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SHORT RESEARCH ARTICLE

Cytological confirmation of ploidy level in a rare twin haploid of coconut (*Cocos nucifera* L.)

Regi J. Thomas, Maya Lekshmi, M. Shareefa*, J.S. Sreelekshmi and Abe Jacob[1]

Abstract

A rare occurrence of a haploid coconut from a twin seedling was observed in a natural population. The haploid nature of the seedling was confirmed using cytology and ploidy analysis. Amplification of tall-specific SCAR marker in the diploid seedling suggested a possible natural cross by pollen from a WCT palm. The haploids identified in the nursery may be a possible route for generating a pure line of coconut that can be used in future breeding programs.

Keywords: Haploidy, Twin seedling, Flow cytometry, Chromosome count, Stomatal index.

Introduction

Coconut *Cocos nucifera* L., belongs to the family Arecaceae with a chromosome number 2n = 32. The main reasons for slow progress in coconut breeding work are prolonged juvenile period, heterozygous nature of the palm, long interval between generations and lack of vegetative reproduction. Although attempts have been made by several researchers, obtaining a pure line from the heterozygous coconut types has still not been achieved.

Development of haploid embryos from *in-vitro* culture of immature anthers of *Datura anoxia* (Guha and Maheswari 1964, 1966) was a major breakthrough in haploidy research. In classical breeding, several cycles of inbreeding are required for pure line production. Spontaneous occurrence of haploids in twin embryos from seeds of sea-island cotton (*Gossypium barbedense*) was reported by Harland (1936). Haploidy research in palms can reduce the time taken to develop a new variety compared to traditional plant breeding techniques (Sunilkumar and Sparjanbabu 2013). Dunwell (2010) reported massive screening for haploids/ doubled haploids in oil palm seedlings. Here, we report the occurrence of haploidy in one of the twin coconut seedlings and the ploidy of the seedling was convincingly confirmed through morphology, cytology, stomatal index and flow cytometry.

Morphological characters of twin seedlings were recorded at 80 days and 365 days after germination. Mitotic chromosome spreads were prepared using the root tip squash technique with fuelgen-stained root tips and observed for suitable chromosome spreads under a light microscope (Carl Zeiss Axioskop 40, Germany). The stomatal count was recorded at 40x magnification. The stomatal length was measured using image processing software ScopeTek Scopephoto image processing software (Version x86, 3.1.475) in µm unit. Ploidy of the leaf samples was determined on a CyFlow Cube 8 flow cytometer (Sysmex: Partex GmbH Otto-Hahn-Strasse 32 D 48161, Germany) following the manufacturer's instructions. Seedlings were screened using the RAPD SCAR marker reported earlier (Rajesh et al. 2013) to differentiate tall and dwarf genotypes.

A case of haploid-diploid twin coconut seedling was reported earlier which was confirmed by chromosome studies (Whitehead and Chapman 1962). The occurrence

ICAR-Central Plantation Crops Research Institute, Regional Station, Kayamkulam Krishnapuram P.O., Alappuzha 690 533, Kerala, India.

[1]Present address: Poomkudy House, Monipally, Kottayam 686 636, Kerala, India.

***Corresponding Author:** M. Shareefa, ICAR-Central Plantation Crops Research Institute, Regional Station, Kayamkulam, Alappuzha 690 533, Kerala, India, E-mail: hishareefa@gmail.com

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Table 1. Observations recorded from the twin seedlings at 80 days and 365 days after sowing

Morphological trait	Diploid seedling			Haploid seedling		
	80 days	365 days	%increase	80 Days	365 days	%increase
Height (cm)	78	94	20.51	55	60	9.09
No. of leaves	4	7	75	3	5	66.67
Leaf width (cm)	15	21.5	43.33	6	6.5	8.33
Collar girth (cm)	8.5	14	64.71	5	5.5	10

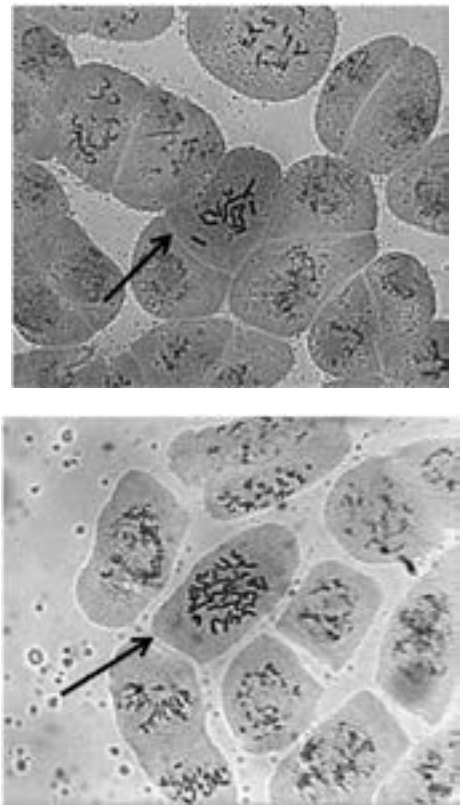
of genetically different twins is very rare and produced as a result of the presence of two or more egg cells in the embryo sac. One of the egg cells fertilizes with pollen and develops into a normal diploid seedling. In contrast, the haploid egg cell developed from synergids/antipodal can develop into a haploid progeny without fertilization.

The twin seedlings used for the present study showed obvious variation in morphology and vigor (Table 1). Remarkable differences were observed in petiole color, the height of seedlings, leaf width and collar girth of the twins. The height increment was 20.5% for diploid seedlings and that of haploid seedlings was 9.1%, indicating the difference in vigor of these twin seedlings. Leaf width and collar girth are considered as traits associated with the vigor of seedlings. The leaf width increment was 43.3% in diploid seedlings, whereas it was only 8.3% in haploid seedlings. Similarly, the collar girth increment was 64.7% in diploid seedlings and that of haploid seedlings was 10%.

Both diploid (2n = 32) and haploid (n = 16) mitotic metaphase chromosome counts were obtained from the root tips collected from the twin seedlings (Fig. 1a, b). During cytological studies on embryo and endosperm of coconut, a very interesting case of a haploid embryo was encountered in a West Coast Tall (WCT) palm which was reported earlier (Ninan and Raveendranath 1965). Cytogenetic studies confirmed its haploid status and the authors suggested that the origin of the haploid embryo resulted probably from haploid parthenogenesis.

Stomatal frequency and stomatal length were used as a measure to assess ploidy (Sascha et al. 2003; Madon et al. 2005). The frequency/density of stomata in putative haploid twin seedlings was found to be more than that in diploid twin (Figs. 2a, b). The average number of stomata in leaf epidermal tissue of putative haploid twin was 10 to 12, whereas it is 5 to 6 in diploid twin seedlings. Stomata length and ploidy are positively correlated. The average stomatal length in diploid twin seedlings was found to be 21.11 μm and 15.35 μm in the putative haploid seedling.

Flow cytometry is an accurate and efficient method to determine ploidy level by measuring DNA content of the interphase nuclei. Sharp histograms of relative fluorescence and small coefficients of variation were obtained for the samples analyzed (Fig. 3). Based on the reference sample,



A

B

Fig. 1. (A). A cell showing haploid (n = 16) set of chromosome. Scale bar represents 10 μm. (B). A cell showing diploid (n = 32) set of chromosomes. Scale bar represents 10 μm

the mean fluorescent intensity of the putative haploid twin (Sample-1) was half that of the diploid twin seedling (Sample-2) and reference sample (Sample-3). Hence, it can be inferred that the ploidy of Sample-2 and 3 are similar and the ploidy of sample-1 is half that of samples 2 and 3.

The amplification of tall specific markers in diploid seedlings (Fig. 4) indicates possible natural crossing by pollen from WCT palm. As the seed nut was from a dwarf palm, there is a possibility that the diploid seedling is a Natural Cross Dwarf (NCD), which was also evident from its petiole color and vigor.

The chromosome count and flow cytometric analysis studies confirmed the haploidy of one of the twin seedlings and this could be due to the formation of embryo directly

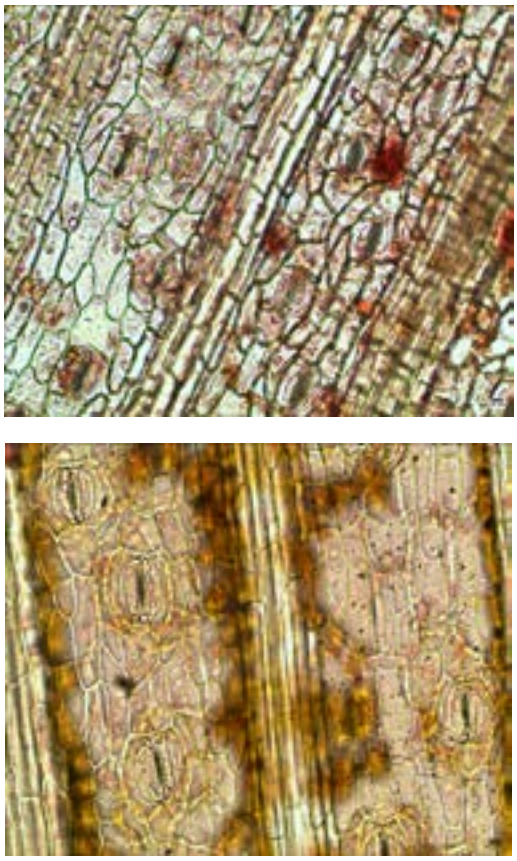


Fig. 2. (a). Leaf section showing small & dense stomata in haploid coconut seedlings. Scale bar represents 10 µm (b). Leaf section showing large & less stomata in diploid coconut seedling scale bar represents 10 µm

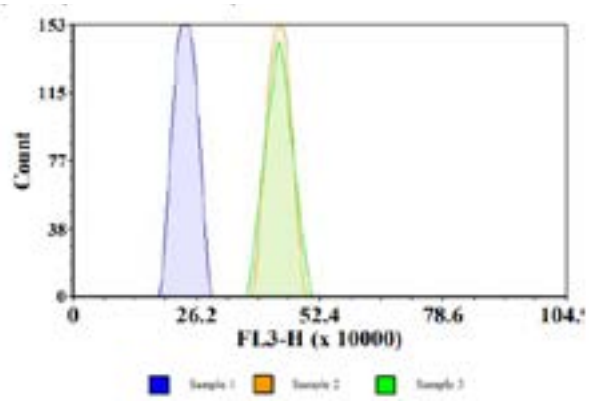


Fig. 3. Histogram of ploidy analysis of leaf samples. Sample 1: Putative haploid twin seedling, Sample 2: Diploid reference sample and Sample 3: Diploid twin seedling

from unfertilized egg cell/synergids/antipodal cells with the pollination stimulus. In the earlier report of haploid twin coconut seedlings (Whitehead and Chapman 1962), haploidy of the twin seedling was confirmed using chromosome count. This is the second report of haploid twin coconut seedling and in addition to morphological characterization, chromosome count and stomatal count, flow cytometric

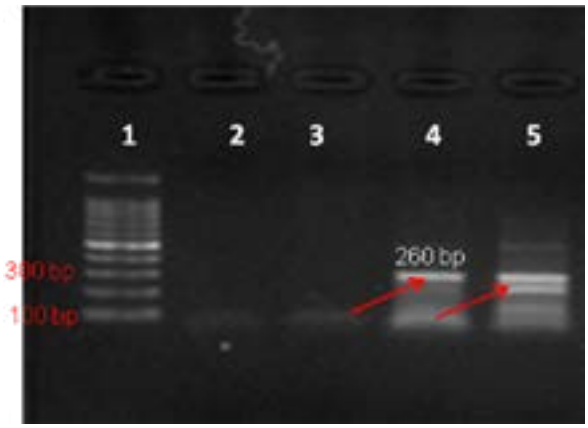


Fig. 4. SCAR marker genotyping. Lane 1 100 bp DNA ladder, Lane2-5 Dwarf, putative haploid twinling, Diploid twinling, WCT

analysis was used for validating the ploidy status of the seedling.

The present report of haploidy in coconut opens up the arena for identifying haploids from seedlings of the WCT variety, which is usually highly heterogeneous in nature. Suppose a haploid seedling is identified in the nursery bed raised from high yielding and root (wilt) disease-free WCT palms. Such haploid seedlings can be used for producing dihaploids/mixoploids using colchicine treatment, as attempted successfully in oil palm (Dunwell et al. 2010). This also becomes a route for the production of purelines of WCT to be used in breeding programme especially for the production of pure F_1 hybrid progenies. Hybridization using doubled haploid WCT as a male parent with resistant CGD mother palms can result in the production of 100% F_1 progenies, which will be truly disease-resistant and high-yielding. Discovering haploids of talls will be of immense breeding value for developing a high-yielding coconut hybrid as cherished by the farming community.

Authors' contribution

Conceptualization of research (RJT); Designing of the experiments (ML, MS); Contribution of experimental materials (AJ); Execution of field/lab experiments and data collection (ML, SJS); Analysis of data and interpretation (ML, RJT, MS); Preparation of the manuscript (RJT, ML, MS).

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