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Polyamine-induced somatic embryogenesis and plantlet regeneration *in vitro* from plumular explants of dwarf cultivars of coconut (*Cocos nucifera*)

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Coconut (*Cocos nucifera* L.), often called the *kalpavriksha* or 'the tree of life', has an important place in the socio-economic milieu of many tropical countries where it is the main source of income and livelihood for rural communities living along the coastal belt. It is chiefly cultivated for the nuts from which copra, coconut oil and coconut fibre are obtained. Presently, the coconut industry in India is burdened with a number of problems such as prevalence of various pests and diseases and senility of existing plantations. Current production of quality planting materials meets only about 20% of the estimated annual requirement of planting material to replace senile and disease ravaged plantations. Due to its long pre-bearing age, long interval between generations and exclusively seed propagated nature, crop improvement in coconut is a difficult and time-consuming programme. Application of biotechnological tools in coconut assumes particular significance due to the problems encountered in conventional breeding.

Standardization of a viable protocol for clonal propagation would open up tremendous possibilities of meeting the requirement for quality, uniform, disease resistant/tolerant planting material and of breaking down productivity barriers. Unfortunately, coconut is a highly recalcitrant species with respect to tissue culture. Various problems encountered during *in vitro* propagation of coconut are intensive tissue browning (due to oxidation of polyphenols), slow *in vitro* response, low rate of somatic embryogenesis and variation in tissue response due to heterogeneity of explants taken from different individuals (Nair *et al*. 1999). A variety of protocols have been developed using a range of explants- immature inflorescence, immature and mature zygotic embryos, young tender leaflets, leaf bases from unopened spindle and plumular tissue, but the protocols lack repeatability (Nair *et al*. 1999).

The dwarf cultivars of coconut are short in stature, growing to a height of 5-7 m with closely arranged leaf scars on their stem. These palms commence bearing in about 3-4 years after planting in contrast to 8-10 years taken by the tall strains. The dwarf palms, unlike the talls, are predominantly self-pollinated owing to the overlapping of the female and male phases in the same inflorescence and hence the variability within a cultivar is comparatively less when compared to tall palms, which are generally cross-pollinated. The present investigation describes the *in vitr*o regeneration of coconut plantlets using plumular tissues from dwarf cultivars of coconut.

Zygotic embryos were scooped out along with a portion of the endosperm using a cork borer from mature, dehusked and split coconuts (11-12 months old) of two dwarf cultivars, viz. Chowghat Green Dwarf (CGD) and Malayan Yellow Dwarf (MYD). The embryos were extracted from the endosperm plug using a scalpel. Under asceptic conditions, the embryos were washed in 50% chlorine water for 20 minutes and then rinsed four times with sterile distilled water. The sterilized embryos were inoculated into plain Y3 medium (Eeuwens 1976) containing 3.0% sucrose, 1g/l activated charcoal and 0.55% (w/v) agar. The pH of the medium was adjusted to 5.8 with 1N NaOH or HCl before autoclaving for 20 minutes at 121°C. The cultures were incubated in the dark for a month at 27±2°C. After a month in the conditioning medium, the plumular ends were sliced out from the embryos and inoculated into Y3 medium supplemented with 2, 4-D (74.66 µM) in combination with TDZ (4.54 µM). The media also contained 3.0% sucrose, 1g/l activated charcoal and was solidified with 0.55% (w/v) agar. The pH of the media was adjusted to 5.8 prior to adding agar and autoclaving. The cultures were incubated in dark at 27±2°C for two months. The calli developed were transferred to media containing 2,4-D (10 mg/l), charcoal (1 g/l) and one of the following growth regulators at different concentrations: spermine (25, 50, 75 and 100µM) and putrescine (1000, 5000, 7500 and 10000 µM). Initially, a

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small scale experiment was conducted with the above treatments. Based on the results obtained, spermine (100 µM) and putrescine (1000 µM) were selected for further experiments.

At each monthly subculture, the 2, 4-D concentration was reduced to 22.62 µM, 4.52 µM and 0.452 µM, while a constant concentration of polyamines was maintained. The charcoal concentration was also reduced to 0.5 mg/l, 0.25 mg/l and 0.1 mg/l with the reduction in 2, 4-D concentration. Plain Y3 minerals without any growth regulators served as control. All the above media were supplemented with 3.0% sucrose and 0.55% (w/v) agar. The cultures were initially incubated in dark at 27±2ºC. The experiments were replicated twice with 15 embryos per replicate. Analysis of variance (one way ANOVA) was used to test if there are significant differences between means obtained with different treatments at the 5% level of significance (Snedecor and Cochran 1975). For percentage data, angular transformation was used ($\sin^{-1}p$, where p is the proportion of the particular character recorded).

Callus initiation was noticed within 4-5 weeks of culture from the perivascular strands of the leaf primordia. After two months of culture in the callus induction medium, the calli were transferred to media containing a polyamine (spermine or putrescence) in combination with 2, 4-D. Within four weeks of culture, the calli could be recognized as embryogenic, non-embryogenic or rhizogenic. The calli were sub-cultured at monthly intervals to media containing lower levels of 2, 4-D. The non-embryogenic and rhizogenic portions were removed during the monthly subcultures, while the embryogenic portions were retained. The embryogenic calli exhibited globular structures within 2-3 weeks of the second subculture (Fig 1A). As soon as somatic embryoid / meristemoid formation was noticed, the cultures were transferred under warm-white fluorescent light with a 16 hour photoperiod. The embryoids germinated (Fig 1B) and plantlets with both root and shoot axes were formed during subsequent subcultures (Fig 1C). Some of the calli also produced protuberances on the surface, which formed green bud spots in light. These gradually grew into well-defined leafy shoots. Observations regarding the percentage of embryogenic calli, somatic embryoids, meristemoids and plantlets were taken at regular intervals.

Significant differences were noticed between varieties for the formation of embryogenic calli (Table 1). Higher percentage of embryogenic calli was formed in CGD compared to MYD. There were also significant differences for interaction between variety and regeneration medium with respect to formation of somatic embryos. In the case of MYD, enhanced somatic embryogenesis was noticed in medium supplemented with spermine compared to putrescine. There was no response for any of the parameters recorded in medium without any growth regulator supplementation (which served as control); hence it was excluded from the analysis.

Somatic embryoids and meristemoids, when formed, were transferred initially to plain liquid Y3 medium without any growth regulators and later to a medium containing BAP (17.77 µM). In meristemoid derived plantlets rooting was induced either by incubation in Y3 media supplemented with IBA (8.3- 41.49µMM) alone or in combination with NAA (5.3 µM) for a period of 3-4 weeks. Root induction as well as further growth was effective when IBA (20.74 µM) and NAA (5.3 µM) were provided together. Plantlets with minimum 2-3 opened leaves and 3-4 primary roots with well developed secondary roots (Fig 1D) were successful for cent percent *ex vitro* recovery in pots. Such plantlets were removed from the culture tubes and washed with sterile water. Before transferring to pots, the plantlets were treated with carbendazim (1%) and thereafter with IBA solution (4.9 mM) for an hour. The potting mixture consisted of sterilized soil, sand and coir dust in equal proportions. Initially, the plantlets were covered with polythene bag for

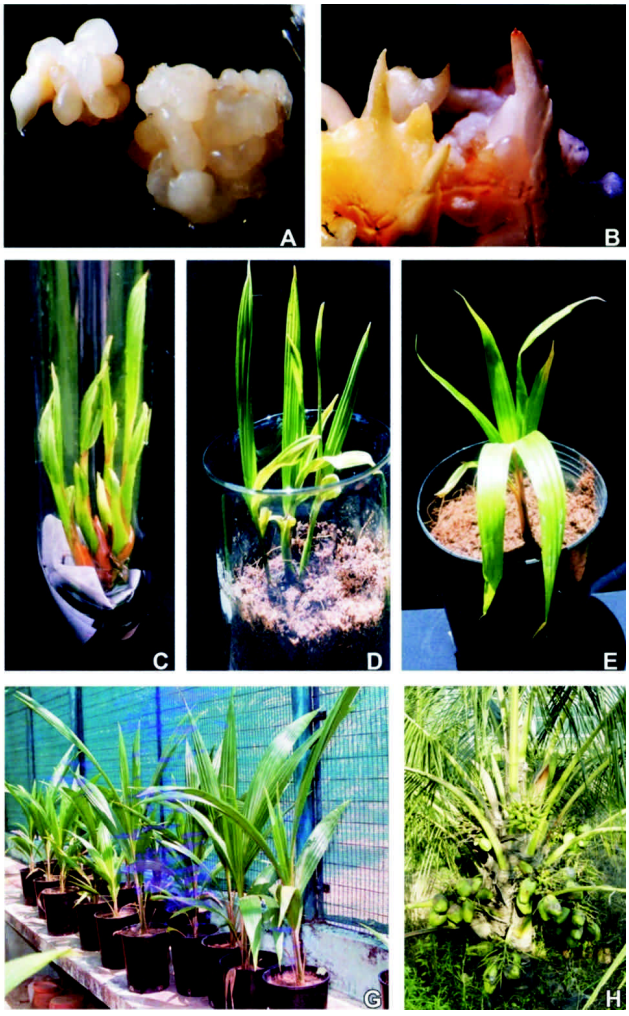

Fig1 Regeneration from plumular explants of DWARF coconut. A. Somatic embryos developed in calli bearing embryogenic structures, B. Germinating somatic embryoids, C. and D. Clump of shoots formed by germinating somatic embryoids, E. Individual plantlet developed from single shoot excised from clumps of shoots, E. Plantlets acclimatized in a green house, F. *In vitro* derived palm established in field in seed bearing stage.

Table 1 Effect of polyamines on regeneration from plumular explants of coconut

		Embryogenic calli	Browning	Meristemoid formation	Rhizogenesis	Somatic embryogenesis
Malayan Yellow Dwarf	Spermine 100 µM	37.23	33.1	10.7	0.0	43.1
	Putrescine 1000 µM	50.75	33.1	10.7	7.48	20.76
Chowghat Green Dwarf	Spermine 100 µM	63.72	14.96	30.9	7.48	26.55
	Putrescine 1000 µM	55.15	26.24	23.9	10.7	43.1
CD (P=0.05) for variety		9.99*				
CD (P=0.05) for variety × regeneration medium						23.87$

Transformation: Angular. *Significant at 5%, $ Significant at 1%

two weeks. Gradually the bags were perforated to reduce humidity and later the bags were removed during the night. After 4 weeks, the bags were removed completely (Fig 1E). Well developed plantlets were acclimatized under green house conditions (Fig 1F) and then successfully established in the field (Fig 1G).

Thus, our data shows that exogenous supply of polyamines can enhance induction of somatic embryogenesis in coconut suggesting an important role for polyamines in somatic embryogenesis. This study also provides clues for the development and establishment of a somatic embryogenesis system for coconut on the basis of modulating cellular polyamine contents, which will be invaluable for genetic enhancement of coconut, a crop which has proved to be recalcitrant to *in vitro* regeneration in spite of decades of research.

Exogenously supplied polyamines have been known to induce cell division, somatic embryogenesis and plant regeneration in many species (Rajesh *et al*. 2003, Minocha *et al*. 2004, De-la-Pena *et al*. 2008, Wu *et al*. 2009). The mechanism of polyamine functions in living cells is probably linked to their chemical and physical interactions with nucleic acids, proteins and phospholipids, and is due to their cationic nature (Smith 1985). Polyamines play a stabilizing role on nucleic acids by binding to phosphate groups, and in particular, they form complexes with DNA - this binding leads to stabilization of the nucleic acid structure or changes in their conformation (Hou *et al.* 2001). There are also reports that polyamines could be involved as second messengers on hormonal regulation of growth and development (Bais and Ravishankar 2002). Polyamines have also been reported to possess a wide spectrum of action with some similarities both with auxins and cytokinins and they modulate morphogenic processes in cooperation with plant phytohormones (Takahashi and Kakehi 2010).

SUMMARY

Regeneration of complete plantlets via organogenesis and somatic embryogenesis was achieved from plumular tissues of two dwarf cultivars of coconut, viz. Chowghat Green Dwarf (CGD) and Malayan Yellow Dwarf (MYD). Significant differences were noticed between varieties for the formation of embryogenic calli. There were also significant differences for interaction between variety and regeneration medium with respect to formation of somatic embryos. Well developed plantlets were acclimatized under green house conditions and then successfully established in the field. The development of an efficient method of cloning coconut using plumular explants (with more rapid development of calli and somatic embryos and greater frequencies of plant regeneration compared with calli from inflorescence or leaf tissues) offers a potential for the development of a long-term *in vitro* means of conserving significant coconut germplasm by cryopreservation of plumular explants. Mass multiplication of elite palms selected on the basis of resistance to root (wilt) diseases of coconut is possible using plumular explants. Plumule cultures can be used for rapid multiplication of proven coconut hybrids. This study can also form a model for future regeneration studies from adult tissues of coconut.

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