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Regular Article

A comparative study of three different methods of shoot meristem excision for induction of embryogenic calli in coconut

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A protocol was standardized to maximize yields of embryogenic calli from shoot meristem culture of coconut. Three different shoot meristem excision methods were tested *viz*., excision of shoot meristem aseptically from *in vitro* germinated embryo after 10-12 days, excision of shoot meristem from *in vitro* germinated embryo subjected to GA3 treatment for five days and excision of shoot meristem from fresh embryo. The primary calli induction after 30 days of culture incubation for the three treatments was 21%, 27% and 79% respectively. Further, the primary calli formed from the shoot meristem excised from fresh embryo gave rise to 56% of embryogenic calli. The calli obtained from the shoot meristem, which were excised from *in vitro* germinated embryo, formed less percentage of embryogenic calli because of the presence of cotyledonary tissues which inhibited the multiplication of meristematic tissues. In the case of shoot meristem extracted from GA3-treated embryos, the percentage of non-embryogenic calli was more compared to the shoot meristem excised from fresh embryo. It was observed that the addition of GA3 in the initial stages of culture inhibited the formation of embryogenic calli and favored direct shoot development. Currently, the shoot meristem excised from fresh embryo is being employed for scaling up the planting material production from released varieties of coconut.

Keywords: Coconut, embryogenic calli, shoot meristem, somatic embryo

Coconut (*Cocos nucifera* L., 2n=32) is cash and subsistence to small holders. As every part of the tree can be made into universally used products (Arunachalalm and Rajesh, 2008), it is popularly known as the "tree of life". The value of coconut palm cannot be assessed only in terms of its potential to produce oil, but as a versatile tree, which play a major role in the wealth, health and welfare of coconut farming community. Most coconut groves worldwide [illegible] because of senescence or because of loss due to diseases. Considering the wide variation observed in coconut populations for productivity, propagation of elite mother palms through somatic tissue culture will go a long way in solving the problem of production of adequate planting materials. Besides, this technique would also help in the rapid propagation of elite hybrids. Tissue culture

of coconut has been carried out in several countries including UK (Wye College), France (IRHO/CIRAD), Philippines (UPLB and PCA), Srilanka (CRI) and Mexico (CICY). A commercially viable regeneration protocol for coconut tissue culture is yet to be perfected.

Studies on plantlet regeneration from immature embryos (Gupta *et al*., 1984), immature leaves (Raju *et al*., 1984; Buffard–Morel *et al*., 1992) and inflorescences (Branton and Blake, 1984) indicate that regeneration is possible, but difficult. Only limited success has been achieved using these explants. In recent years, plumular tissues (shoot meristem) have responded well to *in vitro* interventions and give rise to higher percentage of embryogenic calli and somatic embryos compared to other explants tested. The somatic embryos developed from plumule explants were capable of germination, subsequent development into plantlets and successful transfer to nursery (Chan *et al*., 1998; Rajesh *et al*., 2005, 2014).

Since the high frequency embryogenic calli induction is a crucial step to achieve somatic embryogenesis, an attempt was made in the present research to test the different methods of pre-culture of the embryo for the excision of shoot meristem to maximize the yield of embryogenic calli.

Materials and methods

Plant materials

Mature nuts (11-12 months) were harvested from WCT (West Coast Tall) palms from the farm at ICAR-CPCRI Kasaragod, Kerala India. Embryos with endosperm were excised from the cut-opened nuts using a cork borer and placed in distilled water. The endosperms enclosing the embryos were sterilized with 0.01% $HgCl_2$ for 5 minutes and then rinsed thrice with sterile distilled water to remove the traces of $HgCl_2$. Embryos were extracted from endosperm plugs, surface sterilized with 20% NaClO solution for 20 minutes and subsequently rinsed with sterile distilled water for 5-6 times.

Excision of shoot meristem from mature zygotic embryos

T1 method: Surface sterilized embryos were aseptically inoculated into Eeuwen's Y3 media supplemented with 60 g/l of sucrose. After 10-12 days of incubation in dark condition, shoot meristem were scooped and inoculated into callus induction media (T1) (Fig. 1a).

T2 method: Surface sterilized embryos were aseptically inoculated into germination media (Y3 with 2.8 µM of GA3). After five days of germination, shoot meristems were scooped from the embryos and inoculated into callus induction media (T2) (Fig. 1b).

T3 method: Shoot meristems were directly scooped (Fig. 1c, 1d, 1e, 1f) from the surface sterilized matured embryos in aseptic condition and inoculated into initial calli induction media (T3) (Fig. 1g).

Culture media and condition

Eeuwens (1976) Y_3 was used as the basal medium supplemented with 30 g/l sucrose, 1 g/l activated charcoal with an auxin (2,4-D- 74.6 µM) and a cytokinin (TDZ- 4.54 µM). This media was used as an initial calli induction media (ICIM). After adjusting the pH to 5.8, the media was autoclaved at 121°C for 20 min. The explants were inoculated into ICIM and kept for incubation in dark condition. After one month of culture incubation, cultures were transferred to a same media with gradual reduction of 2,4-D (74.6 µM → 45.2 µM → 22.6 µM). A polyamine (spermine- 50 µM) was also incorporated in these media after first subculture. Subculturing was done once in 30 days. Observations were taken for callus initiation, growth and texture of the cultures.

After 90 days of incubation, friable calli obtained from these cultures were transferred to hormone-free media supplemented with 2.5 g/l of activated charcoal. After 30 days of incubation in hormone-free media, the cultures were transferred to initial regeneration media supplemented with 2,4-D (0.045 µM), BAP (22.2 µM), GA3 (2.8 µM) and glutamine (34.2 µM). The somatic embryos obtained from these cultures were transferred to a regeneration media supplemented with cytokinin BAP (88.8 µM) and IBA (19.6 µM) to recover the normal plantlet.

Statistical analysis

Each treatment was conducted in three replications. The results obtained were analyzed using SAS software and means compared by Duncan's multiple range test.

Results

Effect of three different methods of shoot meristem excision on initial calli induction

In the case of T1, the excised embryos were inoculated to Eeuwen's Y3 media supplemented with 60 g/l of sucrose. After 10 days of incubation in dark condition, shoot meristem was scooped and inoculated into ICIM. In the case of T2, surface sterilized embryos were aseptically inoculated into germination media (Y3 with 2.8 µM of GA3). After five days of incubation, initiation of germination was observed and shoot meristem was scooped and inoculated into callus induction media. In the case of T3, shoot meristem was directly scooped from the surface sterilized matured embryos in aseptic condition and inoculated in to the media containing initial callogenic chemicals. Initial calli was observed (after 30 days) in all the three methods of shoot meristem excision. The percentage of response obtained from T1 (Fig. 2a), T2 (Fig. 2b) and T3 (Fig. 2c) were 21%, 27% and 79% respectively (Table 1). White translucent type of calli was obtained from T3 method.

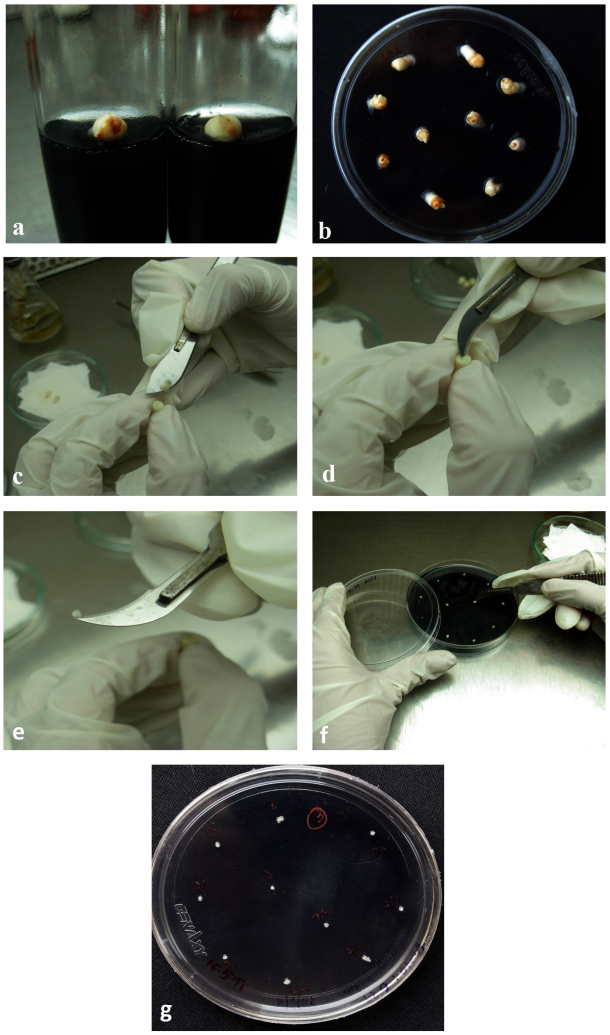

Fig. 1. Three methods of shoot meristem excision. a. Inoculation of embryos to Y3 basal media supplemented with 60 g/l of sucrose (T1 method), b. Inoculation of embryos to Y3 basal media supplemented with GA3 (1 mg/l) (T2 method), c. T3 method- slicing of the embryo, d. Scooping of the shoot meristem, e. Excised shoot meristem, f. Inoculation of shoot meristem to callus induction media, g. Shoot meristems in callus induction media.

Effect of three different methods of shoot meristem excision on embryogenic calli induction

After 30 days of incubation in dark condition, the calli obtained were subcultured to decreased concentrations of 2,4-D. It was noticed that embryogenic calli were initiated in all the three methods. Percentage of formation of embryogenic calli in T1 (Fig. 2d), T2 (Fig. 2e) and T3 (Fig. 2f) methods were 11%, 15% and 56% respectively. In the case of T1, the calli initially obtained turned to compact structures and showed more browning (Fig. 2g). Yellow coloured compact type calli (Fig. 2h) were observed in cultures obtained in T2 treatments. Thus, the formation of embryogenic calli was reduced and some embryogenic calli showed abnormal shoot development (Fig. 2i). The cultures initiated from T3 method showed translucent ear-like shaped calli in the initial stages. Percentage of compact calli was very less compared to other methods. Statistical analysis was done and T1 and T2 methods showed mean values of 0.1000 and 0.1500 whereas T3 method showed a high mean value of 0.5000 (Table 1).

In the case of T1 and T2 methods, somatic embryo formation was not observed. Embryogenic calli obtained from T3 method showed more tendencies towards formation of somatic embryos. In case of T3 method, embryogenic calli (90 days old) were subcultured to hormone-free media supplemented with 2.5 g/l of activated charcoal which was used as maturation media. In this media, formation of proembryos was observed (Fig. 2j). In later stages of development, somatic embryos were initiated from proembryos (Fig. 2k). The somatic embryos formed shoots when transferred to regeneration media [2,4-D (0.045 µM), BAP (22.2 µM), GA3 (2.8 µM) and glutamine (34.2 µM)] (Fig. 2l). Normal plantlets were recovered after 30-40 days of incubation in a media supplemented with BAP (88.8 µM) and IBA (19.6 µM) for the normal recovery of plantlets.

Table 1. Effect of three different methods of plumule extraction for initial calli induction after 30 days of culture incubation and embryogenic calli induction after 90 days of culture incubation

Treatment	Percentage of initial friable calli	Mean (CI)	Percentage of embryogenic calli	Mean (EC)
T1	21	0.2000b	11	0.1000 b
T2	27	0.2667b	15	0.1500 b
T3	79	0.7500a	56	0.5000 a
	CD (P=0.05)	0.1545	CD (P=0.05)	0.1434

Discussion

The present study describes the induction of embryogenic calli by comparing three methods of shoot meristem excision. Shoot meristem tissue has been shown to respond well and more rapidly than immature inflorescence explants in terms of callus formation and the development of embryogenic capacity (Hornung, 1995b). The yields were larger (nearly two-fold for calli and over ten-fold for calli bearing somatic embryo) than those reported with inflorescence (Verdiel *et al*., 1994). It has been reported earlier that the somatic embryos formed from plumule explants has more potential of germination, regeneration compared to inflorescence explants (Chan *et al*., 1998; Rajesh *et al*., 2005, 2014).

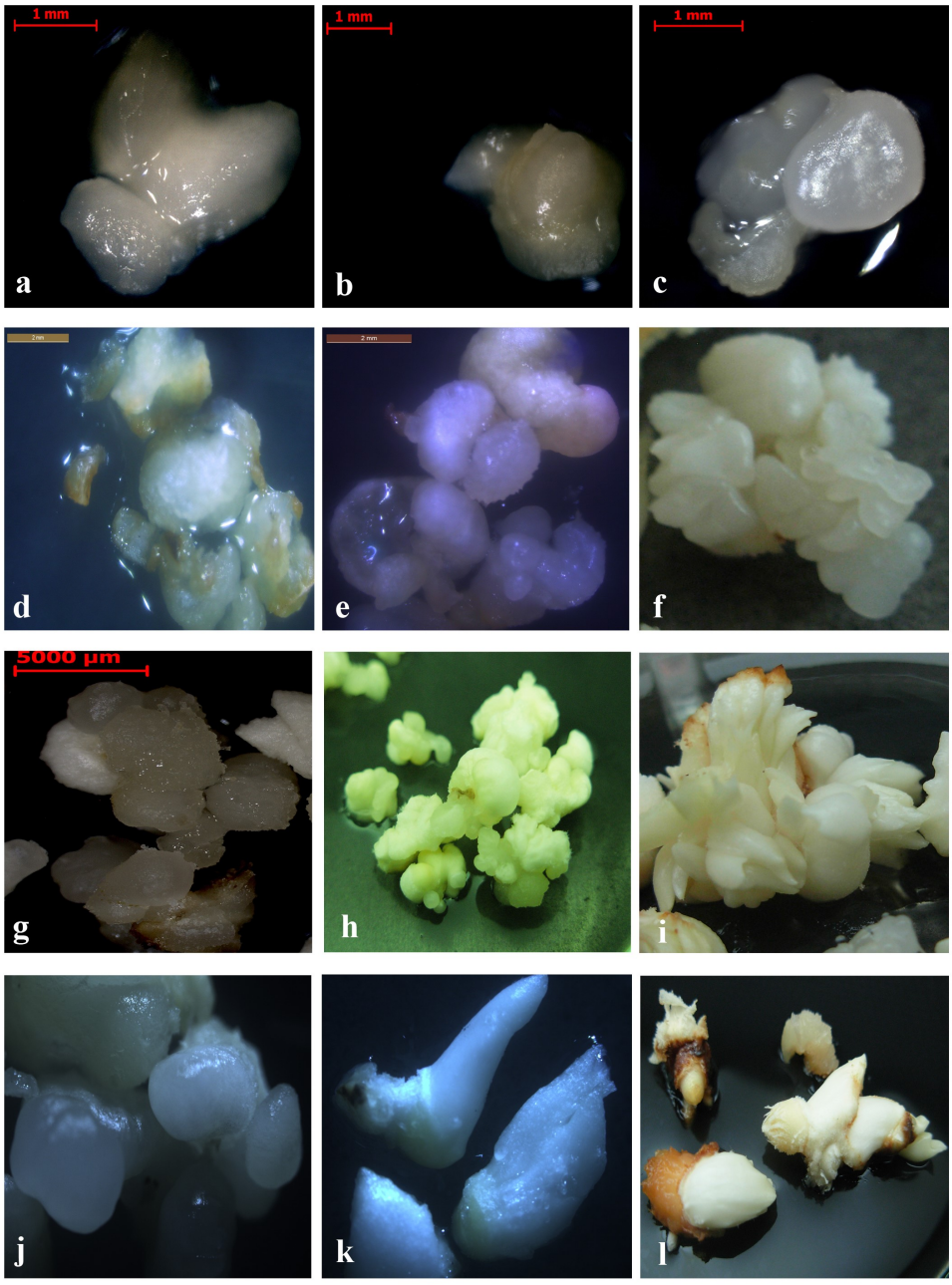

Fig. 2. Embryogenic calli induction from three methods of shoot meristem excision
a. Calli initiation in T1 method after 30 days, b. Calli initiation in T2 method after 30 days, c. Calli initiation in T3 method after 30 days, d. Embryogenic calli induction in T1 method after 60 days, e. Embryogenic calli induction in T2 method after 60 days, f. Embryogenic calli induction in T3 method after 60 days, g. Embryogenic calli turned to compact calli in T1 method, h. Yellowish coloured compact calli formation in T2 method, i. Embryogenic calli showing abnormal development of shoots in T2 method, j. Formation of proembryo in T3 cultures, k. Somatic embryo formation in T3 cultures, l. Germination of somatic embryo in T3 cultures.

Comparison of three methods of shoot excision was tried in this study, to obtain fast embryogenesis and subsequent development into plantlets. In the case of T1 method, the surface sterilized embryos were inoculated into Y3 media supplemented with 60 g/l of sucrose. Karun *et al.* (1999) had earlier reported the effect of sucrose (60 g/l) on coconut embryo culture. From these germinated embryos, shoot meristems were excised and inoculated to ICIM. In this case, initial friable calli was observed in 21% of the cultures. In some of the cultures, compact type of calli was initiated instead of friable type of calli (51%). It was found that the cotyledonary tissues present in the shoot meristem inhibited the embryogenic potential of the tissues. Earlier studies have also revealed that the presence of cotyledonary tissues inhibited apical development of meristematic tissues in date palm (Fki *et al*., 2003).

In the case of T2 method, surface sterilized embryos were aseptically inoculated into germination media supplemented with GA3. GA3 has been reported to promote post germinative development and conversion into plantlets in several species (Evans *et al*., 1996; Rascio *et al*., 1998; Lev-Yadun *et al*., 1999). It was also revealed that, in coconut embryo culture, the use of GA3 improved the germination percentage and the rate of plantlet recovery (Ake *et al*., 2007). For coconut, no significant differences were found on root and shoot length between untreated embryos and embryos treated with different GA3 concentrations (Karun *et al*., 2001). Calli initiation was observed in 27% of the cultures and the formation of embryogenic calli was also quite low (15%). It was observed that GA3 promoted embryo germination but inhibited induction of embryogenic calli. The presence of GA3 during the induction and expression phases of TDZ-induced embryogenesis was significantly detrimental to somatic embryo formation on the hypocotyl explants of geranium (*Pelargonium x hortorum*) (Hutchinson *et al*., 1997a). Miroshnicheko *et al.* (2009), reported the influence of daminozide, an inhibitor of GA3 biosynthesis, on the somatic embryogenesis in immature and mature embryo derived from tissue cultures of wheat. They observed the positive effect of low concentration of daminozide on somatic embryogenesis in wheat culture.

Out of three types of plumule extraction, direct plumule extraction from fresh embryos were found to be more effective in initial calli (79%) and formation of embryogenic calli (56%). Most of the translucent calli showed ear- like shape (Saenz *et al*., 2006). Chan *et al*. (1998) also reported the direct excision of plumule from fresh embryos which resulted in 54.3% of embryogenic calli formation in Y_3 media supplemented with 2, 4-D (100 µM). In T3 method, after the induction of friable calli, the cultures were transferred to a media with gradual decrease of 2,4-D. It was also reported earlier that if the drop in auxin level was too rapid, the maturation of the embryogenic structures usually led to incomplete or deviated forms (Buffard-Morel *et al*., 1995).

Embryogenic calli obtained from T3 method were transferred to hormone-free media supplemented with 2.5 g/l of activated charcoal to remove excess amount of auxin. However, Smith *et al.* (1990) had reported the positive effect of hormone-free media in somatic proembryos production from excised wounded zygotic embryos of carrot. In our study, after 30 days of incubation in hormone-free media, cultures were transferred to initial regeneration media supplemented with 2,4-D, BAP and GA3 along with glutamine. Hamasaki *et al.* (2005) reported the effect of glutamine on shoot

organogenesis in leaf base culture of pineapple, where organogenesis competence could be enhanced to 70% by adding 8 mM of glutamine. The somatic embryos obtained from these cultures were transferred to a regeneration media supplemented with BAP and GA3. Chan *et al.* (1998) had earlier used BAP (50 µM) for the germination of somatic embryos in coconut. After the shoot initiation, cultures were transferred to rooting media supplemented with IBA.

To conclude, results of this study revealed that the shoot meristem excised from fresh embryo showed good response towards embryogenic calli induction and somatic embryo development compared to other methods of shoot meristem excision. The embryogenic calli obtained from these cultures could be utilized for the initiation of suspension cultures.

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