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Callus Induction and Somatic Embryogenesis in *Nypa fruticans* Wurmb Zygotic Embryo

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***Nypa fruticans* has been reported to be a potential source of ethanol. To date, there is no available protocol on tissue culture suitable for *N. fruticans* to produce superior and uniform planting materials for future wide-scale production. This paper investigated the influence on callus induction of various concentrations of 2,4-D and activated charcoal as well as the effects of three orientations of the explant on the media. Sixty-four percent of the explants cultured in full-strength MS media, supplemented with various combinations of different concentrations of 2,4-D and activated charcoal, produced protocorm-like structures in cultures with lower concentrations of 2,4-D combined with high concentrations of activated charcoal. Fifty-two percent of protocorm-like structures in T18 cultures significantly ($p<0.05$) developed white, compact, nodular calli and soft, white, friable calli eight weeks after inoculation. Twenty percent of the explants in the T18 cultures developed somatic embryos sixteen weeks after inoculation. Explants in cultures with higher 2, 4-D concentrations combined with activated charcoal significantly ($p<0.05$) formed brown, hard, compact calli. Treatments devoid of activated charcoal did not induce callus and did not produce somatic embryos. The absence of activated charcoal in the MS media also led to browning of explants within the eighth week of incubation. The orientation of explants on the culture media resulted in callus initiation only in the T18 cultures, although protocorm-like structures were observed in all cultures regardless of the explant orientation.**

Key Words: activated charcoal, callus induction, somatic embryogenesis, 2, 4-D

INTRODUCTION

Nypa fruticans Wurmb, the nipa palm, remains uncultivated and grows mainly as natural stands near estuaries and rivers (Rasco 2010; Tsuji et al. 2011) throughout the Philippines. Its large pinnate leaves are traditionally harvested for use as roof thatches and stabilizers of coastal areas, while its sap is regularly harvested for small-scale vinegar and vodka production (Rasco 2010; Rasco et al. 2012). *N. fruticans* sap contains as much as 60% sugar and its annual yield was reported to be much more than those from sweet potatoes, coconut, tapioca, and sugarcane (Hamilton and Murphy 1988). The depleting petroleum resources and increasing demand for fuel brought on great interest to look into the potential of *N. fruticans* as a source of bio-ethanol, a renewable resource now widely tapped as a fuel blend. The advantage of *N. fruticans* lies in that it is not being widely utilized as a food source, unlike its other popular counterparts. One main constraint however is that *N. fruticans* palms display slow growth in natural populations. The traditional method of nipa production is by harvesting wild seedlings and transplanting them to any available land, which produces non-uniform plants with varied quality of

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sap (Low et al. 2008; Sghaier et al. 2008; Rasco 2010; Rasco et al. 2012). Vegetative propagation through tissue culture has been proven to be useful in cloning uniform (Low et al. 2008; Sghaier et al. 2008) planting materials within a short time (Karunaratne and Periyapperuma 1989; Sghaier et al. 2008). However, to date, there is no published tissue culture protocol for *N. fruticans*.

For *N. fruticans* palms found within Davao City, an approach to produce high quality, uniform planting materials was explored. The study looked into developing a protocol for callus induction using zygotic embryos. The results herein are useful for coming up with a protocol on *N. fruticans* somatic embryogenesis and plant regeneration of these high-yielding and high-quality palms.

MATERIALS AND METHODS

Plant materials

Nypa fruticans fruits were collected from semi-wild stands at Shimric Beach, Talomo and Davao City. These were transported to the laboratory at the Department of Biological Sciences and Environmental Studies, College of Science and Mathematics, University of the Philippines Mindanao. In the laboratory, the fruits were washed with a detergent solution, thoroughly rinsed with tap water, transferred into a pail (24 cm x 28 cm x 28 cm), and soaked in 1% sodium hypochlorite solution for 15 min. Fruits were de-husked in the laminar flow chamber. Using a sterile hacksaw, each de-husked fruit was cut transversely to break open the solid endocarp. Those bearing semi-translucent peripheral endosperms were chosen to obtain the zygotic embryo. Zygotic embryos were immersed in 1% sodium hypochlorite for 10 min followed by three washes with sterile distilled water. These were transferred onto several layers of sterile filter paper to remove excess water. Each was longitudinally halved using a sterile scalpel, ensuring that each half possessed the region of radicle emergence.

Culture medium and conditions

The halved sterile zygotic embryos were cultured on modified Murashige and Skoog (MS) media (Murashige and Skoog 1962) supplemented with 30 $g \cdot L^{-1}$ sucrose and 0.01$g \cdot L^{-1}$myo-inositol, with 6$g \cdot L^{-1}$ pharmaceutical agar as a gelling agent. The pH was adjusted to 5.7-5.8 using a calibrated pH pen and subsequently autoclaved at 121ºC, 105 kPa for 15 min. Thirty mL of the media was dispensed into each petri dish (100mm x 20mm).

Sterile halved zygotic embryos were inoculated onto each dish after media had cooled and solidified. Each dish was sealed with parafilm. The cultures under different treatments were incubated in the dark at 25 ± 2ºC.

Effects of different 2,-D concentrations in combination with various activated charcoal concentrations

A 7 x 3 factorial experiment arranged in a completely randomized design was carried out. The different treatments consisted of five replicates. Each replicate contained five longitudinally halved sterile zygotic embryo explants. A total of 525 explants were used. The effects of various concentrations of 2,4-Dichlorophenoxyacetic acid (2,4-D), i.e., 0 µM, 20 µM, 40 µM, 60 µM, 80 µM, 100 µM, 300 µM, and 500 µM, each in combination with two concentrations of activated charcoal (AC), i.e., 0 $g \cdot L^{-1}$, 0.75 $g \cdot L^{-1}$, and 1.50 $g \cdot L^{-1}$, were evaluated. All longitudinally halved zygotic embryo explants were placed onto the media with incision facing upward. Weekly observations for callus initiation and somatic embryogenesis were conducted for 3 and 4 months, respectively.

Effects of explant orientation and 2,4-D concentrations

A 3 x 5 factorial experiment arranged in a completely randomized design was carried out. Each treatment consisted of triplicates, containing five halved sterile zygotic embryo explants per replicate. A total of 225 explants were used. Three different explant orientations were adopted, viz.: A - longitudinal incisions oriented up, away from the media surface; B - longitudinal incisions oriented face down on media surface; and, C - cross-sectional incisions face down on media surface. The five halved explants in a specific position were separately inoculated onto different modified media, each of which contained one of the five concentrations of 2,4-D, i.e., 0 µM, 40 µM, 60 µM, 80 µM, 100 µM, and 300 µM. The media for all combinations of explant positions and 2,4-D concentrations were supplemented with 1.50 $g \cdot L^{-1}$AC. The effects of explant position and the five 2,4-D concentrations were determined. Weekly observations on callus initiation were carried out for 2 months.

Statistical Analyses

The square-root transformed data were subjected to ANOVA (P= 0.05) using SPSS v14. The means were separated using Fisher's least significant difference (LSD) (P= 0.05).

RESULTS AND DISCUSSION

Effects of different 2,4-D concentrations in combination with various activated charcoal concentrations

Different concentrations of 2,4-D in combination with various concentrations of AC were used to supplement modified MS media to determine which combination was suitable for callus induction and somatic embryogenesis

of longitudinally halved zygotic embryo explants. The results (Table 1) revealed that protocorm-like structures (PLS) developed in nine out of twenty-one treatment combinations. Of these nine treatments, two, viz., T18 and T20, produced calli but only T18 successfully produced somatic embryos. The T18 cultures which were in the MS medium containing 300 µM 2,4-D in combination with 1.50 $g \cdot L^{-1}$ AC produced calli in as much as 52% of the explants.

The formation of PLS was initiated at the base of the radicle and was generally observed as early as 2 wk after inoculation in the following treatments, viz.: T9; T10; T11; T13; T14; T15; T16; T17; T18; T20; and, T21 (Table 1). The production of the PLS displayed by the explants is the first stage in the morphological changes leading to callus induction. Some explants did not exhibit any morphological changes (Fig. 1a). Most of the PLS produced in the other treatments exhibited signs of necrosis 4 wk after inoculation (Fig. 1b). Each PLS initially appeared as a smooth, round, white, ball-like structure, which eventually elongated (Fig. 1c).

Callus was initiated 8 wk after inoculation only in cultures within treatments T18 and T20, which contained supplements of 300 µM 2,4-D with 1.50 $g \cdot L^{-1}$ AC and 500 µM 2,4-D with 0.75$g \cdot L^{-1}$ AC, respectively. The calli initiation occurred at the periphery of the PLS (Fig. 1d). Some of the calli formed in the treatment T18 cultures were soft, friable, white, and non-embryogenic, while others were white, nodular, compact, and embryogenic. These white, nodular, compact, embryogenic calli observed in treatment T18 cultures proliferated 12 wk after inoculation and developed into somatic embryos after 16

Table 1. Effects of different 2,4-D concentrations in combination with various activated charcoal (AC) concentrations on callus induction and somatic embryogenesis in halved *Nypa fruticans* zygotic embryo explants.

Treatments (T)	**2,4-D** (µM)	**AC** ($g \cdot L^{-1}$)	**Protocorm-like structures** ± s.e. frequency (%)	**Callus induction** ± s.e.frequency (%)	**Somatic embryo-genesis** ± s.e. frequency (%)
1	20	0.00	0 a	0 a	0 a
2	40	0.00	0 a	0 a	0 a
3	60	0.00	0 a	0 a	0 a
4	80	0.00	0 a	0 a	0 a
5	100	0.00	0 a	0 a	0 a
6	300	0.00	0 a	0 a	0 a
7	20	0.75	0 a	0 a	0 a
8	40	0.75	0 a	0 a	0 a
9	60	0.75	4.00 ± 4.00 ab	0 a	0 a
10	80	0.75	16.00±7.50 abc	0 a	0 a
11	100	0.75	24.00±19.4 abcd	0 a	0 a
12	300	0.75	0 a	0 a	0 a
13	20	1.50	32.00±13.60 bcd	0 a	0 a
14	40	1.50	32.00±16.20 bcd	0 a	0 a
15	60	1.50	44.00±23.20 cde	0 a	0 a
16	80	1.50	20.00±15.50 abcd	0 a	0 a
17	100	1.50	32.00±20.60 bcd	0 a	0 a
18	300	1.50	64.00±16.00 e	52.00±20.6b	20.00±6.00 b
19	500	0.00	0 a	0 a	0 a
20	500	0.75	40.00±12.60 cde	40.00±12.6b	0 a
21	500	1.50	8.00 ± 4.90 ab	0 a	0 a
P-values		2,4-D	0.882	0.000	0.000
		AC	0.000	0.017	0.000
		2,4-D x AC	0.017	0.000	0.000

Values are means of 5 replicates, consisting of 5 halved zygotic embryo explants per replicate. Data on callus initiation were recorded after 3 months of culture; data on somatic embryogenesis were recorded after 4 months. *Different letters within columns showed a significant difference at P=0.05, as determined by Fisher's LSD.

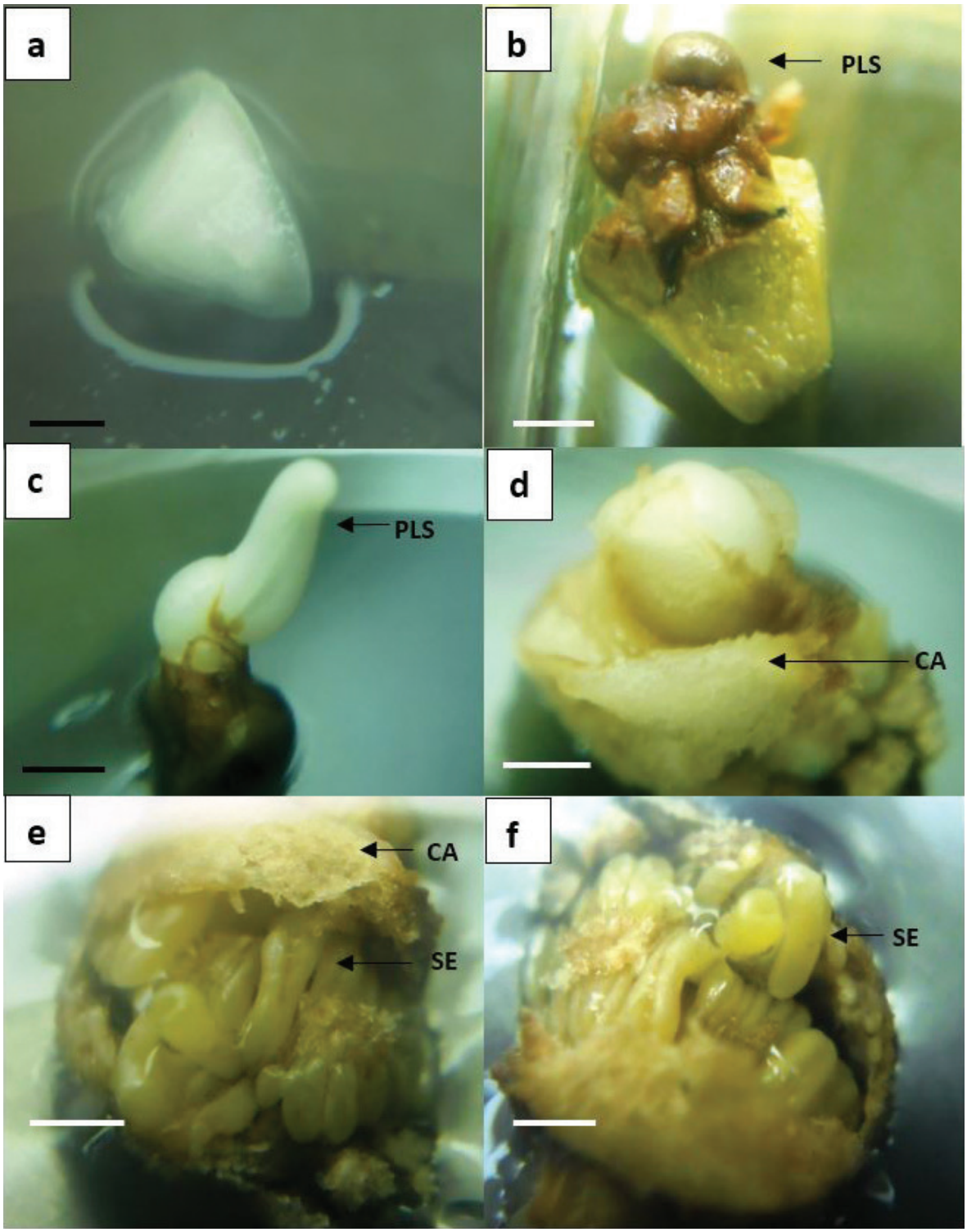

Figure 1. Generally exhibited responses in longitudinally halved zygotic embryo explants inoculated in treatments with different concentrations of 2,4-D in combination with various concentrations of activated charcoal: (a) no discoloration and morphological changes observed; (b) protocorm-like structures (PLS) protruded but became necrotic; (c) PLS elongated; (d) development of white and compact calli (CA); and, (e and f) development of somatic embryos (SE) surrounded by white and compact calli. Scale bar = 5mm.

wk (Figs.1e and 1f). The older cultures of zygotic embryo explants produced more calli as was the case in *Cuminum cyminum* (L.) embryo explants which were reported to have increased occurrences of calli production when the cultures were older (Ebrahimie et al. 2007). It was observed, however, that production of somatic embryos from *N. fruticans* zygotic embryo explants successfully occurred only when the PLS were not elongated.

The calli produced in treatment T20 cultures, on the other hand, were brown, hard, compact, and non-embryogenic. These calli induced in treatment T20 did not develop into somatic embryos, because of the high 2,4-D concentration at 500 µM. High concentrations of 2,4-D were reported to be toxic and lead to cell degeneration during somatic embryogenesis induction in *Cocos nucifera* (L.) cultures (Magnaval et al. 1997). This is likely the case with *N. fruticans* where necrosis of explants and/or browning of calli were observed.

The media in treatments without any AC supplement all turned brown. Phenolic compounds were released and oxidized to form phytotoxic products when the halved explants were placed onto the culture media, causing the media as well as the explants to turn brown (Poudyal et al. 2008). Furthermore, no PLS were observed to develop from the halved zygotic embryo explants inoculated in treatments where the media were devoid of AC. Neither calli nor somatic embryos were induced in the absence of PLS. The cultures supplemented with AC retarded the high incidence of phenolic oxidation and prevented the media from browning as was reported in the study on somatic embryogenesis induced from oil palm immature inflorescence (Teixera et al. 1994).

Some zygotic embryo explants that did not produce calli or somatic embryos but these retained their color as when they were freshly collected (Fig. 1a). In contrast, the other zygotic embryo explants which produced calli and somatic embryos displayed browning. The presence however of AC in these treatments prevented the media from turning brown. Thus, these AC-modified MS media continued to support the induced calli and somatic embryos. A study on somatic embryogenesis in *Acrocomia aculeata* palm using zygotic embryo tissue explants reported that the tissues of the explant in contact with the media turned brown, yet successfully proceeded with the production of somatic embryos (Moura 2009).

The most effective concentration of 2,4-D for inducing callus in *Nypa fruticans* zygotic embryos explants in this study was higher than the concentration needed to produce calli and somatic embryos from coconut zygotic embryo explants. Samosir et al. (1998) reported that in coconut explants, 60 µM to 80 µM 2,4-D were usually required for callus production. Fifty percent of the immature embryos produced calli when they used a combination of 60 µM 2,4-D and 1.25g·L^{-1} AC supplements in their media. In this study, the treatments with 300 µM 2,4-D with 1.50 g·L^{-1} AC and 500 µM 2,4-D with 0.75g·L^{-1} AC successfully induced calli from *Nypa fruticans* zygotic embryo explants. Further, the 300 µM 2,4-D with 1.50 g·L^{-1} AC treatment, i.e., T18, produced 52% calli.

In a study conducted on date palm, another recalcitrant palm species, it was shown that application of low concentration of cytokinin, i.e., BAP, and frequent sub-culturing can increase the rate of embryogenesis as these measures prevented oxidative browning brought about by the presence of high phenols and peroxidase activity (Abohatem et al. 2011). The increase in lipid peroxidase products which concomitantly occurred with the decrease in the antioxidants in the media was indeed associated with plant recalcitrance to tissue culture (Benson and Roubelakis-Angelakis 1993). The future application of the protocol developed in this study may effectively enhance callus induction and somatic embryogenesis by increasing the frequency of sub-culturing. Recalcitrance may be alleviated with the supplementation of appropriate antioxidants in the culture media developed in this study.

Effects of explant orientation and various 2,4-D concentrations

Figures 2 and 3 show the PLS produced and the calli induced in the cultures containing media supplemented with 1.50 g·L^{-1} AC in combination with 2,4-D concentrations ranging from 40 µM to 300 µM. The values, however, did not significantly affect formation of PLS with P values for explants position (EP) (0.602), 2,4-D (0.887), and the interaction between EP x 2,4-D (0.524). Only the trends are represented graphically to demonstrate the degree of PLS production per treatment (Figure 2). As similarly observed in the preceding experiment, only cultures within the T5 and T15 treatments which contained 300 µM 2-4-D produced calli two months after inoculation (Figure 3). However, all values showed no significant differences in terms of P values for EP (0.564), 2,4-D (0.149), and the interaction between EP x 2,4-D (0.783). Additionally, the preliminary experiments which we conducted revealed that the central section of the zygotic embryo wherefrom the radicle emerges was essential for PLS formation and callus initiation. Otherwise, no significant morphological changes in the zygotic embryo explants were observed. A study involving *Cocos nucifera* zygotic embryo similarly revealed that the explant section which included the point of radicle emergence presented the best embryogenic response (Adkins et al. 2005).

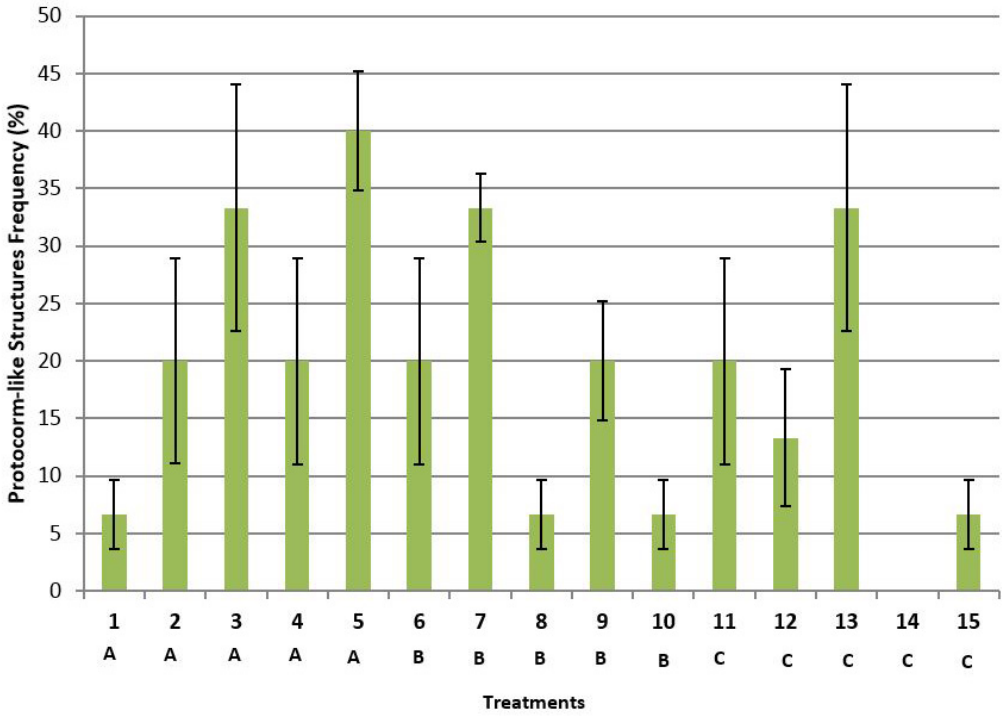

Figure 2. Frequency of protocorm-like structures in *Nypa fruticans* 15 treatments with varying 2,4-D concentrations and explants positions. Treatments (in bold) supplemented with the corresponding 2,4-D concentrations: 40 µM - **1, 6, 11**; 60 µM - **2, 7, 12**; 80 µM - **3, 8, 13**; 100 µM - **4, 9, 14**; 300 µM - **5, 10, 15**; Explant positions: **A**- longitudinal incision oriented up, away from media surface; **B** - longitudinal incisions oriented down on media surface; **C** - cross-sectional incisions face down.

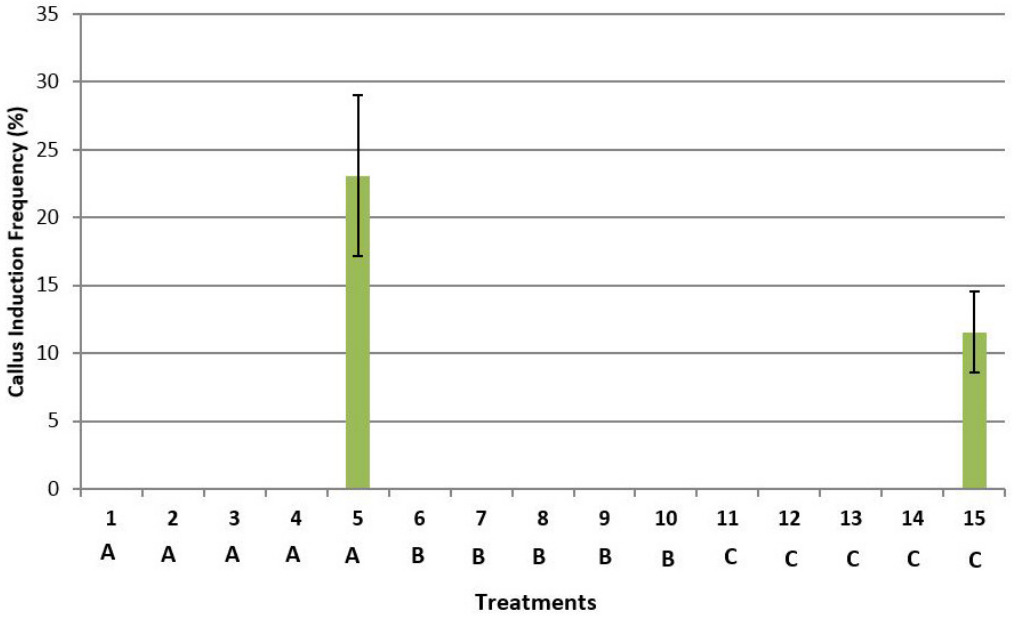

Figure 3. Frequency of callus induction in *Nypa fruticans* in 15 treatments with varying 2,4-D concentrations and explants positions. Treatments (in bold) supplemented with the corresponding 2,4-D concentrations: 40 µM - **1, 6, 11**; 60 µM - **2, 7, 12**; 80 µM - **3, 8, 13**; 100 µM - **4, 9, 14**; 300 µM - **5, 10, 15**; Explant positions: **A**- longitudinal incision oriented up, away from media surface; **B** - longitudinal incisions oriented down on media surface; **C** - cross-sectional incisions face down.

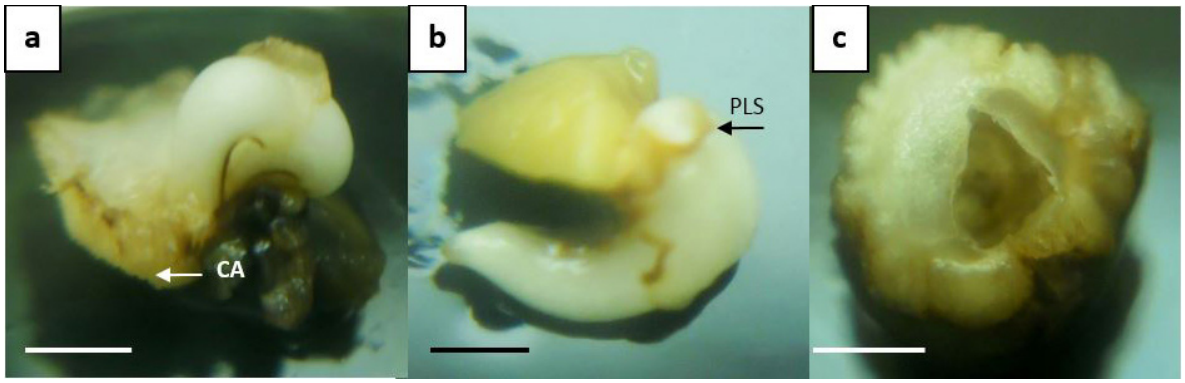

Figure 4. Production of protocorm-like structures and calli in halved zygotic embryo explants in different orientations: (a) longitudinal incision oriented up, away from media surface; (b) longitudinal incision oriented facedown onto media surface; and, (c) cross-sectional incision facedown. Legend: CA – callus; PLS – protocorm-like structures. Scale bar = 5 mm.

CONCLUSIONS

For the formation of zygotic embryos of *Nypa fruticans* important parameters were the following: explants were obtained from fruits which contained characteristically translucent, semi-solid peripheral endosperms and fruit sterilization was best achieved when these were soaked in 1% sodium hypochlorite for 10 min and thoroughly rinsed thrice with sterile distilled water. Explant position did not significantly affect callus initiation. However, the central section of the zygotic embryo wherefrom the radicle emerges was essential to produce protocorm-like structures and to initiate callus formation. The MS media supplemented with 300 µM 2,4-D combined with 1.50 $g \cdot L^{-1}$ AC was the most productive treatment. This treatment resulted in 64% of the halved zygotic embryo explants successfully developing protocorm-like structures, of which, 52% progressed into calli formation while 20% produced somatic embryos.

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