Generation of double haploids in coconut (*Cocos nucifera* L.) plants via anther culture

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ABSTRACT

Coconut breeding is seriously constrained by the highly heterozygous nature of the palm. Although development of homozygous coconut lines is of utmost importance, its achievement by conventional methods is impractical. Studies on generation of dihaploid plants *via* anther culture were undertaken to develop an unconventional method of producing homozygous lines. Anthers collected from coconut inflorescences at a maturity stage of three weeks before splitting were subjected to heat (38 °C) pretreatments for six days prior to culture in modified Eeuwens Y_3 liquid medium supplemented with 100 µM 2,4-dichlorophenoxyacetic acid (2,4-D), 0.1% activated charcoal and 9% sucrose. The calli/ embryoids were produced at a frequency of 22.2%. They were sub-cultured to somatic embryo induction medium (containing 66 µM 2,4-D) followed by maturation medium (without hormones) and germination medium (containing 5 µM - 6-benzylaminopurine and 0.35 µM gibberellic acid). 43% of the well defined, mature, opaque embryoids germinated directly. Histological studies revealed that the calli/ embryoids originated from pollen grains. Ploidy analysis of calli/ embryoids showed that 50% were haploid and the rest were diploid. Diploid plantlets were found to be double haploids by simple sequence repeats (SSR) analysis.

KEY WORDS: anther culture; *Cocos nucifera* L.; double haploids; flow cytometry; histology; SSR markers.

ABBREVIATIONS: BAP - 6-benzylaminopurine; 2,4-D - 2,4-dichlorophenoxyacetic acid; GA_3 - gibberellic acid; DH- double haploid; SSR- Simple Sequence Repeats

INTRODUCTION

Tall coconut varieties are allogamous and exhibit great variation in agronomic characters. Problems encountered with conventional breeding of coconut are the long juvenile phase, low multiplication rate and high heterozygosity that make plant breeding programmes a long and difficult process. Production of homozygous lines will have a tremendous impact on generating new hybrid varieties. To obtain homozygous lines, breeders resort to self or back crossing over several generations taking a minimum of 60 years. Furthermore, 100% homozygosity cannot be achieved by this method.

Generation of double haploids (DHs) by anther or microspore culture offers a method for rapid production of homozygous lines. Androgenesis is the most commonly used technique for the production of DHs by which embryogenesis can be initiated directly from microspores or pollen

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grains (Pechan & Smykal, 2001). It has a great potential in producing double haploid lines and the inbreeding cycle can be shortened to one or two years within a single step. Furthermore, haploid lines express recessive genes and mutant genes that could be useful for incorporation into plant breeding programs.

Following the first report of anther culture by Guha and Maheshwari (1964), production of haploids by androgenesis has been reported in more than 250 plant species, belonging to 100 genera and 40 families (Ochatt and Zhang, 1996). The technique is successful with many annual crop species.

Even though, androgenesis has been reported in many plant species, (Ochatt and Zhang, 1996) it has limited success in woody species (Peixe *et al.* 2004). Very few studies are reported on coconut anther culture. According to Kovoor (1981) only one out of thousands of coconut anthers cultured developed callus. Iyer (1981) obtained multi-celled pro-embryoids in cultured anthers but these structures failed to develop further. According to Thanh-Tuyen and de Guzman (1983), development of embryos from pollen was observed in cultured coconut anthers at a very low frequency of less than 1% and these embryos failed to develop further. Monfort (1985) obtained anther-derived embryos (with root tip and a leaf primordium) at an extremely low frequency. Further development of the embryos into plantlets was not reported. Thus according to these reports, a successful anther culture protocol for coconut has not been developed. The present study was undertaken with the view of developing a protocol for the production of double haploids *via* anther culture of coconut.

MATERIALS AND METHODS

Anthers excised from male flowers of adult coconut palms of the cultivar Sri Lanka Tall were used as explants. The developmental stage of each inflorescence is determined by its position within the crown of the palm. The interval between splitting of two spadices to open the inflorescence is generally four weeks. The maturity stage of the inflorescence from which samples were collected was based on the age of the spadix, in terms of number of weeks before splitting of the spadix. For collection of samples, palms with newly opened inflorescences (0 stage) were selected. The inflorescence to open next, termed -1 inflorescence was forced open and rachillae were collected. The middle portions of rachillae were given a temperature pre-treatment of 38 °C for six days. Then the male flowers were detached and anthers were excised from the filaments. The anthers were surface sterilised by agitating in 2% (w/v) calcium hypochlorite solution under aseptic conditions for 12 min followed by four rinses with sterile distilled water.

Modified Eeuwens Y_3 liquid medium (Fernando and Gamage, 2000), used as the basal medium, was supplemented with 100 µM 2,4-D, 9% (w/v) sucrose and 0.1% (w/v) activated charcoal (BDH acid washed). Ten anthers were cultured in petri plates (100 x 10 mm), each containing 25 ml of culture medium. Cultures were maintained in the dark for nine months at 28 °C. The calli and embryoids produced were sub-cultured into the somatic embryo induction medium (modified Eeuwens Y_3 solid medium with 66 µM 2,4-D) for four weeks followed by somatic embryo maturation medium (modified Eeuwens Y_3 solid medium without any hormones) for four weeks. The embryogenic structures were then transferred and maintained in germination medium (modified Eeuwens Y_3 solid medium supplemented with 5 µM BAP, 0.1 µM 2,4-D and 0.35 µM GA_3). After embryo germination, the cultures were exposed to light (16 h photoperiod; PAR 25 µ $molm^{-2} s^{-1}$).

Histological analysis was conducted, according to Perera *et al.* (2007), to identify the origin of the anther derived embryoids/calli.

The ploidy level of the anther-derived calli/embryoids and plantlets was determined by flow cytometry (Perera *et al.*, 2007). Each sample was measured in two replications, using leaves of embryo-

cultured Sri Lanka Tall coconut palms as the diploid control and hexaploid *Hieracium* as the known standard. Further, diploid plantlets derived from anthers were tested by Simple Sequence Repeats (SSR) marker analysis for homozygosity. Genomic DNA was extracted from leaf tissues and PCR was performed (Perera *et al.*, 2003). One of the SSR molecular markers that was heterozygous for the mother palm was selected to test the diploid plantlets for homozygosity. After performing PCR, each sample (10 µl) was electrophoresed on a 6% (w/v) polyacrylamide gel and the banding patterns of the samples were compared with the donor palm.

RESULTS AND DISCUSSION

Pollen embryogenesis was successfully induced in coconut for the first time. Both direct and callus-mediated embryogenesis was observed consistently under the culture conditions employed. Induction of embryogenic structures was observed after three months of culture initiation and continued up to eight months indicating that the potential for pollen embryogenesis lasts for a considerable period of time. The peak response of anthers was observed five months after culture initiation (Fig. 1). Formation of both embryoids and calli was observed under the same culture conditions.

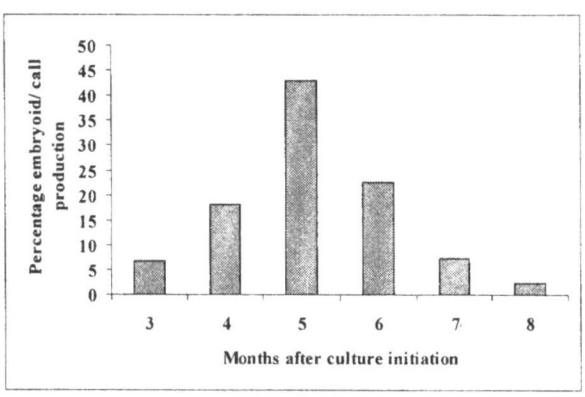

Figure 1. Embryoid/ calli production from anthers over a period of 3-8 months after culture initiation

During direct embryogenesis, the embryoids (Fig. 2a) emerged either through the groove of the anther lobe or by breaking through the anther wall. Embryoids at different developmental stages were observed on the same anther. The mature embryoids were white and opaque with heart or round shape, whereas the immature embryoids were translucent and round in shape. Once the emerging immature embryoids dropped into the medium they formed calli. Calli were off- white and translucent with a frilly appearance (Fig. 2b). The calli/ embryoids were produced at a frequency of 22%.

Upon sub culturing to somatic embryo induction medium, calli, consisting of

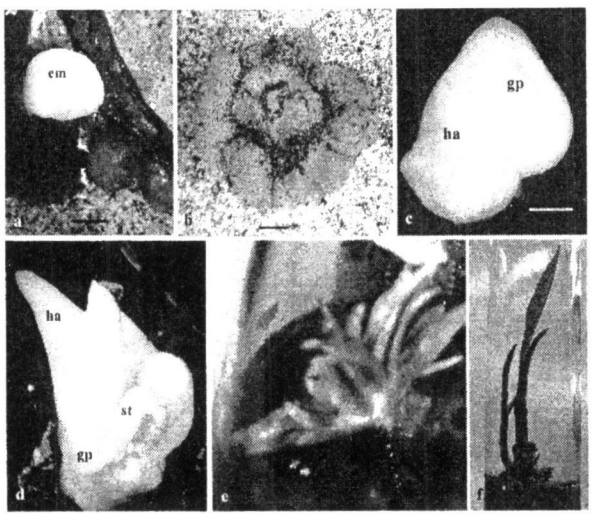

Figure 2. Coconut anther culture. **a.** An embryoid formed on anther (the anther is floating on the liquid culture medium) (Bar=2 mm) **b.** Formation of callus in an embryoid which dropped into the androgenesis induction medium (Bar=0.5 mm) **c.** A developing embryo in hormone free culture medium (Bar=1 mm) [the germination point (gp) is oriented in the haustorium (ha). **d.** Emerging shoot (st) through the germination point (Bar=2 mm) **e.** Germinated embryo **f.** Anther-derived plantlet

translucent masses of globules gave rise to somatic embryos, but at a relatively low regeneration capacity. In contrast, 43% of the well defined, mature, opaque embryoids (Fig. 2c) germinated directly (Fig. 2d) and produced shoots (Fig. 2e). Some of the germinated embryos gave rise to complete plantlets (Fig.2f) after repeated sub-culture to germination medium.

Through histological studies, the pollen-derived pro-embryoids at different stages of development were identified. The results confirmed that the origin of the em-

bryoid/ calli was from the pollen grains. Histological sections revealed that the embryoid is connected to the anther wall by a tiny structure that is only several cell layers in thickness (Fig. 3). Easy removal of embryoids /calli from the anther indicated that it is loosely connected to the tissues inside. Histological sections further illustrated the degeneration of the cells in the anther wall and tapetum that indicates the loss of totipotency in those cells (Fig. 3). This suggested that the calli/ embryoids produced, originated from the viable pollen grains in the pollen sacs of the anthers.

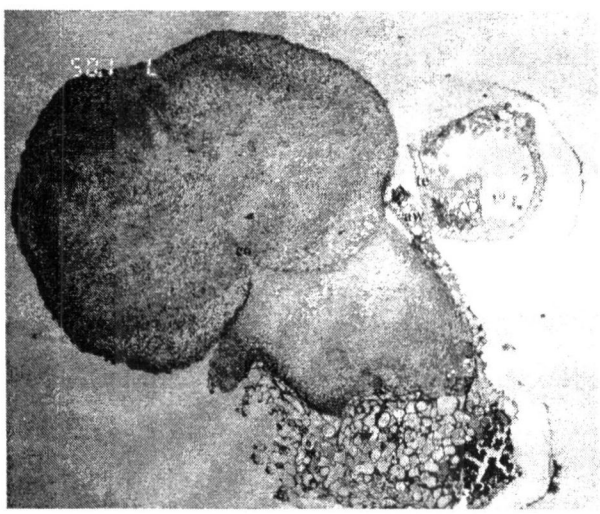

Figure 3. A section of an embryoid developing through the anther wall. Note the tiny connector (Co) which is several cell layers in thickness and the degenerating cells of the tapetum (te) (Bar=200μm).

Ploidy analysis revealed that of the 20 plantlets tested, 50% were haploid and the rest were diploid. Haploid state indicates that the origin of the calli/ embryoids and plantlets were from the pollen grains of the anthers also supported by histological studies. Ten anther-derived diploid coconut plantlets were analysed using SSR markers. SSR loci were used to distinguish between homozygous and heterozygous plantlets among anther-derived diploids. A single primer with the segregating allele in the donor palm is sufficient for distinguishing the population since all the analyzed structures have been derived from the pollen of the same donor palm. The primer CNZ 43 that was found to generate distinguishable and polymorphic fragments, indicating the dominant and recessive alleles, was selected for testing the segregation pattern of the anther-derived structures. No heterozygous diploids were found in the samples tested and all were DHs. The results indicated that diplodization has occurred by spontaneous doubling of chromosomes in anther derived plantlets. Thus the protocol developed in the present study is shown to be effective in producing DH plants of coconut.

In woody species, haploid plant production from anthers or isolated microspores has a very low success (Peixe *et al.*, 2004). The formation of callus from cultured anthers has been reported only in a few species such as apricot (*Prunus armenica*), but plant regeneration has not been achieved (Peixe *et al.*, 2004). Thus, production of double haploids in a woody monocot crop species like coconut can be considered as a significant achievement.

CONCLUSIONS

The present study indicated the feasibility of developing an anther culture protocol for DH plant production in coconut. Histological studies revealed that the origin of calli/ embryoids were from the pollen grains. Ploidy analysis revealed that some of these structures were haploid whereas the others were diploid. SSR marker analysis revealed that all the tested plantlets containing diploid chromosome complement were DHs.

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