Plant Cell Tiss Organ Cult (2008) 92:293–302
DOI 10.1007/s11240-008-9337-5

ORIGINAL PAPER

Androgenic potential in coconut (*Cocos nucifera* L.)

P. I. P. Perera · V. Hocher · J.-L. Verdeil · H. D. D. Bandupriya · D. M. D. Yakandawala · L. K. Weerakoon

Received: 10 October 2007 / Accepted: 16 January 2008 / Published online: 30 January 2008
© Springer Science+Business Media B.V. 2008

Abstract Conditions for induction of androgenesis in coconut cv. Sri Lanka Tall were studied. Anthers collected from inflorescences at four maturity stages were given heat (38°C) or cold (4°C) pretreatments for 1, 3, 6 and 14 days, either prior to or post inoculation. Three different basal media and different anther densities were also tested. Androgenesis was observed only in anthers collected from inflorescences 3 weeks before splitting (WBS) and after a heat pretreatment at 38°C for 6 days. Modified Eeuwens Y_3 liquid medium supplemented with 100 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 0.1% activated charcoal and 9% sucrose was effective in inducing an androgenic response. The lowest anther density tested, 10 per petri plate, was found to be the optimal density. When androgenic calli or embryos were subcultured to Y_3 medium containing 66 μM 2,4-D, followed by transfer to Y_3 medium without plant growth regulators and finally to Y_3 medium containing 5 μM 6-benzyladenine (BA) and 0.35 μM gibberellic acid (GA_3), plantlets regenerated at a frequency of 7%. Histological study indicated that the calli and embryos originated from the inner tissues of the anthers. Ploidy analysis of calli and embryos showed that they were haploid. This is the first report of successful androgenesis yielding haploid plants from coconut anthers.

Keywords Anther culture · Androgenesis · Callus · Embryos · Flow cytometry · Histology

Abbreviations

BA	Benzyladenine
CLZ	Cambium like zone
2,4-D	2,4-dichlorophenoxyacetic acid
GA_3	Gibberellic acid
NBB	Naphthol blue black
PAS	Periodic acid Schiff's reaction
WBS	Weeks before splitting

P. I. P. Perera (✉) · H. D. D. Bandupriya · L. K. Weerakoon
Tissue Culture Division, Coconut Research Institute, Lunuwila 61150, Sri Lanka
e-mail: prasanthi1970@yahoo.com

V. Hocher
Institute for Research and Development (IRD), UMR 1098 BEPC, IRD, BP 64501, 911 Av. Agropolis, Montpellier Cedex 1 34394, France

J.-L.Verdeil
CIRAD, TA40/02 Avenue Agropolis, Montpellier Cedex 5 34398, France

D. M. D. Yakandawala
Department of Botany, Faculty of Science, University of Peradeniya, Peradeniya, Sri Lanka

P. I. P. Perera · D. M. D. Yakandawala
Post-Graduate Institute of Science, University of Peradeniya, Peradeniya, Sri Lanka

Introduction

The coconut palm *(Cocos nucifera* L.), a monocot, is grown as a plantation crop in more than 90 countries for many uses. The tall cultivars are allogamous and exhibit wide variation. Problems encountered with conventional breeding of coconut are its long life span and high heterozygosity that make plant breeding a long, difficult and expensive process. Production of homozygous lines will have a tremendous impact on generating new cultivars through breeding programs. In order to obtain homozygous lines of coconut, breeders need to use self-fertilization or backcrossing that may take 60 years by conventional methods.

Androgenesis has been reported in more than 250 plant species, belonging to 100 genera and 40 families (Ochatt and Zhang 1996). However, in woody species, androgenesis has had only limited success (Peixe et al. 2004). The developmental stage of the microspores is a critical factor that determines the success of anther culture (Peixe et al. 2004; Konieczny et al. 2003; Raina and Iyre 1983). Various stress treatments have been necessary to block gametophytic development and trigger pollen embryogenesis in microspores (Reynolds 1997).

Few studies have been conducted on coconut anther culture. Kovoor (1981) observed callus formation in cultured coconut anthers at a low frequency, while Iyer (1981) obtained many-celled anther-derived proembryos that, however, failed to develop further. Thanh-Tuyen and de Guzman (1983) reported the development of embryos from pollen in cultured anthers at less than 1%. Likewise, these embryos showed no further development. Monfort (1985) obtained a few anther-derived embryos exhibiting root tips and leaf primordia, but these structures did not develop into plantlets. The present study was undertaken to optimize techniques to develop haploid plants of *Cocos nucifera* by anther culture.

Materials and methods

Culture media preparation and in vitro culture conditions

Modified Eeuwens Y_3 medium (Fernando and Gamage 2000) was used as the basal medium (unless otherwise stated). This was supplemented with 100 μM 2,4-D and 9% (w/v) sucrose. The pH was adjusted to 5.8, after adding 0.1% (w/v) activated charcoal (BDH acid washed) and the media were autoclaved at 121°C for 20 min. This liquid medium was used as the standard culture medium for induction of androgenesis in all the experiments unless otherwise stated. Ten anthers (unless otherwise stated) were cultured in petri plates (100 × 10 mm), each containing 25 ml of culture medium. The petri plates were sealed with Parafilm and incubated in the dark at 28°C for 8 months.

Induction of androgenesis

Microspore developmental stage

Anthers excised from male flowers of 15–20 year old coconut palms (cultivar Sri Lanka Tall) were used as the explants. In order to determine the most responsive microspore developmental stage for androgenesis, we selected anthers at different stages of development as described below.

In coconut, a new inflorescence emerges at the tip of the crown every 4 weeks. After emergence, the spathe splits open due to the expansion of the inflorescence. We designated a newly split open inflorescence as stage 0 and the next one immediately below it, that had not yet emerged, as stage −1. Palms bearing stage 0 inflorescences were selected and rachillae bearing male flowers were excised by opening the stage −1 inflorescence. The rachillae were excised again three more times, at weekly intervals, from the same inflorescence. The rachillae were excised from inflorescences at 4, 3, 2 and 1 week before splitting (WBS) and the developmental stages of anthers at the times of excision were designated as 4-WBS, 3-WBS, 2-WBS and 1-WBS, respectively (Fig. 1).

Anther pre-treatments

Cold or heat pre-treatments were applied either before or after inoculation of anthers in the culture medium as described below. The middle portion of the excised rachillae (Fig. 2a and b) bearing male flowers (Fig. 2c), in all four maturity stages, 1-WBS

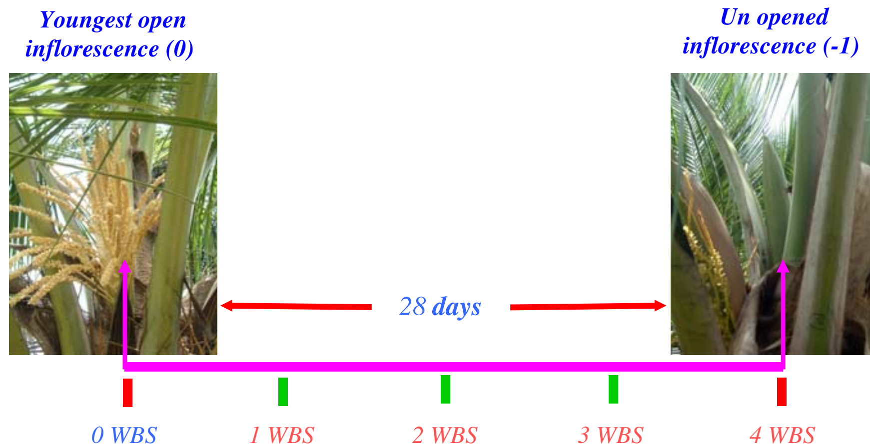

Fig. 1 Sample (male flowers) collection from −1 inflorescence at different stages. Time interval between splitting of two consecutive inflorescences is approx. 4 weeks (28 days). The date of opening of the youngest inflorescence (0 stage) was recorded. Then the −1 inflorescence (that would be expected to open next) was forced open and rachillae were collected at weekly intervals for 4 consecutive weeks. Thus rachillae collected during the 1st, 2nd, 3rd and 4th week belong to 4-, 3-, 2- and 1-WBS stages, respectively

to 4-WBS, was wrapped in wet tissue paper and then in aluminium foil and placed in a refrigerator at 4°C or in an incubator at 38°C for 1, 3, 6 or 14 days in the dark. As a control rachillae were kept at room temperature for the same duration. Anthers were next excised from the filaments (Fig. 2d and e) and surface sterilized by immersion in 2% (w/v) calcium hypochlorite with a few drops of liquid detergent, for 12 min followed by four rinses with sterilized water under aseptic conditions. Anthers were cultured in petri plates each containing 25 ml of standard liquid culture medium mentioned above.

Another set of anthers was cold or heat pre-treated after inoculation by exposure to 4 or 38°C respectively for 1, 3, 6 or 14 days in the dark. Anthers kept continuously at room temperature in the dark were

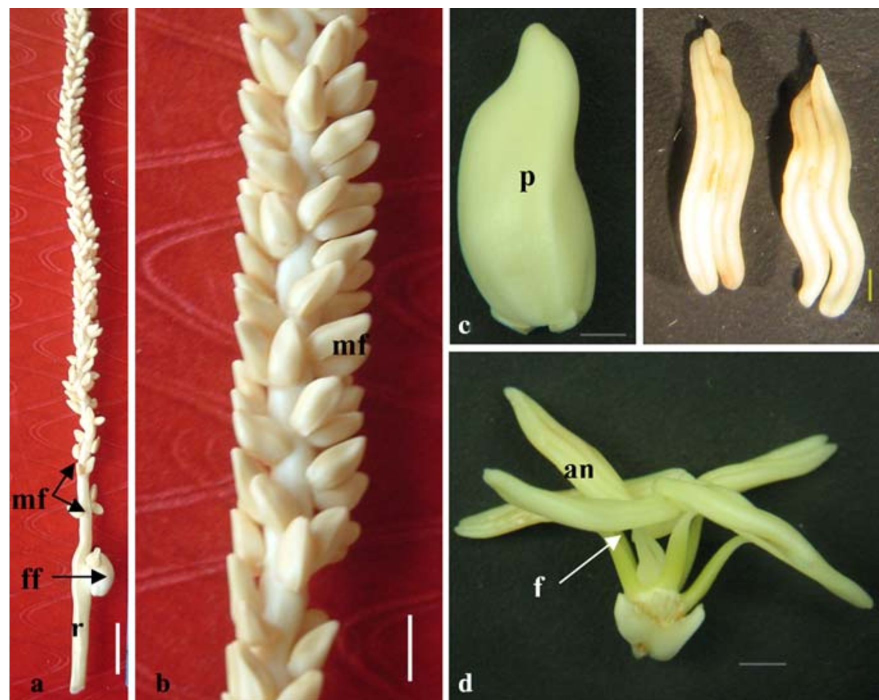

Fig. 2 Isolation of anther from the male flowers of inflorescence at −1 stage (**a**) Isolated rachilla (r) containing male (mf) and female flowers (ff) (Bar = 3cm) (**b**) Close view of the male flowers attached to the middle portion of the rachilla (Bar = 1 cm) (**c**) Isolated male flower. Anthers are totally covered by the petals (p) (Bar = 1.7 mm) (**d**) Anthers (an) attached to the filaments (f) (Bar = 2 cm) (**e**) Excised anthers (Bar = 1.3 mm)

used as a control. After these pre-treatments, all the petri plates were incubated in the dark at 28°C±1 for 8 months.

Culture medium

Three different basal media namely, modified Eeuwens Y_3 medium, MS medium (Murashige and Skoog 1962) and CRI 72 medium (Karunaratne and Periyapperuma 1989) were each supplemented with 9% (w/v) sucrose and 100 μM 2,4-D. Anthers at developmental stage 3-WBS subjected to a heat pre-treatment at 38°C for 6 days, were cultured in ten replicate petri plates containing these three media in liquid form.

Anther density

The number of anthers per Petri plate was varied by culturing 10, 20, 30, 40 or 50 anthers per petri plate in five replicates for each anther density. Anthers at 3-WBS stage, pre-treated at 38°C for 6 days were used. After surface sterilization anthers were cultured in standard culture medium as described above.

Induction of embryogenesis and plant regeneration

The calli and embryos that developed were subcultured after 1 month in modified Eeuwens Y_3 medium with 66 μM 2,4-D for 4 weeks followed by transfer to modified Eeuwens Y_3 medium without growth regulators for 4 weeks. The calli/embryos were next transferred to modified Eeuwens Y_3 medium supplemented with 5 μM BA, 0.1 μM 2,4-D and 0.35 μM GA_3 to induce conversion. All these media were solidified with 0.55% (w/v) agar.

Histological observations

Anthers bearing calli or embryos in standard culture medium (as described above) after 3 months of inoculation were sampled for histological studies. The samples were fixed in FAA solution (50% ethanol:10% formaldehyde:glacial acetic acid = 18:1:1, v/v/v) for 72 h. Next the samples were dehydrated in a graded ethanol series, impregnated, embedded in resin, Technovit 7100® (Heraeus Kluzer GmbH, Germany) to polymerise overnight at room temperature according to a protocol developed by Perera et al. (2007). Sections of 3.5 μm thickness were cut using a microtome (Historange, LKB), double stained with periodic acid Schiff's reagent (PAS) and protein-specific naphthol blue black (NBB) (Fisher 1968) as described by Buffard-Morel et al. (1992). The prepared slides were observed under a light microscope (Leitz DMR, Germany).

Ploidy analysis

Ploidy of calli or embryos obtained 3 months after culture initiation was analysed. Extraction of nuclei and the analysis were done according to the protocol developed for coconut by Sandoval et al. (2003). The ploidy was determined using a FACScan cytometer (Becton—Dickinson, USA) with an argon laser (15 mV) at 488 nm encompassing an emission range of greater than 590 nm. Calli and embryos (approx. 500 mg) were chopped in 2 ml of extraction buffer (Dolezel et al. 1989) containing 3% triton to release the intact nuclei. The suspension containing the nuclei was then filtered through a 36 μm cloth mesh filter to eliminate cell debris. The cell nuclei were stained with propidium iodide (P4170, Sigma) by incorporating 100 μl of propidium iodide stock solution (at 1 mg ml^{-1}) in 300 μl of filtered nucleus suspension. The solution was incubated for 5 min prior to analysis. Ten embryos/callus were analysed. Each sample was measured in two replicates, using leaves of embryo-cultured Sri Lanka Tall coconut palms as the diploid control (internal reference). Ploidy was determined by calculating the ratio of the peaks of the sample and the reference.

Statistical analysis

After 3 months of culture initiation and thereafter, anther-derived structures (calli and embryos) were counted and recorded. The androgenic frequency was calculated as the number of calli/embryos produced per 100 anthers cultured and the data were analysed

using SAS statistical package (SAS Institute 1999). Chi-square or maximum likelihood ANOVA 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

Pollen developmental stage and anther pre-treatment

Androgenesis was induced only in anthers at 3-WBS stage after a heat pre treatment at 38°C for 6 days. These anthers developed calli or embryos at a frequency of 22%. None of the other pre-treatments or other anther developmental stages responded. Embryos emerged from the anthers whereas calli developed from nodules in contact with the culture medium.

The developmental stage of pollen is known to be critical for androgenesis (Zheng 2003). A previous study established the presence of late uninucleate microspores in anthers at 3-WBS stage in inflorescences of Sri Lanka Tall coconut under local environmental conditions (Perera 2003). Anthers bearing late uninucleate microspores have been reported to be optimal for induction of androgenesis in many crop species (Thanh-Tuyen and de Guzman 1983) as found in the present study for coconut.

According to Thanh-Tuyen and de Guzman (1983), anthers at 4-WBS stage with microspores just prior to, during, or immediately after the first pollen mitosis were the most responsive for androgenesis in the coconut cv. Laguna Tall. Despite the difference in the age of the spadices (i.e., 3-WBS or 4-WBS), from which explants were collected, the developmental stage of microspores contained within them seems similar in both cultivars, Sri Lanka Tall and Laguna Tall. The difference in genotype and environmental conditions may have contributed to the variation in splitting of inflorescences in the two cultivars. In other studies reported on coconut anther culture, anthers at first pollen mitosis (Monfort 1985), tetrad stage (Kovoor 1981) and uninucleate stage (Iyer 1981) have given rise to embryos or calli at a low frequency.

In many crops, anther pretreatments have triggered induction of an androgenic response (Zheng 2003). Cold pretreatment has been effective in barley (*Hordeum vulgare*; Hou et al. 1993; Devaux et al. 1993), rye (*Secale cereale*; Immonen and Anttila 1999), wheat (*Triticum aestivum*; Shimada 1981; Konieczny et al. 2003), timothy (*Phleum pratense*; Guo et al. 1999), soybean (*Glycine max*; Rodrigues et al. 2005) and several other crops. High frequency of callus development has been reported in cultured anthers of apricot (*Prunus armeniaca*; Peixe et al. 2004), cabbage (*Brassica oleracea*; Achar 2002), cow cockle (*Saponaria vaccaria*; Kernan and Ferrie 2005) and broccoli (*Brassica oleracea*; Arnison et al. 1990) after applying thermal shocks.

In the present investigation the heat pretreatment that induced androgenesis is comparable to that of Monfort (1985) where a pretreatment of anthers of coconut at 35°C for 1 week, induced development of embryos at a low frequency. Thanh-Tuyen (1985) observed androgenesis in coconut anthers pretreated at 7°C for 7 days or 14°C for 4 days. However, the cold pretreatment given to anthers at 4°C for 1, 3, 6 and 14 days did not induce androgenesis in our study.

Culture medium

The culture medium which provides both nutrients and osmoticum is a key factor that affects androgenesis. Of the three basal media tested, development of embryos and calli at a frequency of 21% was observed only in modified Eeuwens Y_3 medium.

The basal medium CRI 72 has been formulated specifically for coconut tissue culture (Karunaratne and Periyapperuma 1989) and used successfully for callus induction and somatic embryogenesis in explants of coconut such as immature embryos (Fernando and Gamage 2000), plumules (Fernando et al. 2003) and ovaries (Perera et al. 2007). However, it did not induce a positive response in cultured anthers of coconut. The modified Eeuwens Y_3 medium has a higher amino acid (asparagine, glutamine and arginine) content as well as more NH_4Cl, KCl and NiCl compared to CRI 72 medium. This could have contributed to the positive response of anthers cultured in Y_3 medium. It has been reported that glutamine has a stimulatory effect on androgenesis (Guo et al. 1999). The anther culture

medium used by Monfort (1985) also contained high glutamine.

MS medium has been used as the basal medium for induction of androgenesis in many crop species, such as pepper (*Capsicum annuum*; Kim et al. 2004), summer squash (*Cucurbita pepo;* Metwally et al. 1998), rice (*Oryza sativa*; Wong et al. 1983) and asparagus (*Asparagus officinalis*; Peng and Wolyn 1999), even though it was not effective in inducing an androgenic response in cultured anthers of coconut.

The synthetic auxin 2,4-D is reported to be the most effective plant growth regulator to induce androgenesis in many crops, including barley (Zheng and Konzak 1999) and wheat (Ball et al. 1993). Furthermore, it has been effective in inducing callogenesis in different explants of coconut (Weerakoon 2004; Verdeil et al. 1989; Ebert and Taylor 1990). Activated charcoal is another component that has been essential in coconut tissue culture media (Verdeil et al. 1989; Fernando and Gamage 2000; Fernando et al. 2003; Perera et al. 2007). The beneficial effect of activated charcoal is attributed to its adsorption of phenols and other growth inhibitory substances. In anther culture, activated charcoal is reported to remove toxic substances released by non-reactive microspores allowing more embryogenic cells to develop (Chatelet et al. 1999). The optimum concentration of 2,4-D in the culture medium varies with the adsorption capacity of the activated charcoal which also adsorbs 2,4-D (Ebert and Taylor 1990). Successful callogenesis thus requires an optimal combination of 2,4-D and activated charcoal in the culture medium. In the present investigation we used only one level of 2,4-D (100 μM); varying the 2,4-D concentration may lead to more efficient callus or embryo formation. The use of elevated levels of sucrose has been beneficial for pollen embryogenesis in many crops (Konieczny et al. 2003; Ishizaka 1998) as with the anther culture medium used in the present study (9%).

Anther density

The number of anthers per petri plate ($G^2 = 9.26$, $P < 0.05$) had a significant effect on androgenesis (Table 1). The number of calli or embryos that developed was significantly greater at a density of ten anthers compared to 30 ($G^2 = 6.09$, $P < 0.05$) and 50 ($G^2 = 6.74$; $P < 0.05$) whereas no androgenic response was observed at a density of 40 anthers. At higher anther densities, browning of both medium and anthers was observed. The embryogenic potential of the calli or embryos formed at higher anther densities (30–50) was low and they all turned brown at an early stage of development. Cultured anthers release endogenous hormones and substances that may affect embryogenesis (George 1993). Thus the density of anthers in the culture vessel could play an important role in embryogenesis.

Table 1 The effect of anther density on the production of calli/embryos in cultured anthers of coconut

Anther density (No. of anthers per petri plate)	Number of calli/embryos produced per 100 anthers[a]
10	7.9
20	2.5
30	1.6
40	0.0
50	1.8
Contrast	Chi-square
10 vs. 30	6.09*
10 vs. 50	6.74*

[a] Mean of five replicates

Maximum likelihood analysis of variance was significant at $G^2 = 9.26$, $*P < 0.05$

Only the significant contrasts were listed in the table

Zhao et al. (2006) reported that anther density did not significantly affect androgenesis. Arnison et al. (1990) indicated that in broccoli, even though the percentage of responding anthers decreased at lower anther densities, the number of embryos formed per responsive anther was independent of anther density. It was further reported that the optimum anther density for broccoli was 60–120 per 20 ml of culture medium. Embryo formation may be dependent on physical and biochemical factors; at low anther density, a few anthers often remain separated in the medium, whereas at greater anther density, loose groupings and associations may be maintained throughout the culture period (Arnison et al. 1990). In a study conducted in barley, Dunwell (1985) also reported that in liquid medium, at least 60 anthers need to be cultured per ml of medium. In a recent study, Zhao et al. (2006) indicated that there was no significant difference in callus production among the

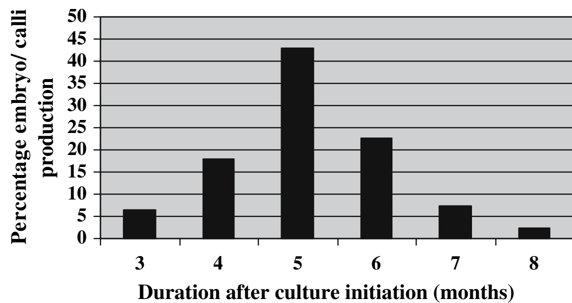

Fig. 3 Percentage embryo/callus production from cultured anthers of coconut 3–8 months after culture initiation

cultures with various densities in purple coneflower (*Echinacea purpurea* L.). The use of petri dishes for anther culture with a shallow liquid phase allows sufficient surface tension to keep the anthers floating on the medium. Furthermore, it also provides highly aerobic conditions for embryo development that could be beneficial in regeneration of green plants (Guo et al. 1999).

Embryogenesis and plantlet regeneration

Induction of callus or embryos was observed after 3 months of culture initiation and continued up to 8 months. After 8–9 months, the embryogenic potential of the anthers diminished, probably due to loss of pollen viability. The peak response of anthers was observed 5 months after culture initiation (Fig. 3).

Both embryos and calli developed on the same culture plate. The embryos were heart-shaped or round and opaque white (Fig. 4a, e). Subculturing embryos onto medium with low 2,4-D followed by

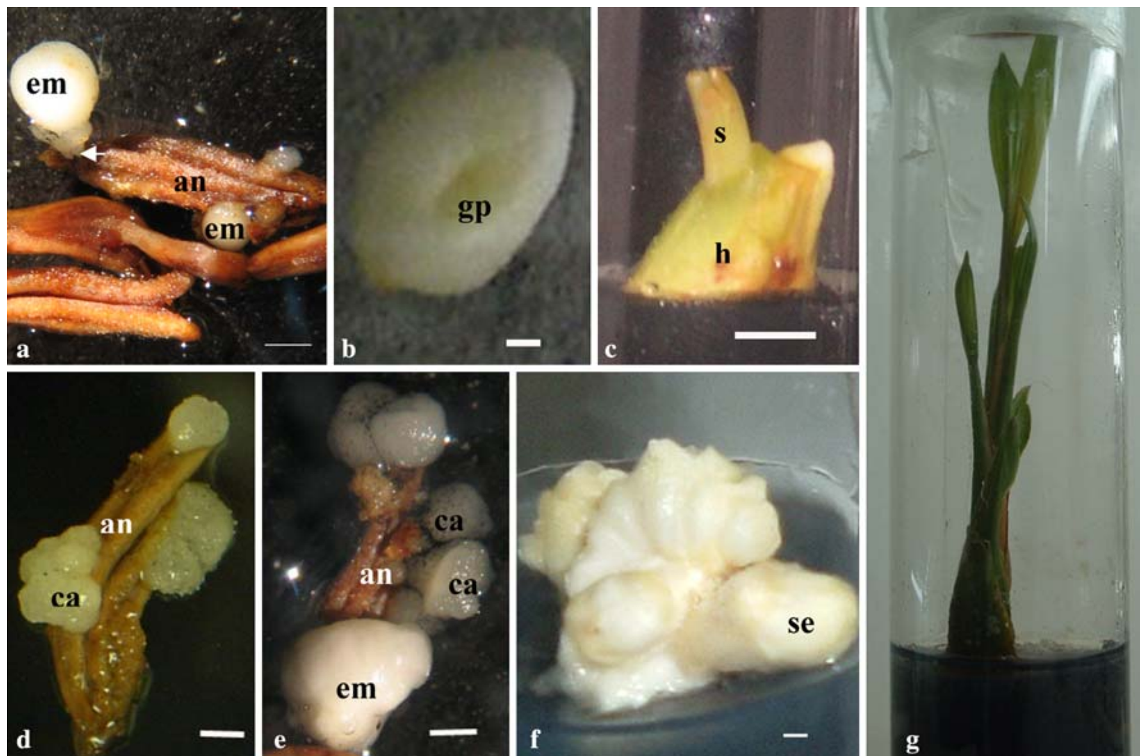

Fig. 4 Plant regeneration via anther culture of coconut (*Cocos nucifera* L.). (**a**) Embryos (em) derived from anther (an). Note the tiny connector (arrow head) between the embryo and the anther wall (Bar = 220 µm). (**b**) Mature embryo with germination point (gp) (Bar = 777 µm). (**c**) Germinating embryo with a shoot (s) emerging by splitting of haustorium (**h**) (Bar = 1 cm). (**d**) Calli (ca) bearing anther (Bar = 1.1 mm). (**e**) An anther bearing both callus (ca) and embryos (em). Note the embryo is opaque white whereas the callus has a translucent appearance (Bar = 1 mm). (**f**) Somatic embryos (se) developed from anther derived callus (Bar = 1.81 mm). (**g**) A complete plantlet derived from anther

transfer to medium devoid of plant growth regulators led to embryo maturation (Fig. 4b). Mature embryos were opaque and for most of them, the germination point could be observed as a depression on the surface (Fig. 4b). However, in some cases, the shoot emerged by splitting of the haustorial tissue of the mature embryo (Fig. 4c). The calli consisted of translucent masses of globules of an off-white colour (Fig. 4d, e). Callus was induced only if the emerging embryos touched the liquid medium (Fig. 4e). When calli were subcultured to medium with low 2,4-D followed by transfer to medium without growth regulators, they developed into somatic embryos (Fig. 4f) that differed morphologically from mature embryos. Some somatic embryos converted into plantlets (Fig. 4g) at a frequency of 7%.

Plants that have regenerated from androgenic callus or embryos have often been albino in many crops, including wheat (Shimada 1981) and barley (Jacquard et al. 2006). However, albino plants were not observed in the present study.

Histological analysis

The initiation of androgenesis from pollen grains could be observed in the pollen sac within the anthers. The embryogenic pollen grains were characterised by higher nucleus to cytoplasm ratio, with depositions of starch and proteins (Fig. 5a). These cells were similar to proembryos (of unicellular origin) that developed from ovary-derived callus in coconut (Perera et al. 2007). Many degenerate pollen grains could also be found within the pollen sac. Pollen derived proembryos at different stages of development (Fig. 5b, c and d) were present. The multicellular structures developed into proembryos and enlarged to fill the interior of the pollen sac. The proembryo gave rise to embryos by a tiny connection (Fig. 5e).

The emerging embryo consisted of a highly meristematic region referred to as the cambium-like zone (CLZ) (Fig. 5e). When each callus or embryo was maintained in the same androgenesis induction

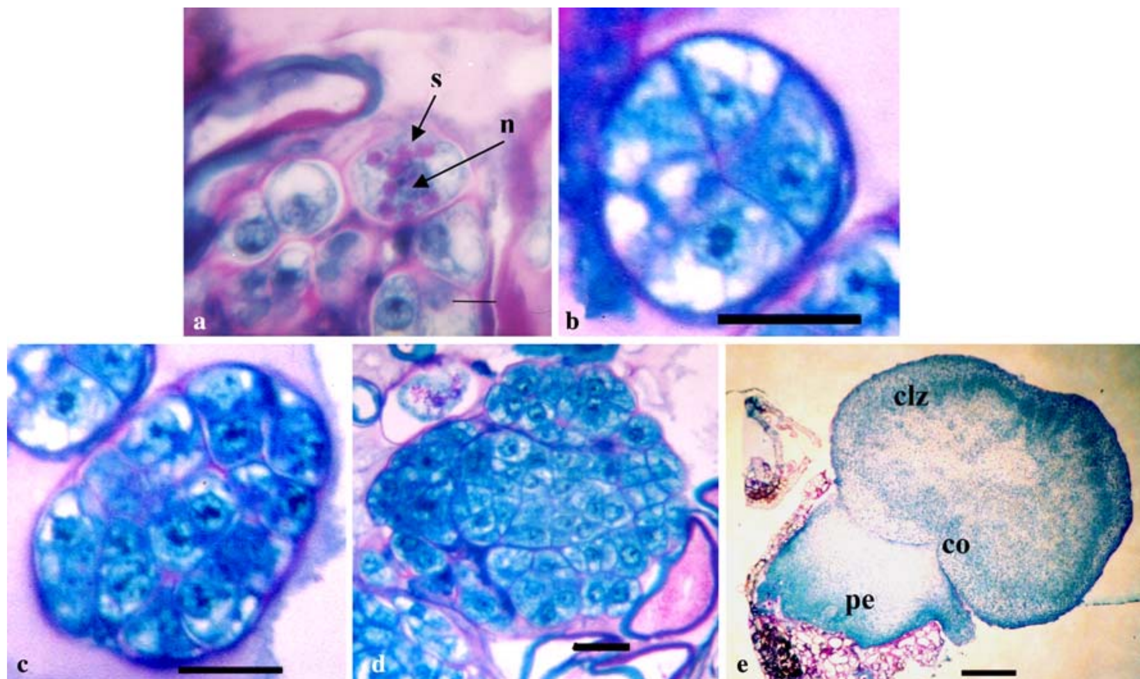

Fig. 5 Histological aspects of embryo and callus formation in cultured anthers of coconut (*Cocos nucifera* L.). (**a**) An embryogenic pollen grain characterized by high nucleus (n) to cytoplasm ratio and high starch (s, in pink color) and protein (in blue color) reserves (Bar = 10 μm). (**b**) Four-celled embryo (Bar = 166 μm). (**c** and **d**) Embryos at different developmental stages (Bar = 166 μm). (**e**) An embryo developing from the proembryo (pe). Note the tiny connector (co) and cambium like zone (clz) (Bar = 200 μm)

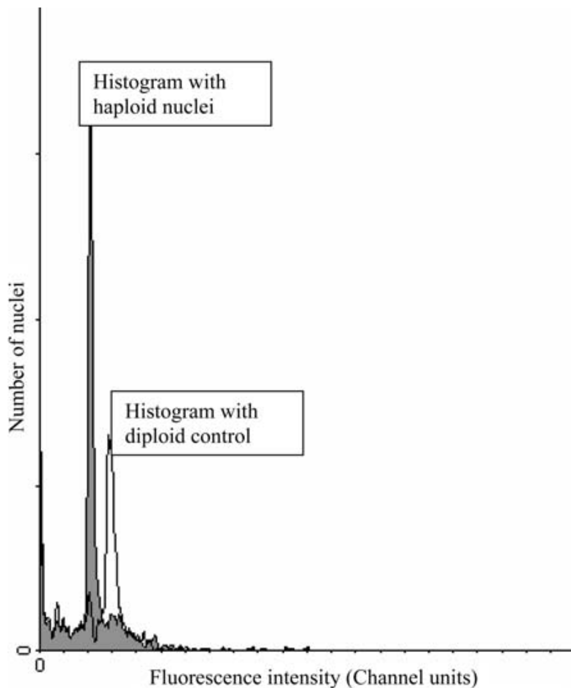

Fig 6 Histogram from flow cytomeric analysis indicates the fluorescence intensity of nuclei isolated from calli/embryos derived from coconut anthers after staining with propidium iodide. Mature leaves (in white) were used as diploid control. Coefficient of variation (CV) was 6.39%. Channel units represent the DNA content of the analyzed nuclei in arbitrary units

medium for another 2–3 weeks, it developed either into an embryo (with differentiation of haustorium, shoot and root poles) or a callus by proliferation of CLZ into more globules.

Easy removal of embryos or calli from the anther indicated that they were loosely connected to the tissues inside. Histological sections revealed that the embryo was connected to the proembryo by a stalk only several cell layers thick (Fig. 5e). Histological sections further illustrated the degeneration of cells in the anther wall and tapetum, indicating that the calli or embryos was likely to have originated only from the viable pollen grains in the pollen sacs.

Flow cytometric analysis revealed that the calli and embryos were haploid ($2n = 1x = 16$) (Fig. 6), confirming their origin from pollen grains. The ploidy was determined by considering the ratio of channel units given for the sample and the internal standard (control). This is further supported by histological evidence described above.

The present study indicated the feasibility of developing an anther culture protocol via pollen embryogenesis with the use of 3-WBS anthers of coconut as the explant. This is the first report of consistent androgenic response in coconut at a relatively high frequency leading to plantlet regeneration.

Acknowledgements Authors acknowledge Mr. C. Duperray (INSERM, Montpellier, France) for the technical assistance in flow-cytometry. We are also thankful to Mr. J. D. J. S. Kularatne for the assistance in statistical analysis. Our special thanks go to Ms. Shantha Ramanayake for reading the manuscript critically.

References

Achar PN (2002) A study of factors affecting embryo yields from anther culture of cabbage. Plant Cell Tissue Organ Cult 69:183–188

Arnison PG, Donaldson P, Ho LCC, Keller WA (1990) The influence of various physical parameters on anther culture of broccoli (*Brassica oleracea* var. *italica*). Plant Cell Tissue Organ Cult 20:147–155

Ball ST, Zhou H, Konzak CF (1993) Influence of 2, 4-D, IAA and duration of callus induction in anther culture of spring wheat. Plant Sci 90:195–200

Buffard-Morel J, Verdeil JL, Pannetier C (1992) Embryogenèse somatique du cocotier (*Cocos nucifera* L.) à partir d'explants foliaires: Étude histologique. Can J Bot 70:735–741

Chatelet P, Gindreau K, Herve Y (1999) Development and use of microspore culture applied to vegetable *Brassica oleracea* breeding. In: Clement C, Pacini E, Audran J-C (eds) Anther and pollen. Springer-Verlag, Berlin, pp 247–259

Compton ME (1994) Statistical methods suitable for the analysis of plant tissue culture data. Plant Cell Tissue Organ Cult 37:217–242

Devaux O, Hou L, Ullrich E, Huang Z, Kleinhof A (1993) Factors affecting anther culturability of recalcitrant barley genotypes. Plant Cell Rep 13:32–36

Dolezel J, Binarova P, Lucretti S (1989) Analysis of nuclear DNA content in plant cells by flow cytometry. Biol Plant 31:113–120

Dunwell JM (1985) Anther and ovary culture. In: Bright SWJ, Jones MGK (eds) Cereal tissue and cell culture. Martinus Nijhoff/Dr W Junk Publishers, Dordrecht, Netherlands, pp 1–44

Ebert A, Taylor HF (1990) Assessment of the changes of 2,4-dichlorophenoxyacetic acid concentrations in plant tissue culture media in the presence of activated charcoal. Plant Cell Tissue Organ Cult 20:165–172

Fernando SC, Gamage CKA (2000) Abscisic acid induced somatic embryogenesis in immature embryo explants of coconut (Cocos *nucifera* L.). Plant Sci 151:193–198

Fernando SC, Verdeil JL, Hocher V, Weerakoon LK, Hirimburegama K (2003) Histological analysis of plant

regeneration from plumule explants of *Cocos nucifera* L. Plant Cell Tissue Organ Cult 72:281–284

Fisher DB (1968) Protein staining of ribboned epon section for light microscopy. Histochemie 16:92–96

George EF (1993) Plant propagation by tissue culture. Part I, the technology. Exegetics Limited, Edington, England

Guo Y-D, Sewon P, Pulli S (1999) Improved embryogenesis from anther culture and plant regeneration in timothy. Plant Cell Tissue Organ Cult 57: 85–93

Hou L, Ullrich SE, Kleinhofs A, Stiff CM (1993) Improvement of anther culture methods for double haploid production in barley breeding. Plant Cell Rep 12:334–338

Immonen S, Anttila H (1999) Cold pretreatment to enhance green plant regeneration from rye anther culture. Plant Cell Tissue Organ Cult 57:121–127

Ishizaka H (1998) Production of microspore derived plants by anther culture of an inter-specific F_1 hybrid between *Cyclamen persicum* × *C. Purpurascens*. Plant Cell Tissue Organ Cult 54:21–28

Iyer RD (1981) In: Rao AN (ed) Proceedings COSTED symposium on tissue culture of economically important plants. National University Singapore, Singapore, pp 219–230

Jacquard C, Asakaviciute R, Hamalian AM (2006) Barley anther culture: effects of annual cycle and spike position on microspore embryogenesis and albinism. Plant Cell Rep 25:375–381

Karunaratne S, Periyapperuma K (1989) Culture of immature embryos of coconut (*Cocos nucifera* L.): Callus proliferation and somatic embryogenesis. Plant Sci 62:247–253

Kernan Z, Ferrie AMR (2005) Microspore embryogenesis and the development of a double haploidy protocol for cow cockle (*Saponaria vaccaria*). Plant Cell Rep 25:274–280

Kim M, Kim J, Yoon M, Choi D, Lee M (2004) Origin of multicellular pollen and pollen embryos in cultured anthers of pepper (*Capsicum annum*). Plant Cell Tissue Organ Cult 77:63–72

Konieczny R, Czaplicki AZ, Golczyk H, Przywara L (2003) Two pathways of plant regeneration in wheat anther culture. Plant Cell Tissue Organ Cult 73:177–187

Kovoor A (1981) Palm tissue culture: state of art and its application to the coconut. FAO Plant production and protection paper 30. FAO, Rome

Metwally EI, Moustafa SA, Ei-Sawy BI, Shalaby TA (1998) Haploid plantlets derived by anther culture of *Cucurbita pepo*. Plant Cell Tissue Organ Cult 52: 171–176

Monfort S (1985) Androgenesis of coconut: embryos from anther culture. Z Pflanzenzuchtg 94:251–254

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Ochatt S, Zhang Y (1996) Haploid plants from pollen grains. Science 163: 85–87

Peixe A, Barroso J, Potes A, Pais MS (2004) Induction of haploid morphogenic calluses from in vitro cultured anthers of *Prunus armeniaca* cv. 'Harcot'. Plant Cell Tissue Organ Cult 77:35–41

Peng M, Wolyn DJ (1999) Improved callus formation and plant regeneration for shed microspore culture in asparagus (*Asparagus officinalis* L.). Plant Cell Rep 18: 954–958

Perera PIP (2003) Cytological examination of pollen development for microspore and anther culture of coconut (*Cocos nucifera* L.) cv Sri Lanka Tall. Cocos 15:53–59

Perera PIP, Hocher V, Verdiil JL, Doulbeau S, Yakandawala DMD, Weerakoon LK (2007). Unfertilised ovary: a novel explant for coconut (*Cocos nucifera* L.) somatic embryogenesis. Plant Cell Rep 26: 21–28

Raina SK, Iyre RD (1983) Multicelled pollen pro-embryoids and callus formation in tea anther culture. J Plant Crops (Supplement):63–67

Reynolds TL (1997) Pollen embryogenesis. Plant Mol Biol 33:1–10

Rodrigues LR, Oliverira JMS, Mariath JEA, Iranco LB, Bodanese-Zanettini MH (2005) Anther culture and cold treatment of floral buds increased symmetrical and extra nuclei frequencies in soybean pollen grains. Plant Cell Tissue Organ Cult 81:101–104

Sandoval A, Hocher V, Verdeil JL (2003) Flow cytometric analysis of the cell cycle in different coconut palm (*Cocos nucifera* L.) tissues cultured *in vitro*. Plant Cell Rep 22:25–31

SAS Institute Inc. (1999) SAS/STAT user's guide, version 7-1. SAS Institute Inc. Cary, North Carolina

Shimada T (1981) Haploid plants regenerated from the pollen callus of wheat (*Triticum aestivum* L.). Jpn J Genet 56:581–588

Thanh-Tuyen NT (1985) Anther culture: its prospects to coconut improvement. Philipp J Crop Sci 10:28–35

Thanh-Tuyen NT, De Guzman EV (1983) Formation of pollen embryos in cultured anthers of coconut (*Cocos nucifera* L.). Plant Sci Lett 29:81–88

Verdeil JL, Buffard-Morel J, Pannetier C (1989) Somatic embryogenesis of coconut (*Cocos nucifera* L.) from leaf and inflorescence tissue. Research findings and prospects. Oleagineux 44:403–411

Weerakoon LK (2004) Coconut tissue and embryo culture in Sri Lanka: current developments and future challenges. In: Peiris TSG, Ranasinghe CS (eds) Proc intern conf coconut res inst Sri Lanka—Part 1 (Review papers and guest presentations). CRI, Lunuwila, Sri Lanka, pp 41–61

Wong C-K, Ko S-W, Woo S-C (1983) Regeneration of rice plantlets on NaCl-stressed medium by anther culture. Bot Bull Acad Sinica 24: 59–64

Zhao F-C, Nilanthi D, Yang Y-S, Wu H (2006) Anther culture and haploid plant regeneration in purple coneflower (*Echinacea purpurea* L.). Plant Cell Tissue Organ Cult 86: 55–62

Zheng MY, Konzak CF (1999) Effect of 2,4-dichlorophenoxyacetic acid on callus induction and plant regeneration in anther culture of wheat (*Triticum asetivum* L.). Plant Cell Rep 19:69–73

Zheng MY (2003) Microspore culture in wheat (*Triticum aestivum*)—doubled haploid production via induced embryogenesis. Plant Cell Tissue Organ Cult 73: 213–230