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CYTOLOGICAL EXAMINATION OF MICROSPORE DEVELOPMENT FOR MICROSPORE AND ANTHER CULTURE OF COCONUT (*Cocos nucifera* L.) *cv* SRI LANKA TALL

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ABSTRACT

Production of double haploids through anther and microspore culture has a considerable potential for shortening the breeding cycle in coconut. The developmental stage of a microspore is a critical factor in androgensis and microspores at late uninucleate stage have been shown to be the most suitable stage for *in vitro* culture for many crop species. Routine cytological examination of microspores for identifying the correct stage for culture is tedious and time-consuming. In the present study, a correlation between the microspore developmental stage and age of the spadix (in Sri Lanka Tall coconut) in terms of weeks-before-splitting (WBS) was established for practical convenience of anther and microspore culture. At the age of eight WBS, anthers consisted of microspore mother cells. Within one week, these cells underwent meiosis and produced tetrads. The release of microspores from tetrads could be observed in six WBS spadices. Microspores at late uninucleate stage, which is reported as the most suitable stage for androgenesis, could be obtained from three WBS spadices.

INTRODUCTION

Cocos nucifera L. is one of the most important perennial crops in tropical countries. It is an open pollinated crop that yields high heterozygosity within a progeny and this presents a major problem in conventional breeding programs due to the long life span of the crop (Thanh-Tuyen and Guzman, 1983a). Therefore double haploids have a great potential to shorten conventional breeding programs. Anther and microspore culture are two techniques for *in vitro* production of double haploids. The developmental stage of microspore within anthers of an individual flower bud is a critical factor in haploid induction.

The uninucleate stage of microspores is the most suitable stage for androgenesis in many crop species (Thanh-Tuyen and Guzman, 1983 a, Metwally *et. al*, 1998). Several studies have been carried out to identify a correlation between the developmental stage of microspore and an external marker. It has been shown that in *Cucurbita pepo* L., male buds having a length of 9-10 mm, contained anthers with mid or late uninucleate

microspores (Metwally *et al.*, 1998). Nitsch (1971), reported that the crucial stage for anther culture of *Nicotiana tabacum* was when microspores are about to undergo the first mitosis and that stage could be recognized from the development of the petals in the floral bud. Sunderland (1974) and Thanh-Tuyen and Guzman (1983 b) established the correlation between age of the spadix in terms of WBS and the developmental stage of coconut microspore. However, microspore development in coconut could vary with the genotype and external environment. Routine examination of microspores prior to *in vitro* culture is tedious and time consuming. A convenient method for collecting anthers at the correct developmental stage without testing each one of them before culturing will greatly facilitate *in vitro* culture work.

Therefore the objective of the present study was to establish a correlation between the stage of the development of microspores and the age of the spadix, of Sri Lanka Tall coconut under local environmental conditions, which will provide a simple, practical method of collecting anthers at the desired developmental stage.

MATERIALS AND METHOD

Experimental material:

According to Menon and Pandalai (1958), most of the tall cultivars of coconut produce 12-14 leaves per annum. Thus it can be assumed that the average number of spadices produced per annum is also 12-14, as each leaf has a spadix at its axil. Therefore the interval between splitting of two successive spadices is about 25-30 days or four weeks.

Flower buds from Sri Lanka Tall coconut were used as experimental material. Palms with a day-old inflorescence ie. few hours after the splitting of the spadix (0 stage) and two unopened spadices aged four WBS (-1 stage) and eight WBS (-2 stage) were selected. Spikelets from unopened spadices were collected at weekly intervals (flower buds were obtained from the middle part of the spikelet), so that sampling could be done from 1-8 WBS. Samples were collected from ten palms over a period of one year.

The date of splitting of an unopened spadix was estimated, from the splitting date of the youngest opened one. In each palm, date of the natural splitting of the youngest open spadix (taken as 0) was recorded, and the following spadices (-1 and –2) were forced open and spikelets were collected at weekly intervals.

Cytological examination:

The florets were taken from the middle part of two spikelets and immediately after collection, they were placed in vials containing cold water at 4 °C (Thanh-Tuyen and Guzman, 1983 b). The vials were kept at 4 °C for two hours. Then the water in the vials was replaced with Farmer's fixative (Absolute alcohol: Glacial acetic acid, 3:1) and kept at 4 °C for another 24 hours. The florets were then transferred and maintained in 70% ethanol until further use.

Three out of six anthers from each fixed floret were squashed in a drop of 4% aceto-carmine on a slide. After removal of debris, a cover slip was placed on the squashed material and examined under the light microscope and photographed.

RESULTS AND DISCUSSION

The preliminary investigations revealed that generally, at a given time, two successive unopened spadices were visible on the crown of a palm. During dry weather, only one unopened spadix could be clearly seen and the second one is hardly visible. However, Thanh-Tuyen and Guzman (1983 b) working with coconut *cv* 'Laguna Tall', reported that three successive unopened spadices are visible on a tree. This could be due to the difference in genotype and environmental conditions.

With regard to the microspore development, a gradient of maturity exists in the flower buds of each rachilla. The floral buds present in the top portion and the basal portion (those attached closely to the female flowers) of the rachilla are more mature than those present in the middle portion. The present study also indicated that all the microspores contained within the six anthers of an individual flower bud were of the same developmental stage.

Cytological observation on microspore development was carried out using spadices of different ages, and the results are summarized in Table 1.

Table 1: Stages of microspore development observed at different ages of the spadix (WBS) in Sri Lanka Tall coconut.

Age of spadix (WBS)	Description of developmental stage
8	Microspore mother cells (MMC)
7	Isolated MMCs and tetrads after meiosis
6	Released oblong shaped microspores in early uninucleate stage
5	Spherical shaped microspores
4	Microspores with vacuoles and thick exine in mid uninucleate stage
3	Spherical microspores with distinct nucleus in late uni-nucleate stage
2/1	First microspore mitosis
1/newly open	Binucleate microspore grains with internal deposits.

Microspore mother cells (MMCs) were observed in spadices aged 8 WBS. These cells were more or less polygonal in outline and compactly arranged in the microspore sac. The cytoplasm of these cells was dense with a large nucleus situated close to the cell wall. These nuclei were spherical with a well-defined nuclear membrane (Fig.1A). These MMCs become separated from each other and rounding off of the cells and thickening of the walls could be observed between 7 and 8 WBS. The cytoplasm of these cells was compact and a space between the cell wall and the cell membrane could be seen (Fig.1B). At this stage, the average width and the length of a microspore mother cell were approximately 49 μm and 42 μm respectively. These microspore mother cells underwent meiosis (Fig.1C and D) and produced tetrads (Fig.1E). Nambiar and Swaminathn (1960) reported that meiosis could be observed in coconut florets collected 50-60 days before opening of spadix.

The daughter cells had thin and uneven cell walls and they were dissociated from the surrounding callose matrix during 6 WBS (Fig.1F). After another week, the cells became more spherical in shape with an even cell wall (Fig.1G). The average length and width of these cells were 46 μm and 33 μm respectively. The cells became spherical in shape due to vacuolation and the cytoplasm was dense at 4 WBS (Fig.1H). According to Tuyen and Guzman (1983 b), once the spores were released from tetrads, they enlarged rapidly and soon became spherical in shape. However, the present study indicated that it takes about two weeks for the spores to become spherical in shape.

As a result of the prominent wall formation, microspores were covered with thick exine and intine. The cytoplasm of the cells became clear and the central nucleus was prominent at 3 WBS (Fig.1I). The first microspore mitosis (Fig.1J) could be observed at 2 or 1 WBS. Just after mitosis, both vegetative and generative nuclei were clear at the centre of the spore (Fig.1

K). One nucleus moves to the periphery of the microspore with further maturing. These binucleate pollen grains with smooth exine were observed at anthesis and a prominent furrow, running along the length of the grain was visible (Fig.1L). The average diameter of a pollen grain was 49 μm.

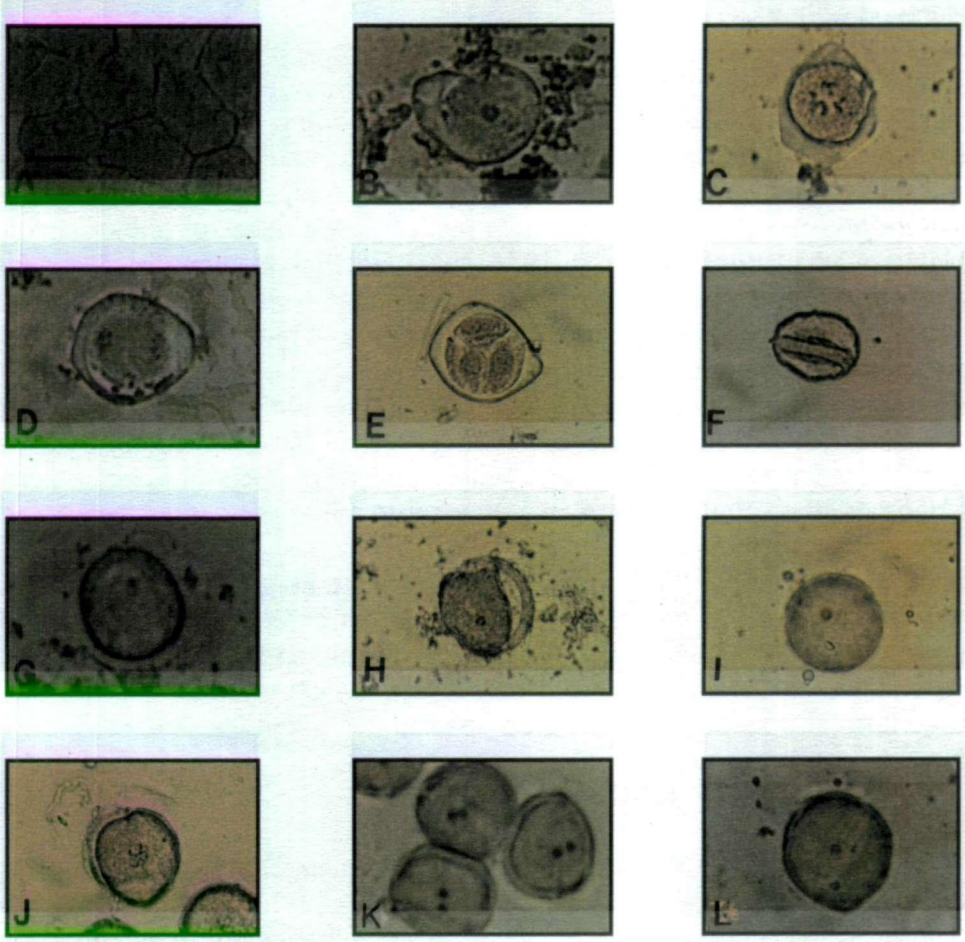

Fig.1. Stages of microspore development in *Cocos nucifera* L. *cv* Sri Lanka Tall. **A.** Compactly arranged MMCs. **B.** A MMC separated from adjoining cells. **C.** A MMC undergoing meiosis in late prophase 1. **D.** A MMC in Telophase **E.** A tetrad. **F.** An oval shaped microspore with thin and uneven cell wall. **G.** An oblong microspore with even cell wall. **H.** A spherical microspore with dense cytoplasm and large vacuole. **I.** A microspore with clear cytoplasm and prominent nucleus. **J.** Microspores undergoing first mitosis. **K.** Binucleate pollen grains with both nuclei at the center of the pollen grain. **L.** A binucleate pollen grain with one nucleus at the periphery (Mag. X 280).

Sunderland (1973) identified three phases in microsporogenesis in higher plants. Phase 1 was meiosis and formation of tetrads whereas phase 2 was dissociation of tetrads and development of individual microspores. The final phase was maturation of microspores into microspore grains. In the present study, these three phases were clearly observed in coconut microsporogenesis.

Many studies have shown that developmental stage of microspore is a critical factor for success in anther culture. Huang and Keller (1989) pointed out that the microspores from late uninucleate to early binucleate stage of *Brassica napus* were highly embryogenic. It was reported that mid or late uninucleate microspores of *Cucurbita pepo* L. were used for anther culture (Metwally *et al.*1998). Nitch in 1971 observed that stamens of *Nicotiana tabacum* cultured at the tetrad stage or when starch was being produced in the microspore grains failed to produce embryos. It has been indicated that androgenic development of *Cyclamen persicum* had occurred frequently in cultured anthers containing microspores at the early uninucleate stage (Ishizaka, 1998). Tuyen and Guzman (1983 b) also indicated that the optimum stage for microspore and anther culture of coconut is just before, during or immediately after the first microspore mitosis.

In the present study, the correlation between the stage of development of microspores and the age of the spadix in terms of WBS was established. Based on the correlation, it can be concluded that spadices of 3 WBS stage are suitable to obtain late uninucleate stage, which is known as the best stage for androgenesis of many crop species.

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