

MICROPROPAGATION OF COCONUT THROUGH PLUMULE CULTURE

S C Fernando, L K Weerakoon and T R Gunathilake

Coconut Research Institute, Lunuwila, Sri Lanka

ABSTRACT

Plumule tissues have found to be more responsive explants for clonal propagation of coconut. In the present study, the feasibility of using plumule explants for clonal propagation of a local coconut variety (Sri Lanka Tall) was assessed. Plumules were cultured in three basal media (MS, Y3 and 72) supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) (24 - 400 μ M) and activated charcoal. Somatic embryogenesis and plant regeneration were induced by incorporating of abscisic acid (ABA) into the medium. Attempts were made to improve plant regeneration by applying high agar concentration-induced water stress, cytokinin or silver nitrate in combination with ABA.

The results revealed that callus can be initiated from plumule explants cultured in all three basal media tested. The callus formation depended on the concentration of 2,4-D in the media and the best effect was observed with the lower level (24 μ M) tested. The plant regeneration frequency observed with ABA treatment was 4.4 %. The combination of ABA with high agar concentration-induced water stress doubled the plant regeneration frequency. About 50 % of the regenerated plants could be successfully acclimatized and field planted.

The results of the study indicate the possibility of regenerating plants from plumule explants of coconut. The method can be used for multiplication of improved seed material. However, the plant regeneration efficiency should be further improved for mass scale propagation of coconut using plumule explants.

INTRODUCTION

The coconut palm is a major plantation crop and the most important palm of the tropics. Due to its economic importance, breeding for crop improvement is of high priority. Coconut is a cross-pollinated and heterozygous plant, which is so far, propagated exclusively through seeds. Clonal propagation of coconut is of prime importance for rapid multiplication and distribution of the best genotypes obtained through conventional breeding. Furthermore, it

would also allow rapid multiplication of selected parental palms for establishing seed gardens and selected individuals, exhibiting resistance to biotic and/ or abiotic stresses, for increasing the yield.

Initial attempts have been made to micropropagate coconut through different explants such as immature inflorescence (Branton and Blake, 1983), tender leaf (Raju *et al.*, 1984) and immature zygotic embryo (Karunaratne and Periyapperuma, 1989). Later studies by Hornung (1995) and Chan *et al.* (1998) have shown that the response of plumules (embryo meristem and first leaves) to *in vitro* culture conditions is better than that of other tissues. Therefore, plumules could be used as a model for clonal propagation of coconut. As plumules are excised from zygotic embryos, they can only produce clones of palms with unknown performance. However, if the embryos are obtained by controlled pollination of selected parents, the clones from these embryos are likely to show many of the desired characteristics of the parent palms (Chan *et al.*, 1998).

This paper reports the progress of clonal plant regeneration of a local coconut variety (Sri Lanka Tall) using plumule explant.

MATERIALS AND METHODS

Plant material

Mature nuts (12-14 month-old) of the variety Sri Lanka Tall coconut were used as the source of mature zygotic embryos.

Callogenesis

Mature embryos were cultured in modified Eeuwens Y3 liquid medium (supplemented with 0.1 μ M 2,4-D, 5 μ M 6-benzylaminopurine (BAP), 6 % (w/v) sucrose and 0.25 % (w/v) activated charcoal [Fluka]), optimized for mature zygotic embryo culture of coconut (Weerakoon *et al.*, 2002). After incubation in dark (at 30 ± 1 °C) for 15-17 days, plumules were excised from these embryos (Fig. 1a). They were crushed and cultured in 28 ml screw capped vials containing 10 ml of culture medium. The effect of three basal media [medium 72 supplemented with 24 μ M 2,4-D (Karunaratne and

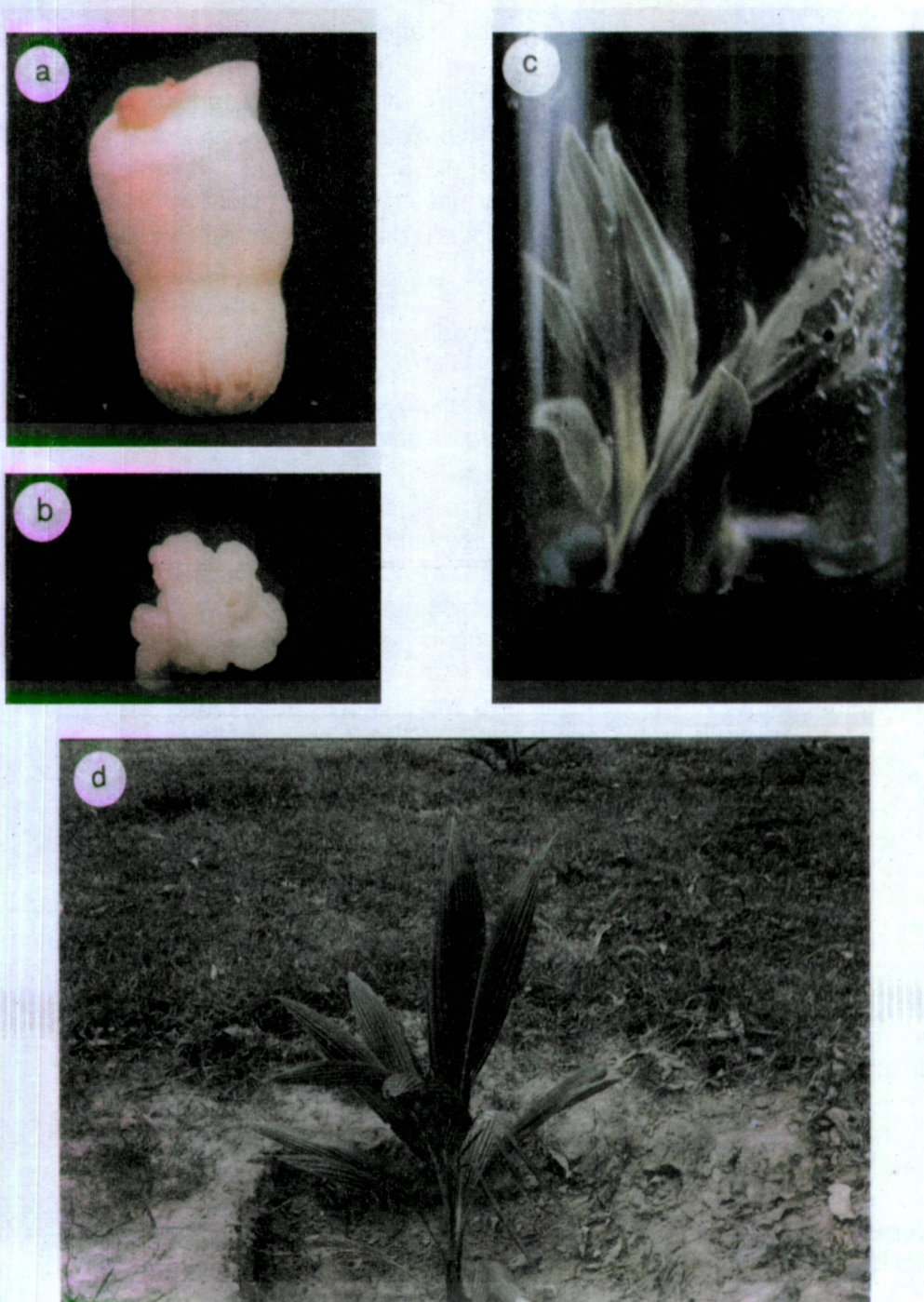


Figure 1: Plant regeneration from plumule explants of coconut. **a** – Mature zygotic embryo (pre-cultured in germination medium for 15 days) ready for plumule excision; **b** – Callus derived from a plumule explant; **c** – Shoots regenerated from plumule-derived callus; **d** - Field-planted plumule callus-derived coconut plant.

Periyapperuma, 1989), MS (Murashige and Skoog, 1962) supplemented with 400 μM 2,4-D (Hornung, 1995) and Y3 (Eeuwens, 1978) supplemented with 100 μM 2,4-D (Chan *et al.*, 1998)] on callogenesis was tested. The 2,4-D concentrations used were based on previous experiments. All the media were solidified with 0.8 % (w/v) agar (Park Scientific) and supplemented with 0.25 % (w/v) activated charcoal (Pharmacos). Cultures were incubated at 30 ± 1 °C for 10 weeks in the dark. Callogenesis was repeated four times each time with 15 replicates.

Somatic embryogenesis and plant regeneration

Embryogenic callus initiated from plumules cultured in medium 72 supplemented with 24 μM 2,4-D was used. Callus separated from the original explants, was subcultured in medium 72 supplemented with 16 μM 2,4-D for 4 weeks before transferring to somatic embryo induction medium. Somatic embryogenesis was induced by subculturing of callus in medium 72 supplemented with 5 μM abscisic acid (ABA) (filter-sterilized) and 6 % (w/v) sucrose for 5 weeks. Maturation of somatic embryos was achieved by transferring them to medium 72 (devoid of hormones) for 4 weeks. Somatic embryo germination was achieved in Y3 solid medium. Regenerated shoots were maintained in Y3 solid for 4-6 months by subculturing at 4-weekly intervals (Fernando and Gamage, 2000). During somatic embryo induction, cultures were maintained in screw-capped vials containing 10 ml of media supplemented with 0.8 % (w/v) agar and 0.25 % (w/v) activated charcoal (Pharmacos) whereas during somatic embryo maturation and germination, they were maintained in 100 ml flasks containing 50 ml of media supplemented with 0.8 % (w/v) agar and 0.25 % (w/v) activated charcoal (Fluka). Cultures were maintained at 30 ± 1 °C in the dark. Regenerated shoots were maintained at 16-h photoperiod (intensity $17 \mu\text{mol m}^{-2} \text{s}^{-2}$) until they were ready for transferring to *ex vitro*. If necessary, roots were induced on shoots by a pulse treatment of 500 μM indole acetic acid (IAA) as described by Fernando and Gamage (1994-1995).

In the present study, attempts were made to improve plant regeneration frequency by testing the effect of high agar concentration-induced water stress (2 % agar in somatic embryo induction medium during the initial 2-week period), 5 μM cytokinin (BAP or isopentyl adenine [2iP]) and 1-10 μM AgNO₃ in combination with ABA. The experiments were repeated at least three times with 30 replicates per treatment.

***Ex vitro* establishment of regenerated plants**

The fully developed plants with about three leaves and well-developed root systems were de-flasked, rinsed well with water and drenched in a mixture of fungicide (0.025 % benlate) and diluted nutrient solution for a few hours

before transfer to clear polypropylene bags containing sterilized potting mixture (river sand and compost 1:1) moistened with a diluted nutrient solution. The bags were initially kept sealed (for maintaining high humidity) in the glass house. After one week, the cover was gradually removed to reduce the relative humidity. The plants were watered regularly and a liquid fertilizer (Maxicrop™) was sprayed every 10 days. In addition, a solution containing minerals of Y₃ medium (Eeuwens, 1978) was applied at two weekly intervals. After 6-8 months, plants were repotted in larger polybags containing a potting mixture of top soil, compost, river sand, dried cow dung and coir dust (2:1:1:1:0.5). The plants were kept in a shade-house for about 3-4 months before transferring to direct sunlight. During this period, a liquid fertilizer (Maxicrop™) was sprayed on to plants at 10-day intervals and 20 g of young palm mixture (urea: saphos phosphate: muriate of potash, 2:3:2) was applied to each plant at two weekly intervals. After exposure to direct sunlight for about 1-2 months, they were ready for field planting. Several plants were transferred to field following the recommendations of the Coconut Research Institute of Sri Lanka (CRISL).

Statistical analysis

Analysis of Variance was carried out to test the differences among treatments.

RESULTS AND DISCUSSION

Callogenesis

Coconut somatic embryogenesis usually occurs through a callus phase. Therefore, production of embryogenic callus is a prerequisite for successful plant regeneration. Among several other factors, the presence of an auxin in the medium is critical for successful callogenesis (Auge, 1995). The most commonly used auxin in coconut tissue culture media is 2,4-D and its concentration depends on the explant and concentration of activated charcoal in the culture medium (Verdeil and Buffard-Morel, 1995). In the present study, the effect of culture media previously used for plumule culture of exogenous coconut varieties [MS supplemented with 400 µM 2,4-D (Hornung, 1995) and Y3 supplemented with 100 µM 2,4-D (Chan et al., 1998)] and immature embryo culture of Sri Lankan coconut varieties [medium 72 supplemented with 24 µM 2,4-D (Karunaratne and Periyapperuma, 1989)] on callogenesis from plumule tissues of Sri Lanka Tall coconut was tested. The study was extended to assess the effect of medium 72 supplemented with higher level (100 µM) of 2,4-D and media MS and Y3 supplemented with lower level (24 µM) of 2,4-D.

The results revealed that the callusing frequencies were higher and comparable in all the three basal media supplemented with lower (24 μM) concentration of 2,4-D. Increase in the concentration of 2,4-D up to 100 or 400 μM resulted in a decrease in callusing (Table 1). Despite the statistically non-significant decrease in callusing frequency in Y3 supplemented with 100 μM 2,4-D, callus initiated in high 2,4-D containing media showed a poor growth compared to that of lower 2,4-D media.

Table 1: The effect of different culture media on callogenesis in plumule explants of coconut

Basal medium	2,4-D concentration (μM)	Callusing frequency (%)
72	24	55.2
	100	40.9
MS	24	49.4
	400	35.5
Y ₃	24	54.3
	100	46.7
Significance		p<0.05
CV (%)		17.3
LSD		12.1

In this study, callusing frequencies in plumule explants cultured in previously published plumule culture media (MS+ 400 μM 2,4-D and Y3+ 100 μM 2,4-D) were lower (35.5 and 46.7 % respectively) than those reported by Hornung (1995) (75 %) and Chan et al. (1998) (60 %). In the present study, the published media were tested with high levels of 2,4-D but without the specific type of activated charcoal, which had been used by the relevant research groups. Different types of charcoal have different adsorption capacities, which results in variations in active auxin concentration in the medium. Therefore, the use of high levels of 2,4-D in the presence of unspecific type of charcoal could be the reason for the low callusing frequencies observed. This was further confirmed by the increase of callus production in those media by reducing the concentration of 2,4-D. With agreement of the results of this study, Saenz et al. (1999) reported that callusing in plumules cultured in medium described by Chan et al. (1998) could be increased up to 100 % by using a different type of charcoal. Furthermore, the use of different coconut genotypes for studies of different research groups might also have contributed to the variable results.

Somatic embryogenesis and plant regeneration

Generally, somatic embryogenesis in coconut callus has been induced by gradual reduction of auxin concentration with a corresponding increase in the cytokinin level in the medium (Buffard-Morel et al., 1995). In contrast,

somatic embryogenesis in coconut has also been induced by an early increase in 2,4-D concentration followed by a gradual reduction (Verdeil *et al.*, 1994) or a rapid decrease in 2,4-D concentration and application of high concentration of cytokinin (Chan *et al.*, 1998). However, research conducted at the CRISL revealed that an alternate treatment, removal of auxin and incorporating ABA to the culture medium, give rise to more consistent plant regeneration from coconut callus (Fernando and Gamage, 2000). Similarly, Samosir *et al.* (1999) reported improved somatic embryogenesis in coconut with the use of ABA.

In this study, embryogenic callus (Fig. 1b) treated with 5 μ M ABA, became more compact. After 5 weeks in the medium with ABA, whitish globular structures were visible. Upon transfer to the medium devoid of hormones for four weeks, some of the embryogenic structures elongated whereas the others developed into haustorial tissues or fused shoot-like structures. On repeated subculture to the Y₃ medium, the somatic embryos produced shoots (Fig. 1c).

Incorporating ABA to the culture medium resulted in normal development of somatic embryos that can develop into complete plants. However, the plant regeneration frequency was low (4.4 %). Among the treatments tested, only the application of high agar-induced water stress in combination with ABA had a positive effect and it doubled (9.0 %) the plant regeneration frequency (Table 2). The use of ABA in combination with osmotically active substances has resulted in improved somatic embryogenesis and plant regeneration in coconut (Samosir *et al.*, 1999), rubber (Linossier *et al.*, 1997) and several other crops. The moisture stress, induced by application of high agar concentration in the medium has also shown encouraging results in plant regeneration of rice (Jain *et al.*, 1995). The effect of such a stress has not been tested for coconut previously. The enhanced results observed in this study might be due to promotion of the expression of embryo-specific genes, improved embryo development and increased production of storage reserves caused by moisture stress.

Table 2: Effect of high agar concentration - induced water stress on plant regeneration

Treatment	Plant regeneration (%)
ABA	4.4
ABA + high agar-induced water stress	9.0
Significance	P= 0.01
CV (%)	31.8
LSD	0.3

An embryogenic callus clump that regenerated plants produced an average number of 1-2 plants. The clonal plants were maintained in Y3 medium until they produced 2-3 leaves and a good root system. When shoots did not produce roots spontaneously, the application of an IAA pulse resulted in successful rhizogenesis in more than 90 % of the treated shoots. When the complete plants were transferred to the potting medium for acclimatization, about 50 % of plants survived and they could be transferred to field successfully (fig. 1d).

The results of this study indicated the possibility of regenerating clonal plants of the local variety Sri Lanka Tall using plumule explants. The concentration of 2,4-D in the callusing medium was found to be the critical factor determining success in callogenesis. Application of ABA in combination with high agar level-induced water stress improved plant regeneration frequency. The resulting plants could be acclimatized and field planted successfully. This method can be applied for multiplication of improved seed material. However, the plant regeneration efficiency of the current protocol should be further improved for mass scale propagation of coconut using plumule explants.

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