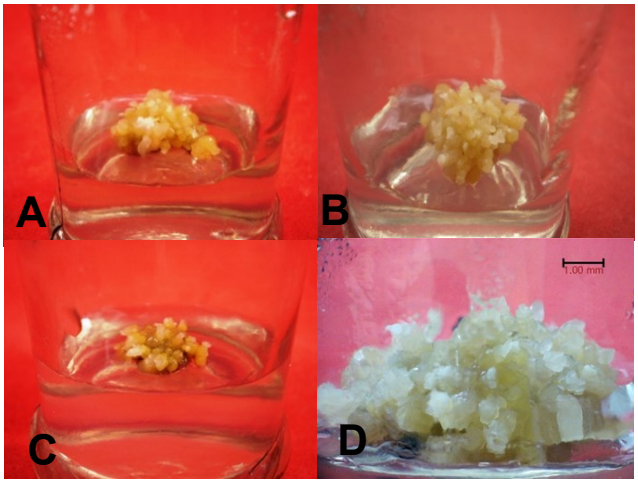

Fig. 3. Abaca 'Inosa' embryogenic complexes (ECs) in various stages of development. (A) 2 mo; (B) 3 mo (C) 4–5 mo and (D) 6–7 mo after inoculation onto INIBAP ZZss medium.

zeatin (Table 1; GFMs vs. EAGs). The cell suspensions were initiated using ECs with at least 10 somatic embryos. M2 (Côte et al. 1996) as a reference medium was also included.

After 1 wk, a heterogeneous suspension formed and comprised of individual cells or cell aggregates and portions of the dissociated callus. The color was pale yellow to bright yellow, indicating embryogenic potential (Strosse et al. 2003). Fine embryogenic suspensions were maintained by using a small pipette tip or by occasional sieving when cell aggregates have become larger than 2 mm^3. Doubling time was about 1 mo before another subculture was required. On average, GFMs 2–4 (Table 1) had higher total cell count over a 16-d period compared with GFM 1 (control; Table 1) and the rest of the treatments. GFM 2 recorded an increasing trend in total cell count and had the highest cell count, but not the other treatments (Fig. 4).

However, GFM2 cell suspensions had a pale grey color suggestive of poor embryogenic capacity. Under the microscope, GFM 2 cells were starch-filled and highly vacuolated, a likely indication of the non-embryogenic nature of these cells. Pale white suspensions are undesirable because they signify a high proportion of non -regenerable cells rich in starch (Strosse et al. 2003). When transferred to the regeneration medium, these cells would eventually turn black and undergo necrosis or would have a low regeneration rate (Sutherland 2006). GFM 3, GFM 4, and GFM 5 had almost the same response as GFM 2, although GFM 4 and GFM 5 had lower cell count on the 16th day. EAG 1-3 turned brown in less than 1 mo and were unfit for further evaluation. GFMs 1-5 were maintained for 3 mo and observed for changes in cell morphology, but the cells remained elongated, highly vacuolated, and starch-filled, which is typical of non-embryogenic cells. Hence, the experiment was terminated.

Cells in M2 medium (Côte et al. 1996) were generally unresponsive throughout the 3-mo observation period. Total cell count was lower than GFM1 (control) and most of the treatments (Fig 4). Nonetheless, we kept the cultures in M2 medium and chanced upon a cell line that was bright yellow and formed distinct cellular aggregates characteristic of highly proliferating suspension after 7 mo without subculture (Fig. 1A and 6). It was, therefore, a serendipitous occurrence to get a cell line with potentially high regeneration ability after a prolonged period. We have since used this as a source of daughter cell lines for our succeeding experiments.

Although cell suspensions tend to be stable for up to 4 yr (Strosse et al. 2003), old cell cultures can progressively show decreased regeneration ability. Banana *in vitro* cultures kept for too long in a liquid medium typically exhibit symptoms of morpho-physiological abnormalities and show increased somaclonal variation after repeated subcultures (Schoofs et al. 1998). However, given the time- and labor-consuming steps in establishing cell suspensions, the practice of keeping long-term cell cultures can be cost-effective. In other words, it is impractical to commence

Table 1. Media formulations used for the establishment of cell suspension cultures of abaca 'Inosa' from embryogenic complex. (All components are in mg L^{-1} except when otherwise stated.)

Culture Media	MS Basal	Sucrose (g L^{-1})	2,4-D	IAA	Zeatin	BAP	*myo*-inositol	Malt Extract	Biotin	Glutamine	Coconut Water (%)	Ascorbic Acid
GFM1	Half-strength	30	1	-	0.219	-	-	-	-	-	-	100
GFM2	Half-strength	45	1	-	0.219	-	-	-	-	100	-	100
GFM3	Half-strength	45	1	-	0.219	-	-	-	-	100	10	100
GFM4	Fullstrength	45	1	-	0.219	-	-	-	-	100	-	100
GFM5	Fullstrength	45	1	-	0.219	-	-	-	-	100	10	100
EAG1	Fullstrength	45	-	1	-	2.5	100	-	-	-	-	-
EAG2	Fullstrength	45	-	2	-	5.0	100	-	-	-	-	-
EAG3	Fullstrength	45	-	3	-	7.5	100	-	-	-	-	-
M2*	Fullstrength	45	1	-	-	-	-	100	100	100	-	-

*Côte et al. (1996)

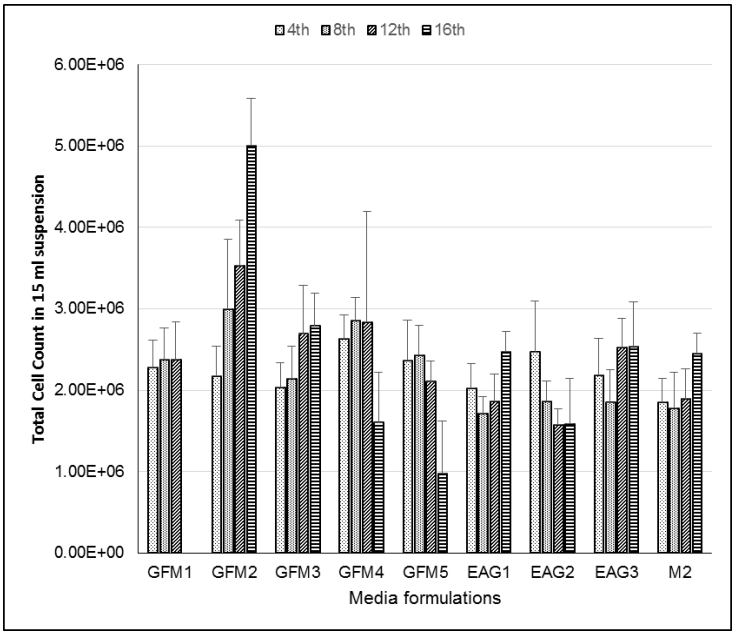

Fig. 4. Total cell count of abaca 'Inosa' cell suspension over a 16-d period, taken every 4 d using a hemocytometer. Error bars represent the s.e. of the mean (n=5).

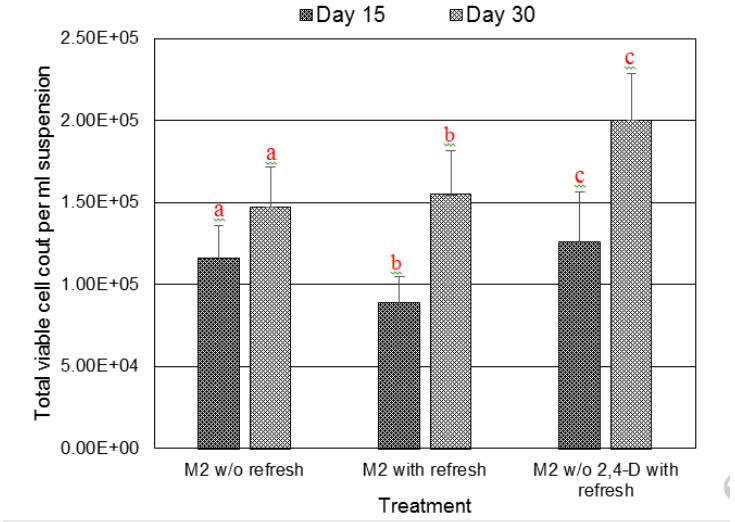

Fig. 5. Total viable cell count of abaca 'Inosa' cell line in response to a gradual reduction of 2,4-D in liquid M2 medium. Cell count data were taken on days 15 and 30. Repeated measures ANOVA detected differences due to natural cell proliferation (p=0.0265) rather than due to treatment (p=0.03128). Values represent the mean + s.e., n=9. Bars with the same letters are not statistically different using LSD at α=0.05.

new suspension cultures frequently, primarily if laboratory resources are constrained, as is the case in the majority of tissue culture laboratories throughout the country if our protocol were to be replicated. Therefore, decreased regeneration rates and possibly unwanted mutations are accepted risks. Moreover, since extended culture maintenance is unavoidable, regular quality testing is ideal, given that abaca cell suspensions are subcultured every 1–3 mo to maintain high proliferative capacity.

Furthermore, the chance of getting Type V cells, characterized by cells and cellular aggregates having large nuclei, which are often vacuolated and have visible, large amyloplasts (Georget et al. 2000), increases in aged cultures or extended contact with 2,4-D. These cell types usually go through necrosis and do poorly in the germination medium. Indeed, we have consistently observed these cell types in past experiments, which represent a setback for succeeding steps. The reduction or withdrawal of 2,4-D serves as a stimulus for the development of somatic embryos (Jimenez 2005). Thus, we designed an experiment to test the hypothesis that a gradual reduction of 2,4-D can yield cells and cell aggregates that are more primed to undergo somatic embryogenesis.

Preliminary data indicate that as 2,4-D levels decrease in the medium, an increase of 42% and 16% can be attained (on the 15th and 30th days, respectively) in total viable cell count compared to maintaining a constant level of 2,4-D over 1 mo (Fig. 5), although statistical analyses revealed that the increase is not due to the treatment (p=0.3128) but instead on the natural course of cell multiplication across time (p=0.0265).

Another method was tested using glutamine, an organic nitrogen source. The glutamine concentration in M2 liquid medium was increased to 400 ppm (from 100 ppm) to evaluate if it could aid in the easy transition of cells into somatic embryos once plated in a semi-solid germination medium. This has been shown to work in banana (Husin et al. 2014). A higher level of glutamine (400 ppm) in the M2 medium gave a 16% advantage in total viable cell count versus the standard 100 ppm on the 15th day. The total viable cell count in the higher glutamine concentration was significantly lower than the standard M2 medium (Fig. 6). However, the difference was due to cells multiplying over time (p=0.0002), rather than the glutamine treatment (p=0.5299). In date palm, the combination of 0.1 mg g L^{-1} 2,4-D and 6.7 x 10^{-4} glutamine appeared to have better embryo development (Zouine and El Hadrani 2007). Improved conversion of cellular aggregates into somatic embryo was also aided by higher glutamine supplementation in banana (Husin et al. 2014). However, this has yet to be proven in abaca.

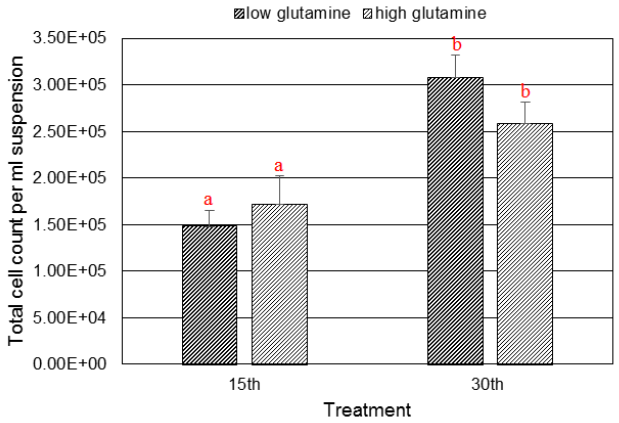

Fig. 6. Total cell count of abaca 'Inosa' cell lines in 100 ppm (low) and 400 ppm (high) levels of glutamine. Data were taken on days 15 and 30. Repeated measures ANOVA indicate increase in cell count (p=0.0002) that is due to natural cell multiplication but not because of glutamine (p=0.5299). Values represent the mean + s.e., n=9–10. Bars with the same letters are not statistically different using LSD at α=0.05.

Induction of Pro-embryogenic Masses (PEMs) and Somatic Embryogenesis from Cell Suspension Cultures

Effect of the gradual withdrawal of 2,4-D and glutamine on viable cell count and PEM formation

For good quality cell suspensions, a time gap of 15–45 d is enough to obtain numerous translucent somatic embryos in hormone-less semi-solid medium (Strosse et al. 2004; Georget et al. 2000). In this study, cells and cellular aggregates in semi-solid MS basal medium formed discrete, yellow nodular structures, or pro-embryogenic masses (PEMs; Fig. 7) instead of discrete, individual somatic embryos. However, the frequency of PEMs was low and non-regenerable. So we explored how cells would respond to a progressive decrease in 2,4-D levels (Jimenez 2005) as well as increased glutamine while in suspension and by plating them on hormone-less regeneration media (MS, ½ MS, and M2). We were able to assess PEM formation, albeit only qualitatively since embryos did not develop within 3 mo.

PEMs in T2 (refreshment with M2 every 4 d) and T3 (refreshment with M2 without 2,4-D every 4 d) had a firmer appearance and intense yellow color, especially those in either MS or M2 (Fig. 8). Cells subjected to 400 ppm glutamine and plated on MS or M2 medium also formed bright yellow and compact PEMs (Fig. 9). Embryos were not obtained from any of these treatments, however. Further, we tested manipulating the sucrose level, with increased glutamine concentration, and adding $AgNO_3$ in M3 regeneration medium (Côte et al. 1996) could stimulate somatic embryo formation.

Effect of sucrose, $AgNO_3$, and glutamine on the formation of PEMs and development of somatic embryos in M3 regeneration medium

M3 regeneration medium was developed for embryo development in 'Grand Nain.' It has high nitrogen concentration from the basic Schenk and Hildebrand media and amino acids, a complex proportion of cytokinin, and two carbohydrate sources (Côte et al. 1996). Unfortunately, embryo development was not visually evident in any of the 33 treatments even after 3 mo of continued incubation. In 'Grand Nain,' embryos that formed were kept on M3 medium for 80 d (Côte et al. 1996). Instead, two in vitro responses were observed in the first 1–1.5 mo after plating. It includes intense blackening/necrosis and the formation of pro-embryogenic masses (PEMs). The same responses were observed when combinations of IAA, ascorbic acid, and $AgNO_3$ in MS basal media were evaluated (data not shown), which suggests that (high) nitrogen or cytokinin are not promotive or unnecessary anyway.

Sucrose also did not favor somatic embryo formation in abaca, although many sources point to the role of sucrose in enhancing somatic embryogenesis in black spruce (Tremblay and Tremblay 1995), orchids (Tokuhara and Mii 2003), strawberry, and carnation (Karami et al. 2008).

Results from these experiments concur with the initial findings from the gradual reduction of 2,4-D from M2

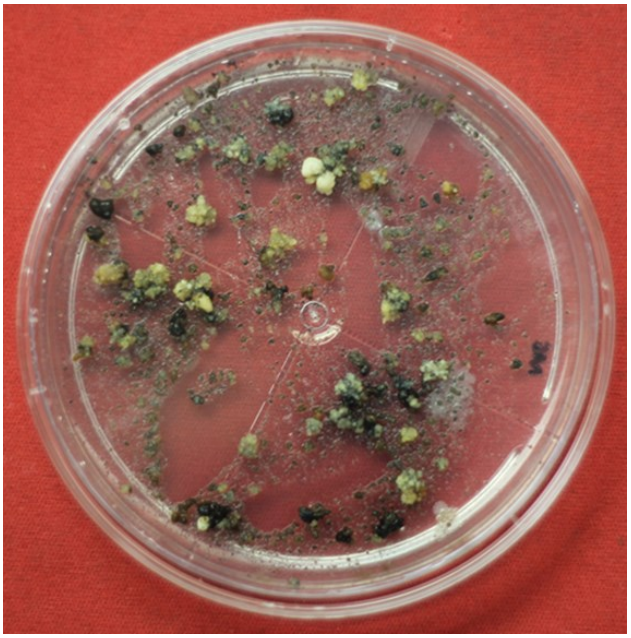

Fig. 7. Induction of pro-embryogenic masses (PEMs) can be achieved in a hormone-free MS basal medium. However, M2 cell lines do not transition into embryos quite easily based on our earlier trial, likely because of the extended exposure to 2,4-D.

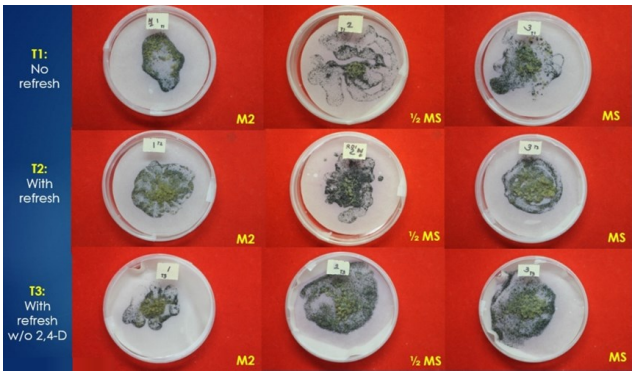

Fig. 8. Effect of gradual reduction of 2,4-D on the formation of pro-embryogenic masses (PEMs) in abaca 'Inosa' in different basal media.

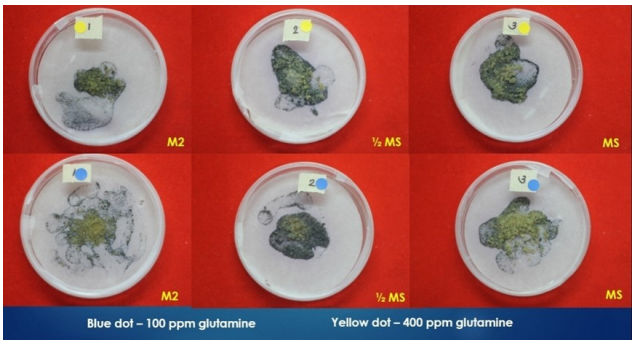

Fig. 9. Effect of increased glutamine levels on the formation of pro-embryogenic masses (PEMs) in abaca 'Inosa' in different basal media.

liquid medium (Fig. 5) and increased glutamine levels (Fig. 6) that somatic embryogenesis can be challenging to achieve given the many influencing factors at play, including the possibility of an over-aged cell line.

Overall, the results from the previous experiments were unsatisfactory in inducing somatic embryo development. A hypothesis is that these cells/PEMs acquire embryogenic competence but lose it if kept in the same medium composition without transfer to hormone-free basal medium. However, the duration of induction, whether a few days or weeks, is not ascertained.

Proliferation of Pro-embryogenic Masses (PEMs) and Induction of Shoot Formation

PEMs obtained from the experiment on the gradual reduction of 2,4-D from M2 liquid medium plated onto solid M2 medium were kept for 3 more months (6 mo in total) and were transferred to fresh medium of the same composition on its sixth month and incubated in the dark. Two months later, PEMs were transferred to P5 medium (Strosse et al. 2006) under standard conditions. White, opaque structures formed within a few weeks (Fig. 10A). The formation of opaque white structures from PEMs (nodular calli, Fig. 10B and 10C) parallels the development of plantlet development in plantain cv. Spambia (Sholi et al. 2009), in *Musa acuminata* Colla (Youssef et al. 2010), and in *M. acuminata* cv. Grand Naine (Remakanthan et al. 2014). The conversion efficiency from putative abaca somatic embryos to regenerated plantlets, however, remains low (ca. <1% per mL cell suspension) compared to reports by Jalil et al. (2003) and Strosse et al. (2006) who also used scalp-derived calli as starting materials. The growth of shoot-like structures (Fig. 10D–10F) followed 2–3 more weeks of incubation in P5 medium under standard conditions. Complete abaca plantlets regenerated after transfer to abaca shoot multiplication medium (Fig. 10G).

SUMMARY AND CONCLUSION

A procedure based on the established INIBAP protocol (Strosse et al. 2003) for banana using embryogenic complexes (ECs) was adapted for abaca. The ECs were induced in INIBAP ZZss using scalps generated from 3–5 mm^3 meristematic buds successively cultured in P5 medium in the dark. Individual somatic embryos were observed in discrete regions of the EC, starting 1–8 mo after inoculation. However, "ideal callus" formation described in the literature as friable embryogenic callus bearing translucent proembryos (Strosse et al. 2003; Sutherland 2006) was not observed. ECs with at least 10 individual somatic embryos were then used to commence cell suspension cultures exploring nine different media formulations. The most promising was the M2 medium (Côte et al. 1996). Out of nine flasks in M2, one developed into the characteristic bright yellow of potentially embryogenic cultures. Although they showed an increasing trend in total cell count, the other media formulations only yielded cells that were starchy, elongated, and highly vacuolated and may be regarded as non-embryogenic. Experiments to induce somatic embryogenesis from established cell suspensions by gradual reduction of 2,4-D in the liquid medium before plating onto a semi-solid medium, as well as the inclusion of higher levels of glutamine to the liquid medium, were tested. The withdrawal of 2,4-D can enhance the frequency of PEM formation on M2 and MS-based solid medium. $AgNO_3$ also had a beneficial effect. PEMS developing on $AgNO_3$-containing medium, with or without ascorbic acid, had a promotive effect on PEM formation. This, in combination with the withdrawal of 2,4-D before plating, had significantly improved the recovery of potentially regenerable PEMs. PEMs transferred onto P5 medium eventually developed mature somatic embryos, appearing as opaque white lobes on the surface of PEMs. These then regenerated into whole plants after successive passages in abaca multiplication medium.

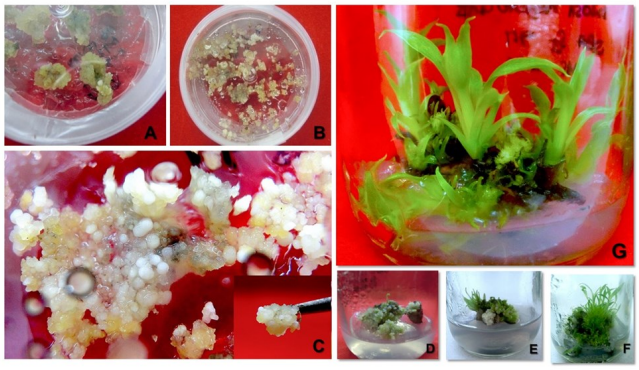

Fig. 10. Stages of conversion from pro-embryogenic masses (PEMs) to mature somatic embryos to plantlets. PEMs in M2 media in P5 medium for induction of somatic embryogenesis (A). Mature somatic embryos (opaque white structures) (B, C). PEMs bearing mature somatic embryos (C, inset) in abaca shoot proliferation medium (D), which later developed into shoots (E, F and G). The development from PEMs to fully-grown shoots took at least 6 mo.

Finally, protocols for establishment of cell suspensions and plantlet regeneration in banana abound in literature. Meanwhile, procedures on abaca cell suspension are very limited (e.g., Aspuria et al. 1997; Aspuria 2003; Aspuria and Garcia 2018), and they remain incomplete and largely unproductive because of the issues of browning, recalcitrance, and the tedious work involved in generating and maintaining cell suspensions. Here, we showed that the embryogenic complex method that proved to be successful in *Musa* (Strosse et al. 2003) can be adapted for abaca. More importantly, our results demonstrate that *in vitro* SE occurs in abaca and presents the opportunity to introduce biotechnological approaches that may help enhance our abaca genetic resources. It is a goal which has been precluded by the absence of a suitable transformation and plant regeneration protocol.

Still, significant questions remain: "When are abaca cells competent enough to undergo somatic embryogenesis?", "What cues do abaca somatic cells need to transition to embryogenesis smoothly?", and "Why does abaca seem to be recalcitrant to somatic embryogenesis?". Basic studies at the physiological, morphogenetic, and molecular levels may be required to answer these questions and lead to advances in the methodology.

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