Variations among Anthers and their Orientation during *in Vitro* Culture for Inducing Androgenesis in Coconut

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ABSTRACT

Tall coconut varieties are allogamous and variation within population is very high. Production of improved coconut hybrids by conventional breeding is hampered by its long life span and high heterozygosity. Production of homozygous lines will accelerate the current breeding program. Induction of microspore embryogenesis is the easiest and quickest way to produce a homozygous line. In the present study the effect of the genotype and anther orientation on androgenesis induction in coconut was investigated. Out of six palms tested, a positive androgenic response was obtained with only one palm. All the anthers cultured in a Petri plate did not elicit an androgenic response and the highest percentage of anthers that showed an androgenic response was 40%. The orientation of anthers did not affect the percentage of responding anthers and the frequency of calli/embryo formation. The calli/embryos emerged from both upper and lower surface of the anthers when they were placed with their abaxial surface 'down'. When the anthers were positioned with their abaxial surface 'up', calli/embryos emerged only from the upper surface. Both the percentage of embryo formation (91.3%) and germination (17.4%) were significantly higher and when the orientation was 'up'. Therefore, it could be suggested that the genotype and the orientation of the anther in culture medium greatly affect the success rate of the anther culture in coconut.

Key words: *Coconut, androgenesis, dihaploids*

INTRODUCTION

Genetic improvement of coconut for increased productivity is a priority research area. The existing variation between the Tall and Dwarf varieties and within the Talls could be used to produce recombinants with desired characters (Batugal and Bourdeix, 2005). Production of new varieties of coconut is difficult due to factors, common to other perennial crops such as inherent heterozygosity, long juvenile phase, inbreeding depression, long life cycle, lack of a good method of vegetative propagation and time and cost limitations (Baudouin *et al.*, 2005). Tall coconut varieties are allogamous and exhibit high variations in desired characters. Production of homozygous lines will have a tremendous impact on coconut breeding to generate new varieties.

Self or back crossing may take a minimum of 60 years to produce lines which are still not 100% homozygous. Generation of double haploids by *in vitro* methods offers a way for rapid production of homozygous lines. Androgenesis is a technique for production of double haploids by induction of embryogenesis in microspores or pollen grains (Peachan and Smykal, 2001). The technique has been successful with a number of annuals such as *Brassica* spp. (Achar, 2002; Arnison *et al.*, 1990), *Triticum aestivum* (Ball *et al.*, 1993; Chu and Hill, 1988), *Oryza sativa* (Chaleff and Stolarz, 1981) and *Hordeum vulgare* (Devaux *et al.*, 1993). However, in woody species, androgenesis is reported to be very low (Peixe *et al.*, 2004). Although callus formation from cultured anthers has been reported in a number of fruit species such as *Prunus armenica* (Peixe *et al.*, 2004) and *Prunus persica* (Hammerschlag, 1983), plant regeneration has not been achieved.

Reports on coconut anther culture showed limited success (Iyer, 1981; Iyer and Raina, 1972; Kovoor, 1981; Thanh-Tuyen and de Guzman, 1983; Monfort, 1985). Perera *et al.* (2008) reported consistent haploid/ doubled haploid plant production via coconut anther culture for the first time. Critical factors that affect androgenesis such as pollen developmental stage, culture medium, stress inducing pre-treatment and anther density in cultures were identified (Perera *et al.*, 2008). This protocol was further refined by studying the effects of growth regulators, physical state of the culture medium and the type and concentration of energy source (un-published).

Genotype of the donor plant is a key factor that controls the potential of androgenic competence (Jones and Petolino, 1987; Achar, 2002). This affects the frequency of embryogenesis, the quality of the embryos and plant regeneration competence (Lionneton *et al.*, 2001). This genotypic effect has also been shown in many other crops such as *Phleum pratense* (Guo *et al.*, 1999), *Brassica juncea* (Lionneton *et al.*, 2001), *Solanum tuberosum* (Wenzel and Uhrig, 1981) and *Avena sterilis* (Rines, 1983). In some cases, hybrids were more responsive than cross pollinated ones as described in *Brassica oleracea* (Achar, 2002). The orientation of anther on culture medium has shown to have an effect on androgenesis in a number of crop species (Shannon *et al.*, 1985; Arnison *et al.*, 1990). Thus the present study was undertaken to determine the effect of the genotype of mother palm and the anther orientation on androgenesis in coconut.

MATERIALS AND METHODS

Androgenesis induction

Male flowers bearing anthers at 3 weeks before splitting (WBS) stage (Perera, 2003; Perera *et al.*, 2008) were used. The developmental stage of each inflorescence is determined by its position within the crown of the palm. The interval between split opening of two spadices 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 split opening of the spadix. For collection of samples, palms with newly opened inflorescences (0 stage) were selected. The -1 inflorescence (that is to be split open next) was forced open and rachillae were collected. Rachillae collected from the selected adult coconut palms of the variety Sri Lanka Tall were incubated at 38°C for six days and surface sterilised as described by Perera *et al.* (2008). Modified Eeuwens Y_3 medium (Karunaratne *et al.* 1985) was supplemented with 100 μM 2,4-D, 9% (w/v) sucrose and 0.1% (w/v) activated charcoal (BDH acid washed, UK) for induction of androgenesis. Ten anthers were cultured in Petri plates (100 x 10 mm), each containing 25 ml of culture medium. Petri plates were incubated in the dark at 28 ±1°C for eight months and kept undisturbed.

Effect of genotype

Genotype effect of the mother palm on androgenesis was evaluated by cultureing anthers collected from six coconut palms (P_1-P_6), located at Bandirippuwa Estate, Lunuwila. Ten Petri plates were used for each genotype (palm). The number of anthers that produced calli/ embryos was recorded.

Effect of anther orientation

Anthers were placed on the culture medium (solidified with 0.65% agar) with their abaxial surface up or down. Three replicates for each treatment were used. The number of anthers bearing embryos/ calli, number of embryos/ calli produced and the number of embryos that germinated were recorded.

Plant regeneration

The calli and embryos were sub-cultured into embryo induction medium with lower level of 2,4-D (66 μM), followed by maturation medium devoid of any growth regulators, and germination medium containing 5 μM 6-benzylaminopurine (BAP), 0.1 μM 2,4-D and 0.35 μM gibberelic acid (GA_3). All culture media were solidified with 0.65% (w/v) agar. Sub-culturing was done at monthly intervals. All the cultures were maintained in dark at 28±1°C until embryos germinated, and they were exposed to light (16 h photoperiod; PAR; 25 μmol m^{-2} s^{-1}) afterwards.

Statistical analysis of data

The androgenic frequency was calculated as the number of calli or embryos produced per 100 anthers cultured. 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 orthogonal contrast coefficients (Compton, 1994).

RESULTS AND DISCUSSION

Effect of the palm

Out of anthers cultured from six palms, the anthers of only one palm (P_3) showed a positive androgenic response. Since coconut is an open pollinated crop, the palm to palm variation is extremely high within a population. It has been suggested that the androgenic potential is a heritable trait (Peachan and Smykal, 2001). Crossing of non-responding genotypes with responding genotypes has resulted in F_1 plants with either high or low androgenic response, indicating a complex inheritance of the trait (Rines, 1983; Wenzel and Uhrig, 1981). This type of crossing in South African wheat germplasm has suggested that several genes may be involved in regulating the androgenic response (Ascough *et al.*, 2006). Torp *et al.* (2000) suggested that regeneration of green plants is due to a heritable trait located at four QTLs within the wheat genome. It is well established that the condition of the mother plant influences the response in anther culture (Bohanec *et al.*, 1993). However, due to the size of the palm, it is quite difficult to control the growth condition of the mother palm in coconut. Thus, further efforts are needed to identify more palms that would elicit an androgenic response. A combination of pre-treatments to the anthers such as starvation and heat stress had induced androgenesis in tobacco (Touraev *et al.*, 1997) and a similar approach could be applied to coconut too.

Exogenous and endogenous phyto-hormone balance influences the regeneration potential. Genotypes which are not too sensitive and which do not require highly balanced phyto-hormone levels are the ones which react more frequently for androgenesis (Wenzel and Uhrig, 1981). Pre-conditioning the culture medium with cultured ovaries or anthers (Zheng *et al.*, 2002; Huang and Keller, 1989) or co-culture of anthers with fresh ovaries (Hu and Kasha, 1997) was beneficial in inducing pollen embryogenesis in recalcitrant genotypes. The response of isolated microspores of recalcitrant cultivars in barley has been largely improved by utilizing ovary co-cultivation (Li and Devaux, 2001). The stimulatory effect could be attributed to readily available nutrients or 'nurse factor', accumulated in the ovary-conditioned medium (Zheng *et al.*, 2002). Such attempts will be useful in improving the currently developed anther culture protocol in coconut. It is also pertinent to study the androgenic response in different varieties of coconut.

Variation in anther response

The results also revealed that only some anthers produced embryos/ calli. The highest percentage of anthers (per Petri plate) that responded to androgenesis was 40% (Fig. 1). This was comparable to the results obtained by Rodrigues *et al.*, (2005) who attributed it to the physiological condition of the explants at the beginning of culture.

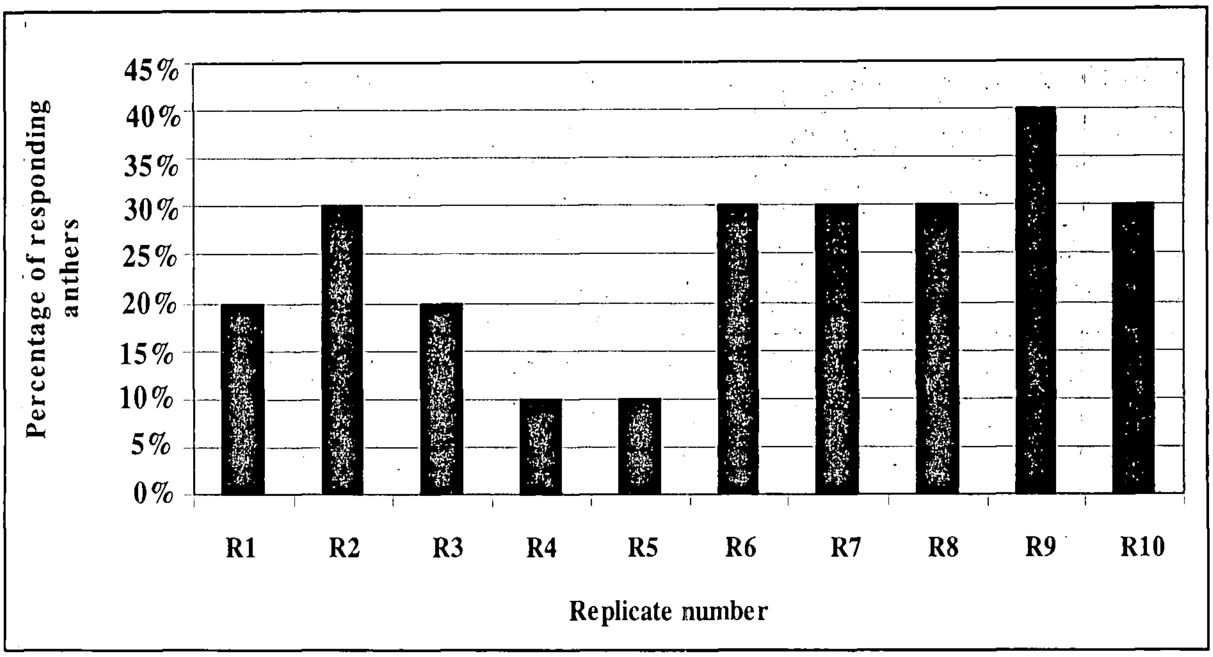

Fig. 1. *Variation in anther response to androgenesis in a coconut palm.*

A maturity gradient exists in the male flowers along the rachillae. Even though the male flowers attached to the middle portion of the rachilla were used for *in vitro* culture, there could still be a variation in their pollen developmental stage. This may have affected their response to androgenesis. The inflorescence that was used to obtain anthers was selected based on the maturity of the youngest opened inflorescence. As described earlier, most suitable developmental stage of pollen grains for the induction of androgenesis was in anthers obtained from inflorescence at -1 stage. This criterion is subjective and may not be uniform in all sample collections. Further, the palms from which the samples were collected were not grown under controlled conditions. Thus physiological condition of the palm and micro environmental variations in the crown could affect the correlation between the pollen developmental stage and the time of split opening of inflorescence. This in turn could contribute to the variation seen in anther response. The genotype of the microspore itself may affect the androgenic response, since each pollen grain is likely to be genetically different due to the heterozygous nature of the mother palm.

Effect of anther orientation

Maximum likelihood analysis of variance (MLAOV) revealed that the orientation of anthers does not significantly affect the percentage of responding anthers and the frequency of calli/ embryo formation. It was also observed that calli/ embryos emerged from both upper and lower surface of the anthers when they were placed with their abaxial surface 'down'. When the anthers were positioned with their abaxial surface 'up', calli/ embryos emerged only from the upper surface (Fig 2a). The results further revealed that the frequency of embryos was significantly higher over calli with the 'up' orientation (G^2=20.67 $p<0.0001$) (Table 1).

When the growing embryogenic structures touched the culture medium containing 2,4-D, they tend to develop into callus rather than embryos. Since 2,4-D favours rapid cell proliferation and callus formation, exposure to a high concentration of 2,4-D could lead to formation of callus. Studies on anther orientation in broccoli showed that embryo formation was enhanced when the orientation was abaxial surface 'up' (Arnison *et al.*, 1990). In barley, placing the anthers sideways (with

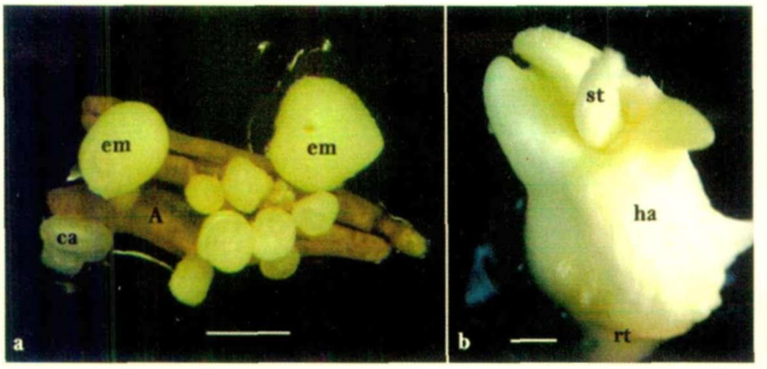

Fig. 2. ***Androgenesis in coconut. a.*** *An anther (A) bearing a callus (ca) and direct embryos (em).* ***b.*** *Germinating embryo. Note the formation of shoot (st) and root (rt) from the haustorial (ha) tissue. Bar=2mm.*

Table 1. *Effect of anther orientation on androgenic response*

Anther orientation[1]	Percentage of anthers responded[2]	Number of calli/embryos per 100 anthers	Percentage of embryos	Percentage of germinated embryos
Up (T_1)	53	230	91.3	17.39
Down (T_2)	47	255	41.8	1.96
MLAOV	NS	NS	20.67***	4.73*

one locule in contact with the medium and the other locule up) was favorable for embryo formation whereas callus was formed predominantly with the locule facing 'up' (Shannon *et al*., 1985).

The percentage germination of embryos (Fig 2b) could be correlated to the number of embryos produced as the embryo germination was high with higher production of embryos. Both the percentage of embryo formation and germination were significantly higher when the orientation was 'up' (G^2=4.73; $p<0.05$; G^2=20.67; $p<0.0001$) (Table 1). Powell *et al.* (1988) also reported that the 'up' position (with one lobe in contact with the medium) significantly increased the green plant regeneration in barley (*Hordeum vulgare* L.) whereas the orientation did not significantly affect the percentage of responding anthers.

CONCLUSIONS

The results of the present study revealed that the genotype is a critical factor that affects androgenesis and out of six palms tested, a positive androgenic response was obtained with only one palm. Only a limited number of cultured anthers in a petri plate elicited an androgenic response. The orientation of anther was also found to be important for successful androgenesis. Both the percentage of embryo formation and germination were significantly higher when the orientation of anther was abaxial surface 'up'.

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