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Somatic Embryogenesis and Plant Regeneration from Unfertilised Ovary Explants of Coconut (*Cocos nucifera* L.)

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ABSTRACT. *Unfertilized ovaries excised from immature female flowers of adult coconut palms (variety Sri Lanka Tall) were cultured in CRI 72 medium supplemented with 100 µM 2,4-dichlorophenoxyacetic acid (2,4-D) for callus induction. Five culture procedures including the standard procedure were tested for induction of somatic embryogenesis and plant regeneration. In the standard procedure, the calli were transferred to CRI 72 medium with 5 µM abscisic acid (ABA) and high agar concentration (2%) (w/v) for two weeks, CRI 72 medium with 5 µM ABA and normal agar concentration (0.55%) (w/v) for further three weeks, maturation medium (CRI 72 medium without any hormones) for four weeks and germination medium [modified Eeuwens Y_3 medium supplemented with 5 µM 6-benzyl aminopurine (BAP) and 0.1 µM 2,4-D]. In the other four procedures, modifications to the standard procedure were done by eliminating certain steps and incorporating 5 µM 2-isopentyl adenine (2iP) into the germination medium. In all five procedures, very poor shoot formation was observed but could be improved by incorporating 0.35 µM gibberelic acid (GA_3) into the germination medium. The highest percentage of shoot regeneration (85%) was achieved by sub-culturing the embryogenic calli directly into the modified Eeuwens Y_3 medium devoid of any growth regulators for four weeks followed by sub-culturing into germination medium supplemented with 5 µM 2iP, 0.1 µM 2,4-D and 5 µM BAP and finally to germination medium with 0.35 µM GA_3. The results clearly indicated the positive effect of 2iP and GA_3 on plant regeneration in ovary-derived calli of coconut.*

INTRODUCTION

Vegetative propagation of superior coconut palms is a promising possibility for increasing production and homogeneity in coconut plantations. Tissue culture remains the only approach to achieve this goal. Although, the problems associated with cloning coconut has

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been addressed by a number of research groups worldwide in the past two decades, the success has been very limited. This is due to the poor response of coconut tissues to *in vitro* culture conditions and thus it is classified as one of the most recalcitrant species to regenerate *in vitro* (George and Sherrington, 1984).

A range of tissues of coconut including shoot tips (Weerakoon *et al.*, 2002), immature inflorescence (Hornung and Verdeil, 1999) and tender leaf (Pannetier and Buffard-Morel, 1982) have been used for *in vitro* culture and different procedures have been adopted. Out of all explants, the most extensively studied are the immature inflorescence and leaf. A small number of clonal plants have been regenerated from these two explants through a process of somatic embryogenesis that appears to be the most promising technique for cloning coconut. According to personal experience, defining the most suitable developmental stage for immature inflorescence culture is very difficult and considerable damage is inflicted to the palm during collection of explants. Hornung (1995) indicated that the callusing frequency in immature inflorescence explants is low (usually less than 30%) and the results are not consistent. In the case of leaf explants, low frequency of callusing (< 20%) was reported from leaves collected from mature palms (Pannetier and Buffard-Morel, 1982) and collection of explants also causes damage to the palm.

The feasibility of using the unfertilized ovary of coconut as an explant for plant regeneration *via* somatic embryogenesis was reported for the first time by Perera *et al*. (2007). Suitable culture conditions for consistent callus production and complete somatic embryo formation have been developed (Perera *et al*., 2007). ABA and BAP have been used for somatic embryo induction, maturation and plant regeneration (Perera *et al*., 2007). Even though histological studies revealed that many of the somatic embryos formed were complete with shoot and root poles (Perera *et al*., 2007) the regeneration efficiency was still low.

Incorporation of 2iP (Weigel and Hughes, 1985; Levi and Sink, 1991) and GA_3 (Ghosh and Sen, 1991) has enhanced plant regeneration efficiency in a number of plant species. Thus, in the present study attempts were made to improve the plant regeneration frequency (by incorporating 2iP and GA_3) and develop an efficient ovary culture protocol for coconut.

MATERIALS AND METHODS

Culture conditions

Unfertilized ovaries were excised from immature female flowers of -4 stage (considering the most recently opened inflorescence as 0 stage, the next inflorescence to open was referred to as -1 and -4 would open approximately 4 months later) of adult coconut palms (variety Sri Lanka Tall), and cultured in CRI 72 medium (Karunaratne and Periapperuma, 1989) supplemented with 100 µM 2,4-dichlorophenoxyacetic acid (2,4-D) for callus induction (Perera *et al*., 2007). Well developed calli were first transferred to CRI 72 medium containing a lower level of 2,4-D (66 µM) for four weeks. In addition to the standard procedure (P_1) (Fernando and Gamage, 2000; Perera *et al*., 2007), four new procedures (P_2 to P_5 as listed below) were tested for induction of somatic embryogenesis and plant regeneration in callus initiated from ovary explants with the aim of enhancing the plant regeneration efficiency.

As indicated below, in the standard procedure (P_1, control), the calli in low-2,4-D medium were transferred to CRI 72 medium with 5 µM ABA and high agar concentration (2%) (w/v) for two weeks followed by transfer to CRI 72 medium with 5 µM ABA and normal agar concentration (0.55%) (w/v) for further three weeks in order to induce somatic embryogenesis. The embryogenic structures developed were then sub-cultured into a maturation medium (CRI 72 medium without any hormones) for four weeks followed by germination medium [modified Eeuwens Y_3 medium (Karunaratne *et al*., 1985) supplemented with 5 µM BAP and 0.1 µM 2,4-D]. In the other four procedures, modifications to the standard procedure were done by eliminating certain steps as given below and incorporating 5 µM 2iP into the germination medium.

P_1. ABA + high agar in CRI 72 medium → ABA + normal agar in CRI 72 medium → maturation medium (CRI 72 medium without hormones) → germination medium (Y_3 with 2, 4-D and BAP) (Standard procedure; Control)

P_2. ABA + normal agar in CRI 72 medium → maturation medium (Y_3 without hormones) → germination medium (Y_3 + 2iP + 2,4-D + BAP)

P_3. ABA + normal agar in CRI 72 medium → maturation medium (Y_3 without hormones) → germination medium (Y_3 + 2,4-D + BAP)

P_4. Maturation medium (Y_3 without hormones) → germination medium (Y_3 + 2iP + 2,4-D + BAP)

P_5. Maturation medium (Y_3 without hormones) → germination medium (Y_3 + 2,4-D + BAP)

In the case of P_1, P_2 and P_3, the cultures containing ABA were maintained in the dark at 28 °C. Once the embryogenic structures were transferred to germination medium (in all the 5 procedures), the cultures were maintained in light (16 h photoperiod, PAR; 25 $\mu molm^{-2}s^{-1}$) at 28 °C. Each sub-culturing was done at monthly intervals.

The experiment was repeated two times with a minimum of 8 replicates (8 calli). The number of shoots produced in each procedure was recorded. In all the five procedures tested, very poor shoot formation was observed even after sub-culturing the embryogenic structures into the germination medium for four times. Thus all the embryogenic structures were transferred to a new germination medium (without 2iP) supplemented with 0.35 µM GA_3 (filter-sterilized) to induce shoot formation. Once a good root system was developed, the plants were transferred to a liquid germination medium and maintained in the same medium until transplanting in soil.

Statistical analysis

The number of shoots produced in each treatment was recorded and the data were analysed using SAS statistical package (SAS Institute, 1999). Chi-square or Maximum likelihood analysis of variance was conducted using the Proc CatMod procedures of PC-SAS. Treatment means were compared using SE, 95% confidence intervals or orthogonal contrast coefficients, where appropriate (Compton, 1994).

RESULTS AND DISCUSSION

The well developed calli consisted of a translucent mass of globules in off white colour. When the calli were transferred onto low 2,4-D medium, the translucent appearance of the globules gradually disappeared and later turned opaque. The somatic embryos obtained from ovary derived-calli were identified based on morphological features including a creamy colour and elongated shape. The morphological characteristics of these somatic embryos were found to be similar to those obtained from other coconut explants such as immature inflorescence and plumule (Verdeil *et al.,* 1994; Chan *et al.*, 1998).

In a previous study, it was shown that 75% of the histologically-analyzed embryogenic structures which were selected morphologically as good somatic embryos contained the shoot pole (Perera *et al*., 2007) indicating that poor shoot regeneration was not mainly due to the absence of a shoot pole as indicated by Fernando *et al.* (2003). It could be due to a hormonal imbalance or other factors hindering proper germination of somatic embryos and shoot formation. Therefore, different culture procedures were tested to improve shoot formation and growth.

In all 5 procedures (P_1- P_5) tested, very poor shoot formation was observed even after repeated sub-culturing of somatic embryos into the germination medium. GA_3 has been used at a concentration of 0.35 µM to enhance germination of zygotic embryos of coconut (Weerakoon *et al.*, 2002). Thus all the embryogenic structures (obtained by the 5 procedures) were sub-cultured into a germination medium supplemented with 0.35 µM GA_3. Shoot formation (Figure 1a & b) was improved by GA_3 application and the number of shoots increased with continuous exposure to GA_3 for all subsequent sub-culturing into the germination medium. The percentages of shoot production in each procedure are summarized in Table 1.

Figure 1. Developing shoots in germination medium containing GA_3. a. A shoot emerging from a somatic embryo **b.** A complete plantlet with a well developed root system

As shown in Table 1, a higher shoot regeneration was obtained with the four new procedures tested (P_2, P_3, P_4 and P_5) when compared to the control P_1 (2.7%). The results clearly indicated the positive effect of 2iP on shoot regeneration. The highest percentage of shoot regeneration (85%) was achieved with P_4 where ABA was not used for induction of somatic embryogenesis. Based on the results, it can be seen that induction of somatic embryogenesis begins when calli are first transferred to the medium containing low 2,4-D. Maturation of embryos occurs in hormone-free medium and early exposure of matured embryos to 2iP seems to enhance shoot regeneration. The results indicated that sub-culturing the somatic embryos directly into the germination medium containing a high level of cytokinin (5 µM 2iP together with 5 µM BAP) could give rise to shoots at a high frequency (85%) ($p<0.01$). Even though ABA has been used for induction and maturation of somatic embryos in coconut (Fernando and Gamage, 2000; Perera *et al.*, 2007), the present results suggest that it could reduce the conversion of somatic embryos into shoots.

2iP has successfully been used to improve the regeneration efficiency in a number of plant species. It has been reported that 1.4 or 1.5 µM 2iP has induced somatic embryos in herbaceous monocots such as *Asparagus officinalis* (Levi and Sink, 1991) and *Hordeum vulgare* (Weigel and Hughes, 1985) in the presence of an auxin. Different concentrations of 2iP (in combination with an auxin or another cytokinin) have also been used for the development of somatic embryos in a number of crop species including *Asparagus officinalis, Allium fistulosum x A. cepa*, *Tiriticum aestivum, Zea mays* and *Saccharum officinarum* (Ozias-Akins and Vasil, 1982; Srinivasan and Vasil, 1986; Conger *et al.*, 1987; Levi and Sink, 1991).

Table 1. Shoot regeneration achieved by different culture procedures.

Culture procedure [1]	Number of calli cultured	Percentage of shoot regeneration (No. of shoots per 100 pieces of calli)
P_1	42	2.7
P_2	45	11.0
P_3	27	7.4
P_4	16	85.0
P_5	17	6.8
Contrasts		Chi square
P_1 vs P_4		31.33****
P_2 vs P_4		40.47****
P_3 vs P_4		40.61****
P_4 vs P_5		40.22****

[1]In all five procedures (P_1- P_5), after repeated sub-culturing into the relevant germination media, embryogenic structures were sub-cultured into the germination medium containing 0.35 µM GA_3.
Maximum likelihood analysis of variance was significant at 0.0001 (G^2=125.96)

2iP has also been used to induce somatic embryogenesis in a number of woody plants species but with limited success. In two of the woody species, *Actinidia chinensis* (Fraser and Harvey, 1986) and *Coffea canephora* (Hatanaka *et al.*, 1991), the effective concentration of 2iP for induction of somatic embryogenesis was 24.6 µM and 5 µM respectively. Plant regeneration has been achieved in some palm species such as *Elaeis*

guineensis (Thomas and Rao, 1985) and *Phoenix dactylifera* (Tisserat, 1979; Tisserat, 1982) with the use of 2iP. In coconut, plantlet regeneration from cultured immature inflorescence explants has been reported on MS medium supplemented with 5 µM BA and 5 µM 2iP with the presence of 2,4-D (Branton and Blake, 1983). The results of this study revealed that incorporation of GA_3 into the germination medium improved the shoot regeneration frequency. Spiegel-Roy and Vardi (1984) also reported that GA_3-supplemented media stimulated germination of *Citrus* somatic embryos.

CONCLUSIONS

The study presents that somatic embryogenesis is induced by transferring callus into a low 2,4-D-containing medium. Maturation of somatic embryos could be achieved in a hormone-free medium and early exposure of somatic embryos to cytokinins promoted shoot regeneration. The presence of GA_3 in a germination medium was also found to be critical for successful plant regeneration.

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