ANTHER CULTURE: ITS PROSPECTS TO COCONUT IMPROVEMENT[1]

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ABSTRACT

Formation of pollen embryos in cultured anthers of coconut (*Cocos nucifera* L.) points to the eventual possibility of regenerating homozygous coconuts, in one or two generations, via *in vitro* culture. Various factors related to the success of experimental androgenesis are reviewed. Among these, the most responsive pollen stage for coconut anther culture, suitable nutritional supplies in culture medium and anther pre-treatments have been determined empirically. Nevertheless, a number of experimental problems remain to be solved before it would be possible to predict, and eventually standardize, conditions favorable for androgenic haploid production in recalcitrant species, including the coconut.

In view of the usefulness of haploids, prospects to utilize them for coconut improvement are discussed in three general ways: (1) shortening the time requirement for the production of homozygous lines, (2) speeding up the fixing of desirable genotypes and (3) facilitating the induction and selection of economically important mutants.

INTRODUCTION

Coconuts are grown extensively in the tropics and millions of people depend on the crop for their livelihood. However, the productivity of coconut trees is generally low despite the many efforts made to improve the yield potential.

The long lifespan and heterozygosity of coconut are the limiting factors in coconut improvement through breeding. Production of in-bred lines for this purpose thus becomes difficult and time-consuming. Unconventional methods must therefore be evolved to shorten the in-breeding cycle of the coconut crop.

The success in haploid plant production by anther and pollen culture in quite a number of plant species following the pioneering work of Guha and Maheshwari (1964, 1966) on *Datura*, has aroused the desire to obtain homozygous lines within one or two generations in perennial crops through the use of haploids. A naturally-occuring haploid embryo in coconut palm has been reported with authentic evidence of its haploid chromosome number (Ninan and Raveendranath, 1965). It was therefore, suggested that haploid formation in coconut could be induced by *in vitro* culture techniques. Recently anther culture of coconut has been conducted in an attempt to induce haploid formation in large quantities. A report by Thanh-Tuyen and de Guzman (1983a) on the formation of pollen embryos at advanced stages in cultured anthers of *Cocos nucifera* L. is a strong indication of the eventual possibility of the regeneration of coconut haploid plants via *in vitro* culture.

DISCUSSION

ANDROGENESIS IN COCONUT

1. Current Status

Anther culture has been successful in about 247 species belonging to 88 genera and 34 families of dicots and monocots (Maheshwari et al., 1983). Of these, only three or four species of angiosperm trees have been attempted at successfully with the induction of androgenic embryos and plants. Apparently, woody species are exceptionally difficult materials for *in vitro* manipulation. The coconut is an example of such recalcitrant material.

An attempt by Iyer and Raina (1972) to induce embryo or callus formation from pollen in cultured anthers of coconut was reported to be unsuccessful. Almost a

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decade later, Iyer (1981) obtained multi-celled-pro-embryoids within the exine of coconut microspores in cultured anthers. No further developments have yet been reported.

Radojevic, as cited in Kovoor (1981) obtained callus from cultured coconut anther. Unfortunately only one out of a thousand cultured coconut anthers produced callus. Another "symptom" of androgenesis was also observed (Haibou, cited in Kovoor, 1981) with the extrusion of globular pollen from cultured coconut anthers.

In the Philippines, Thanh-Tuyen and de Guzman (1983a) attempted to induce haploid formation in coconut via anther culture. Various stages in the development of pollen embryos were observed in cultured anthers. More interestingly, the androgenic origin of the embryos was demonstrated. Despite the low frequency (less than 1%) of pollen embryo formation, the achievement indicates strongly the possibility of inducing androgenic haploids in coconut through direct embryogenesis.

2. Experimental Androgenesis

a. **The responsive pollen stage for coconut anther culture.** The tetrade and uninucleate stages have been observed as optimum for coconut anther culture by Haibou (cited in Kovoor, 1981) and Iyer (1981), respectively. On the other hand, Thanh-Tuyen and de Guzman (1983b) reported that the stage just before, during or immediately after the first pollen mitosis is the most responsive for the formation of pollen embryos in coconut.

Literature has indeed shown that, in most species, the suitable stage for culture is when the pollen is at the verge of mitosis. The peak of androgenic response seems to coincide with the intense metabolic activities in the microspores at this stage (Vasil and Nitsch, 1975). In-depth studies on the control point of differentiation of a pollen grain into an embryogenic cell have revealed pollen dimorphism, which is, according to Heberle-Bors and Reinert (1980), the formation of pollen *in situ* that is competent for androgenesis aside from the normal gametophytic pollen in nearly mature anthers. The former are generally smaller and lightly staining while the latter are larger and densely staining. The two types of pollen can be separated by density gradient centrifugation (Wernicke et al., 1978). It has been confirmed from isolated pollen culture experiments in tobacco that it is the former type which forms embryos (Rashid and Reinert, 1980, 1983).

Further experiments on tobacco have shown a correlation of pollen dimorphism with pollen sterility and an *in vivo* shift in sex balance towards femaleness (Heberle-Bors, 1982a). The phenomena can be regulated by temperature changes at early stage in flower development, that is, before meiosis. Thereafter, the pollen becomes irreversibly determined (Heberle-Bors, 1982b).

Pollen dimorphism has been shown to occur in rye (Wenzel and Thomas, 1974, Sunderland, 1978), barley (Dale, 1975), tobacco (Horner and Street, 1978), *Anemone coronaria*, *Tradescantia bracteata* (Sunderland, 1977), oats, *Nicotiana knightiana* (Sunderland, 1978), and wheat (Tan and Halloran, 1982). In coconut, two different types of pollen with respect to acetocarmine staining have also been observed (Thanh-Tuyen, unpublished) and experiments are being carried out to relate this with *in vitro* pollen embryogenesis.

In a study at the biochemical level on various classes of pollen grains in henbane, Raghavan (1981) reported an evidence for the transcription of new informational type RNA during the induction of pollen embryogenesis, which is lacking in other grains ready to germinate.

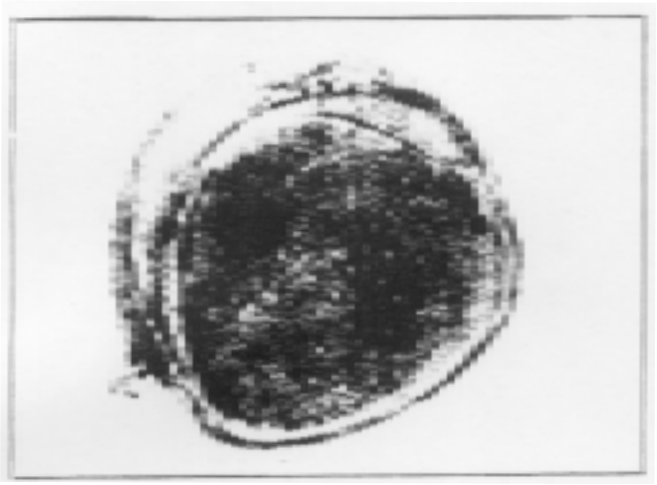
b. **Nutritional factors.** The nutrient requirement for inducing a switch from the gametophytic to androgenetic development in coconut are relatively simple. Modified Blaydes (1966) and B_5 (Gamborg et al., 1968) media supplemented with 6%-9%, 15% coconut water, 2 ppm naphthaleneacetic acid and 0.5% activated charcoal were found suitable for coconut pollen embryo formation (Thanh-Tuyen and de Guzman, 1983a). A close look into the mineral composition of these media indicates that a relatively high level of NO_3 - and low concentration of NH_4+ in their formulations favor the formation of pollen embryos.

The beneficial effect of high nitrate and low ammonium concentrations for haploid production of cereals has been reported by Chu et al. (1975). B_5 medium also includes high content of chlorine, which is known to promote the growth of cultured embryos and *in vitro* seedlings of coconut (Miniano and de Guzman, 1978). In addition, the high level of potassium ions in Blaydes medium seems to favor, to some extent, the induction of pollen embryo formation. The Y_3 medium formulated by Eeuwens (1976), using a very high concentration of iodine, was also tested. Anthers cultured on Y_3 medium responded very well with regard to the percentage of embryonic pollen grains formed, but the latter did not develop further into embryos. Surprisingly, there was no significant differences in terms of anther response to the variations in the availability/concentration of various organic substances, e.g. coconut water, potato extract, auxins, added to any one of the mineral formulations mentioned above. Further studies on the role of growth hormones and other organic constituents in coconut haploid plant regeneration are being undertaken.

c. **Anther pre-treatments.** Cold treatment of excised anthers or flower buds is believed to improve anther response in culture (Nitsch and Norreel, 1972) and has been successfully employed in a good number of species (Maheshwari et al., 1980). In coconut, cold treatment given to flower buds before the excision of anthers was more effective in retaining or preserving pollen viability in culture, than when given to cultured anthers. In anthers exposed to 7°C, the peak response was observed after 7 days of cold treatment, while at 14°C, anthers cold-treated for 4 days gave the maximum response (Thanh-Tuyen,

unpublished). A similar trend was also observed with tobacco anthers by Sunderland and Roberts (1979). The authors concluded that the enhancing effect of cold treatment is not only restricted at temperatures as low as 3-5°C but higher temperatures, e.g. 10-15°C, could also be effective as long as the duration of pretreatment is reduced appropriately.

The stimulatory effect of cold pretreatment to androgenesis has been attributed to a number of factors such as dissolution of microtubules (Jensen, 1974), alteration in the first pollen mitosis (Nitsch, 1974) or preservation of higher ratio of viable pollen capable of pollen embryogenesis (Sunderland, 1978). Cold treatment of flower buds may also act to help the differentiation of the embryogenic class of pollen grains of tobacco by repressing their gametophytic differentiation (Rashid, 1983) or by lowering the abscisic acid content of anthers (Johansson et al., 1982), which is considered to be inhibitory for androgenesis. Bernard (1980) and Huang and Sunderland (1982) reported an increase in the relative yield of green versus albino plantlets after cold treatment.



a. A two-celled microspore derived from a symmetrical mitotic division

b. A pro-embryo with more than 10 cells still enclosed in the exine.

c. A heart-shaped embryo.

d. A torpedo-shaped showing distinct shoot and radicular poles.

Fig. 1. Pollen embryogenesis in cultured anthers of coconut

3. Development of Pollen Embryos

As reported by Thanh-Tuyen and de Guzman (1983a), coconut pollen embryogenesis starts with symmetrical pollen mitosis to give rise to two equal and identical nuclei (Fig. 1a). This is considered to be one of the more common pathways of androgenesis (Maheshwari et al., 1980). Through this pathway, no vegetative and generative cell is differentiated. However, the staining characteristics of the two identical nuclei are similar to those of typical vegetative ones. The continued division of the two identical nuclei led to the formation of multicellular pro-embryos. Fig. 1b shows a pro-embryo with more than ten cells still enclosed within the exine. Still at the microscopic level, the exine of embryonic grains burst out to expel embryos at the heart-shaped (Fig. c) and torpedo-shaped (Fig. 1d) stages. The rupture of the exine to liberate the embryo, as shown in Fig. 2, is a strong evidence to support the androgenic origin of the embryos. Morphologically, coconut pollen embryos at the torpedo stage possess a distinct bipolarity with shoot and radicular poles. The development of one cotyledon is consistently suppressed in the heart and torpedo-shaped embryos. This is obviously one of the characteristics of monocot embryos.

Fig. 2. Androgenic origin of the torpedo embryo (E = embryo; P = Pollen).

Fig. 3. An unorganized multicellular grain.

Direct embryogenesis appears to be the principal pathway of haploid formation in coconut. Although unorganized multicellular grains, as shown in Fig. 3, were occasionally observed, probably indicating the early stage of pollen callus formation, no further developments were noted. In terms of genetic stability, plants derived from pollen via direct embryogenesis are preferable to those regenerated from callus. The former is mostly limited to the *Solanaceae*, while the indirect mode of development is quite common in other plant taxa (Reinert and Bajaj, 1977). In coconut, the successful development of pollen embryos via direct embryogenesis is a very encouraging sign in attempts to produce haploid plants by anther culture. It is believed that once a cell has been turned into an embryo, plant regeneration almost always follows (Jensen, 1980). Yet the regeneration of pollen-derived coconuts from cultured anthers still remains a challenge.

EXPERIMENTAL PROBLEMS

One of the most serious problems encountered in coconut tissue culture is the variation in culture response from one tree, or batch of collection, to another. Commonly, in coconut tissue and embryo culture, the desired growth proliferation is not always obtained in different batches of cultures that have been subjected to exactly the

same culture conditions (de Guzman, 1980). In coconut anther culture, Radojevic (cited in Kovoor, 1981) reported callus formation in only one out of a few thousand cultured anthers. A similar phenomenon was also observed by Thanh-Tuyen (unpublished) in experiments subsequent to the initial success obtained in the induction of microspore embryo formation.

The formation of embryos in cultured anthers of coconut, as reported by Thanh-Tuyen and de Guzman (1983a), is considered to be a response of the microspores to *in vitro* culture conditions. This statement is supported by the observation that among anthers extracted from one inflorescence, pollen embryogenesis was in those cultured on B_5 medium, while those on Bourgin and Nitsch (1967) medium degenerated (Thanh-Tuyen, unpublished). Moreover, the formation of coconut pollen embryos was induced in anthers of two inflorescences of the same age, collected in the same batch and inoculated onto the suitable media of Blaydes and Gamborg et al. These facts rule out the possibility of the existence of spontaneous or *in vivo* pollen embryogenesis, which naturally occurs in anthers, as observed in *Narcissus biflorus* (Koul and Karihaloo, 1977).

Thanh-Tuyen and de Guzman (1983a) speculated regarding the factors governing the switch from gametophytic to sporophytic pathway of pollen development in cultured anthers of coconut. From the results, the authors attributed the triggering of androgenesis to internal factors within the anther-supplying palm or within the anthers themselves. The effects of genotype and physiological conditions of the donor plants on androgenesis are well known in other plant species (Maheshwari et al., 1980; Maheshwari and Tyagi, 1981). Such effects are manifested in the different behavior of species, cultivars and even individuals. Attempts to induce haploid formation have thus recently been geared towards manipulations of growth conditions of the donor plants.

Increase in androgenic response has been reported after various treatments to the donor plants (Maheshwari et al., 1982). Among these, light intensity (Fourughi-Wehr and Mix, 1979), light duration (Heberle-Bors and Reinert, 1979) and low temperature (Foroughi-Wehr and Mix 1979; Keller et al., 1983) are well-known regulatory factors. Further studies with *Nicotiana tabacum* var. Badischer Burley have confirmed the environmental control on pollen embryogenesis and that the response is predetermined before excision and culture of the pollen or anthers (Heberle-Bors and Reinert, 1981). Short-day and/or low temperature (15-18°C) treatments of the donor plants result in differentiation of embryonic pollen *in vivo* at higher frequency (Heberle-Bors, 1982a; Rashid, 1983). Likewise, formation of *in situ* embryogenic pollen grains and subsequent *in vitro* pollen embryogenesis have been induced by treatments with feminizing agents (Heberle-Bors, 1983).

For coconut, unlike herbaceous species, the bulk of the tree and its long lifespan render the exposure of the palm to the controlled environment in the greenhouse or the growth chamber impractical, if not impossible. To a great extent, this fact accounts for variation in anther response among individual trees not only in coconut but perhaps also in other perennial woody species, where successful manipulation *in vitro* cultures are still very limited.

Nevertheless, as the regulation of haploid induction has been well-studied and understood, it would be possible to predict conditions favorable for androgenic haploid production from even recalcitrant species, including the coconut.

PROSPECTS TO COCONUT IMPROVEMENTS

The significance and value of Angiosperm haploids in plant research have long been recognized (Kimber and Riley, 1963). In the past decade, with an appreciation of their usefulness in crop improvement, a great deal of efforts have been devoted to the induction of haploid formation in large quantities, via anther and microspore culture (Sunderland, 1974, Wernicke and Kohlenbach, 1976, Kao, 1981, Nitsch and Norreel, 1972, Sunderland and Robert, 1977, Bajaj, 1978, Wenzel et al., 1975, Wernicke et al., 1978). The potential of haploids in various facets in development of noble cultivars of crop species has been outlined in a number of reviews (Vasil and Nitsch, 1975, Straub, 1977, Reinert and Bajaj, 1977, Sink and Padmanabhan, 1977, Acharya and Ramji, 1977, Keller and Stringam, 1978, Maheshawri et al., 1980, 1982, 1983, Vasil, 1980, Snape, 1981, Collins and Genovesi, 1982) and their impact in crop genetic improvement has culminated in four recent symposia (Kasha, 1974, Davies and Hopwood, 1980, Hermsen and Ramanna, 1981, 3rd Int'l Symposium on Haploidy, China, 1984).

There has been no attempt to cover in this paper the discussion on all possibilities in utilizing haploids for coconut improvement. However, as far as the problems in conventional breeding of this palm are concerned, coconut haploids would be useful in three general ways: (1) shortening the time requirement for the production of homozygous lines. (2) speeding up the fixing of desirable genotypes and (3) facilitating the induction and selection of mutants.

Haploid production via anther/pollen culture and subsequent diploidization greatly cuts short the time required for obtaining pure lines. By this means, the production of homozygous lines of selected characters in coconut would be hastened as compared to conventional ways that require many generations of in-breeding. Successful crossing of anther-derived pure lines will guarantee hybrid vigor.

Hybridization of heterozygous parents, which is quite common in coconuts, usually results in a variety of gene combination. New combinations of characters, which otherwise cannot be detected until the F_2 and, in the homozygous form, until the F_3 or even later generations, may already be selected among plants which develop from haploid pollen grains of an F_1 hybrid. When these are diploidized, the new combination is available as a fertile pure line. Once desirable genotypes are fixed, they can be propagated rapidly by asexual methods. Success in clonal propagation of coconut is not far away, and progress has been reported recently with regard to the development of organized structures in callus derived from coconut explants (Branton and Blake, 1983).

Another advantage of haploids is the expression of genes within a single genome, which eliminates the complexity of a diploid. Indeed, pollen embryogenesis is a better system to induce, detect and analyze mutants as large numbers of haploid cells are available, which have the potential of developing into plantlets (Maheshwari et al., 1983). Notable success has been reported in obtaining several nitrate reductase deficient and auxotrophic mutants utilizing protoplasts and cultured cells from pollen-derived haploids of *D. innoxia, Hyoscyamus muticus, Nicotiana plumbaginifolia* and *N. tabacum* (King, 1983). Selection has also been made for several useful resistant variants/mutants employing haploid cells, including those for better nutritional quality in rice (Schaeffer and Sharpe, 1981).

Such an advantage can be exploited in induction and selection of desirable mutants of coconut that are resistant to important pests and diseases, with high oil and protein content of the endosperm or with fatty acid composition particularly high in a certain fatty acid, e.g. lauric acid.

The formation of pollen embryos from coconut anthers holds great promise for the production of haploids in large quantities for use in crop improvement. Although a number of problems encountered in the process remain to be resolved, the eventual success in coconut haploid plant regeneration will open up a new avenue for the improvement of the performance of coconut palms in the near future.

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