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POLLEN FERTILITY AND FEMALE FLOWER ANATOMY OF MICROPROPAGATED COCONUT PALMS

FERTILIDAD DE POLEN Y ANATOMÍA DE LA FLOR FEMENINA DE PALMAS MICROPROPAGADAS DE COCOTERO

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RESUMEN

The increasing demand in México for disease resistant coconut palms (*Cocos nucifera* L.) requires massive multiplication of improved or selected genotypes. This could be achieved through micropropagation. A reproducible micropropagation protocol via somatic embryogenesis, based on the use of plumule explants has been previously reported. The present study reports the pollen fertility and female flower structure of palms obtained from micropropagation and planted in the field. After two years under nursery conditions and two and half years in the field, the palms showed development of reproductive organs. When compared with sexually propagated palms, there were no differences in the number of inflorescences, number of female flowers and rachillae per inflorescence, pollen grains number, its viability and percentage of germination. Ovary anatomy of micropropagated palms was similar to those of seed palms. This is the first report on coconut micropropagated palms that reached sexual maturity in the field. These results show the potential of coconut micropropagation to produce true-to-type palms.

Index words: *Cocos nucifera*, micropropagation, pollen, inflorescences.

RESUMEN

La demanda cada vez mayor de palmas de cocotero (*Cocos nucifera* L.) en México resistente a enfermedades requiere de la multiplicación masiva de genotipos seleccionados o mejorados. Esto podría ser logrado por medio de la micropropagación. Previamente se ha reportado un protocolo de micropropagación reproducible utilizando plúmulas como explantes. El presente estudio reporta la evaluación de la fertilidad del polen y la estructura de la flor femenina de las primeras palmas micropropagadas en el campo. Después de dos años en vivero y dos años y medio en campo, las plantas presentaron el desarrollo de los órganos reproductivos. Cuando se compararon palmas obtenidas por semillas, no hubo diferencias en el número de inflorescencias, numero de flores femeninas y raquillas por inflorescencias, el número de granos de polen, su viabilidad y porcentaje de germinación. La anatomía del ovario de las palmas micropropagadas fue similar a aquellas de palmas obtenidas por semilla. Este es el primer reporte sobre palmas de cocotero micropropagadas que han alcanzado la madurez sexual en el campo. Estos resultados muestran el potencial de la micropropagación de cocotero para producir palmas fieles al tipo.

Palabras clave: *Cocos nucifera*, micropropagación, polen, inflorescencias.

INTRODUCTION

The coconut palm (*Cocos nucifera* L.) is important as a cash crop and for subsistence in most Latin American and Caribbean countries, but several problems are currently affecting this crop. A very pressing problem is the phytoplasma-associated disease lethal yellowing (LY) that has killed millions of palms. So far the most effective way to control LY is by replanting with resistant palms. Therefore it is necessary to identify resistant palms and to propagate them faster than nature does. Micropropagation methods are needed since seed propagation will take decades to produce the amounts of plants required in countries like México, due to the large area that needs replanting.

Coconut micropropagation studies based on somatic embryogenesis started about 35 years ago, focused mostly on the use of inflorescence explants which have proved to be very recalcitrant (Verdeil and Buffard-Morel, 1995). Alternatively, the Centro de Investigación Científica de Yucatán (CICY) in collaboration with the Imperial College, London (Wye Campus) and L'Institut de Recherche pour le Développement/Centre de Cooperation Internationale en Recherche Agronomique pour le Developpement (IRD/CIRAD, France) using plumule as explants, developed a micropropagation protocol to promote somatic embryogenesis, with an improved efficiency for the formation of calli, embryos and plantlets, the last ones being successfully transferred to *ex vitro* conditions (Chan *et al.*, 1998; Sáenz *et al.*, 1999). Therefore, it is important to learn if these plantlets present any alterations for instance in development of flowers and their performance, as it has been reported for oil palm female flowers obtained through somatic embryogenesis (Tregear *et al.*, 2002).

The objective of the present work was to evaluate pollen fertility and female flower structure of micropropagated coconut palms, and compare these features with those of palms obtained from seed germination.

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MATERIALS AND METHODS

Establishment of the micropropagated palms in the field

Regenerated plantlets from Malayan Green Dwarf (MGD) coconut plumule explants were obtained after 18 months of *in vitro* culture as described in Chan *et al*. (1998). They were acclimated in nursery conditions for two years (Chan *et al*., 1998), and then transplanted to field conditions at San Crisanto North Coast of Yucatán State (21° 20' LN, 89° 09'LW).

The trial consisted of a batch of nine micropropagated palms and, for comparison purposes, a batch of ten palms germinated from seed of the same variety. When the first plants started producing flower structures (aprox. five and half years old), the following traits were analyzed: number of inflorescences, number of rachillae and females flowers per inflorescence, pollen count, pollen fertility and anatomy of female flowers.

Pollen count

Three male flowers from the middle rachillae were collected from each of three palms. Each flower was placed in 5 mL of a 0.1 % Tween 20 solution and stirred during 30 min; for pollen visualization, a drop of a toluidine blue solution (0.5 %) was added. An aliquot of 1 mL of this volume was placed in a cell counter (Sedgewick rafter cell) for pollen counting using a light microscope (100 X). The amount of pollen per male flower was calculated using the equation PG = APG x 100 x 5 (according to manufacturer's instructions). PG is the number of pollen grains in a male flower; APG is the average number of pollen grains in a cell, calculated from the individual numbers of 10 cells chosen randomly; 100 is the number of cells contained in the cell counter; and five is the volume of the solution where the pollen of each flower was placed initially. Three flowers were analyzed per inflorescence (the most recently open one) and one inflorescence per palm.

Pollen viability

This trait was evaluated by staining the pollen grains with a solution of triphenyl tetrazolium chloride (TTC: 15 % sucrose in 10 mL distilled water and 0.05 g of TTC; Kearns and Inouye, 1993). A drop of the TCC solution was placed on a glass slide, pollen from a male flower was spread onto the surface of the solution and incubated in darkness at 27 °C. After 4 h the pollen grains were visualized under a light microscope (100 X) and the pollen stained in red were considered viable. For each slide assay the pollen from one male flower was used, and ten stereomicroscope fields were analyzed per slide. Five flowers were analyzed per inflorescence and one inflorescence per palm.

Pollen germination

Pollen germination was tested on a medium containing: 15 % w/v sucrose, 100 mg L^{-1} H_3BO_3, 86 mg L^{-1} $Ca(NO_3)_2.4H_2O$, 40 mg L^{-1} $MgSO_4.7H_2O$, 20 mg L^{-1} KNO_3 and 0.1 % w/v agar (Kearns and Inouye, 1993). *In vitro* pollen germination was evaluated using the hanging-drop method. A drop of medium was spread on a glass slide and let it to solidify; pollen was spread onto the agar surface and incubated in darkness at 27 °C. After 6 h a drop of toluidine blue (0.5 %) was added to visualize the germinated pollen grains. A pollen grain was considered germinated if it produced a tube longer than the diameter of the grain (Roberts *et al*., 1983). For each slide assay, the pollen from one male flower was used and ten microscope fields (100 X) were analyzed per slide. Five flowers were analyzed per inflorescence and one inflorescence per palm.

Histological procedures

Samples for histology were prepared according to Buffard-Morel *et al*. (1992) Tissue samples were fixed in 4 % paraformaldehyde in 0.2 M phosphate buffer (pH 7.2) for 24 h under vacuum. Samples were dehydrated in a stepwise manner (1 h per step) using 30, 50, 70, 80 90, 96 and 100 % ethanol in water. This was followed by impregnation with JB-4 (Polyscience, USA) resin for 3 d. Cross sections (5 μm) were made with high profile microtome blades (Polysciences) and stained with toluidine blue (0.5 %) for 5 min. Toluidine blue stains RNA purple, DNA blue or blue green, and ligning and some polyphenols green or blue green (Yeung, 1984).

Statistical analysis

To determine the statistical difference in number of pollen grains and pollen fertility between the two treatments, a Student's t test was carried out using the Sigma Stat package (Jandel Scientific Software, Chicago, USA). For the number, viability and germination of pollen, each from three sampled palms, the corresponding averages and standard deviations were calculated.

RESULTS AND DISCUSSION

After 2.5 years in the field, both the coconut micropropagated palms and seed palms started to develop floral organs (Figure 1A). A spadix with its characteristic spear-like shape (Figure 1B) formed with each emerging leaf. When spadices opened, the enclosed inflorescences

presented rachillae with male flowers and female flowers (Figure 1C and 2D).

Inflorescence analyses

The numbers of inflorescences in the three micropropagated palms were 10, 9 and 7, and in the three seed palms were 10, 9 and 6 (Table 1). The number of rachillae/inflorescence showed small differences among palms within each batch and between batches, with average values of 20.7 ± 2.0 for micropropagated palms and 20.2 ± 1.9 for seed palms (Table 1).

Table 1. Reproductive structures of coconut palms obtained by micropropagation or from seed.

Number of reproductive structures	Micropropagated palms	Seed propagated palms
Inflorescences	8.6 ± 1.5	8.3 ± 2.0
Female flowers/inflorescence	5.5 ± 3.1	4.1 ± 2.3
Rachichae/inflorescence	20.7 ± 2.0	20.2 ± 1.9

Data are means of three palms of each propagation method (n=3) ± standard deviation. No statistical differences were found between the propagation methods (Student's t test P≤ 0.05).

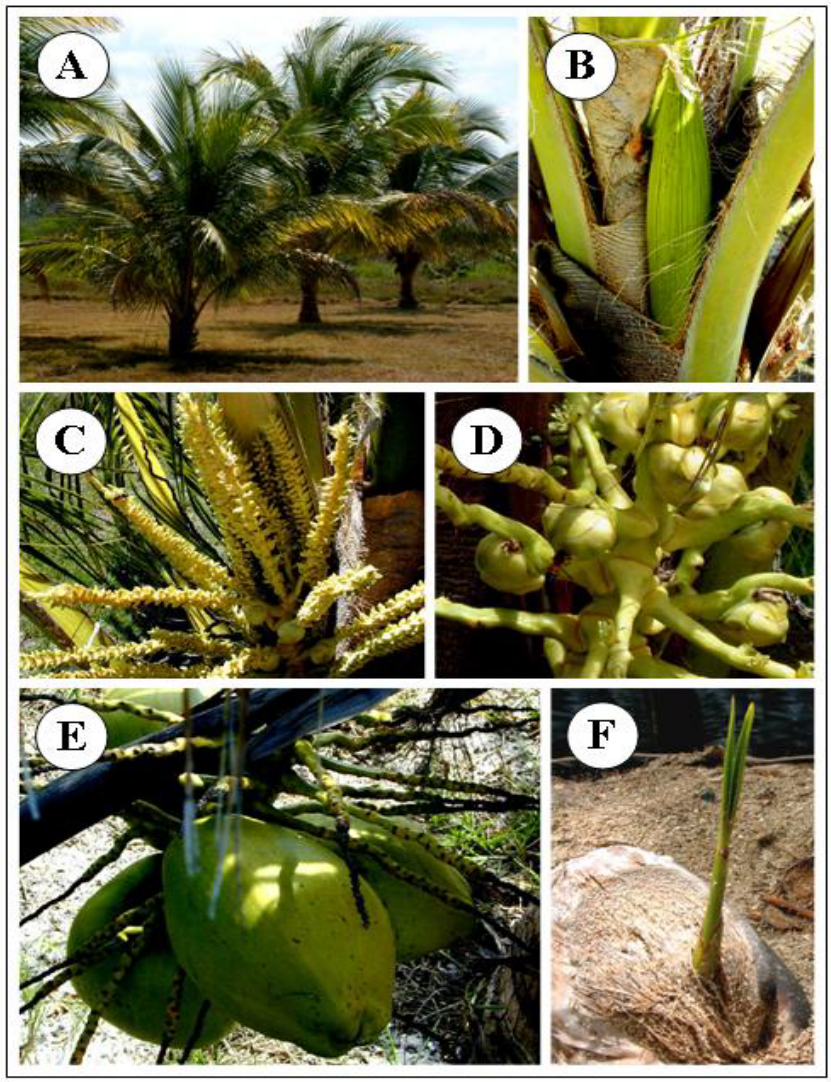

Figure 1. Micropropagated coconut palms obtained via somatic embryogenesis from plumule explants. Palms growing in the field (A); spadix (B); open inflorescence showing rachillae with male flowers and female flowers (C); close up of female flowers (D); coconuts growing (E); and coconut seed germination (F).

The numbers of female flowers showed variation among palms within each batch, but all palms were producing female flowers (Table 1). Production of male flowers was more uniformly established than production of female flowers; however, a similar pattern was observed between, the micropropagated and the seed palms.

Female flowers from micropropagated plants were exposed to open pollination and formed fruit with normal appearance (Figure 1E) which could germinate (Figure 1F) under field conditions.

Count of pollen grains

The number of pollen grains was quantified in male flowers from micropropagated flowering palms and palms obtained through seed germination. Analyses showed that the average of grains per male flower was 22 426 ± 10 215 in the micropropagated palms and 24 972 ± 12 197 in the palms germinated from seed (Table 2). The averages from both types of palms were similar but there was a large variation in pollen population among flowers of each group as indicated by their standard deviation (Table 2). It is expected that young palms show a large variation in pollen population, characteristic that may decrease in the future. Large variation in pollen number seems to occur commonly in plants, according to Jürgens *et al*. (2002) who studied this feature in 79 plant species.

Table 2. Pollen grains population, viability and germination of micropropagated and seed coconut palms

Pollen parameters	Micropropagated palms	Seed propagated palms
Population of pollen grains	22426 ± 10215	24972 ± 12197
Pollen viability (%)	84.4 ± 9.5	93.4 ± 8.9
Pollen germination (%)	40.8 ± 14.9	40.2 ± 15.6

Data are means of three flowers of three palms of each propagation method (n=9) ± standard deviation. No statistical differences were found between the propagation methods (Student's t test $P \leq 0.05$).

Pollen fertility

Pollen fertility is usually assessed in two ways, by staining with TTC or by germination in culture medium (Piven *et al*., 2001; Song, 2001). The analysis with TTC showed that the percentage of viability of pollen grains was 84.3 % ± 9.5 in the case of micropropagated palms and 93.4 % ± 8.9 in seed palms (Table 2), showing no significant differences between groups. Pollen from micropropagated palms was 40.7 % ± 14.9 and 40.2 % ± 15.6 for pollen grains of seed palms (Table 2), with no significant differences between the two groups of palms.

Within both groups of palms, the number of pollen grains that did not germinate was above one half of those that stained with TTC. Since the evaluation of viability with TTC staining did not indicate the pollen grain fertility, it is suggested to use the pollen germination method for *Cocos nucifer*a. According to Song (2001), pollen germination data usually shows large variation whereas that of viability does not, results which are similar to the present research. Variation coefficient for the viability test was 9-11 % whereas for the pollen germination test was 36-38 %.

Histology and fertility of female flowers

Female flowers from both micropropagated palms (Figure 2C and 2D) and seed palms (Figure 2A and 2B) showed a tricarpellar structure, with three ovules, a pachychalasa with numerous vascular bundle. The mycropyle and embryo sac were also observed (Figure 2B y 2D). No abnormalities were observed in ovaries of both types of palms; in contrast oil palms from somatic embryogenesis showed abnormalities in their floral development, involving an apparent feminization in flowers of both sexes, called "mantled" phenotype (Tregear *et al*., 2002). In severe cases there was a formation of supernumerary pseudocarpels that resulted in partial or complete sterility, thus affecting oil production (Tregear *et al*., 2002).

More than 40 years have already been dedicated to the research for the development of protocols for coconut palm clonal propagation, and advances in the efficiency and reliability of coconut somatic embryogenesis have been achieved recently (Pérez-Nuñez *et al*., 2006). Branton and Blake (1983) reported the production of some clonal plants, but none could be successfully established (Blake, 1990). More recently, Verdeil *et al*. (1994) showed the production of plantlets from isolated somatic embryos of different genotypes, although there are no further reports of whether they became established in field conditions or not. There is only one report of a single palm produced by Unilever in the UK that was established in the field in Solomon Island (Blake, 1990), but no further reports have appeared on its performance or survival.

There are other laboratories that have produced clonal coconut palms and established them in the field (Anitha Karum[1] and Luckmini K. Weerakoon[2], Personal communications), but so far, no reports were found. So, to the best of our knowledge this is the first reported study that evaluates whether field grown micropropagated coconut palms present abnormal morphology. We also developed an advanced *in vitro* coconut propagation protocol that produces

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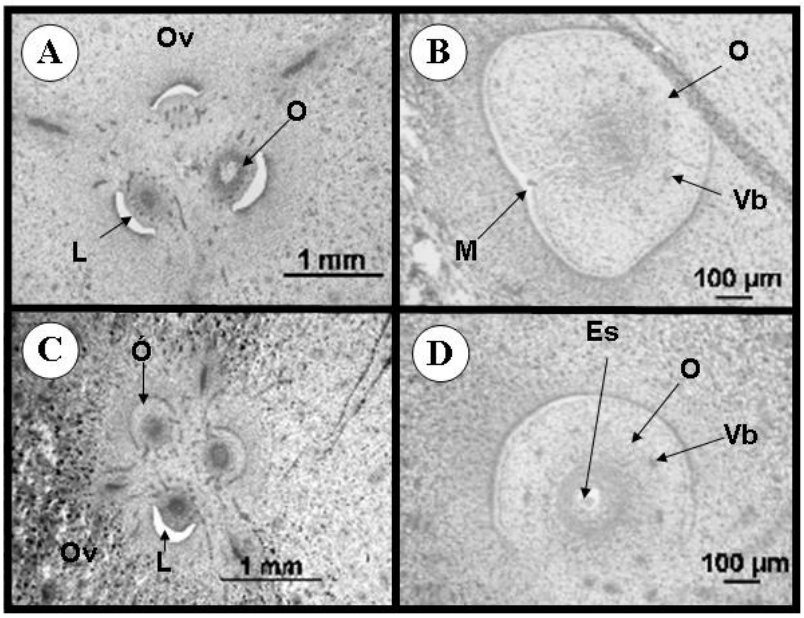

Figure 2. Floral anatomy of coconut female flowers. Ovary cross sections from a seed palm (A) and from a micropropagated palm (C). Ovary longitudinal sections of a seed palm (B) and from a micropropagated palm (D). Es = embryo sac; M = micropyle; O = ovule; Ov = ovary; L= locule; Vb = vascular bundle.

tens of thousands of somatic embryos from a single plumule (Pérez-Nuñez *et al*., 2006). Learning about the true-to-typeness morphology and performance of the plants in the field is of great interest because of its potential application for coconut mass production in the near future.

CONCLUSIONS

This is the first report on micropropagated coconut palms that have been established in the field, reaching sexual maturity without abnormal morphology, indicated by the number of inflorescences, rachillae, female flowers, pollen population, fertility and ovary structure, as compared with palms obtained from seed. Although this is a preliminary study, these results show the potential of coconut micropropagation to provide normal plants with reproductive capacity.

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