Cruz de Santana, Marlucia; Magalhães Souza, Margarete; Santana Pereira, Telma Nair; Lopes Teixeira, Silvio
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Universidade Federal de Santa Maria
Santa Maria, Brasil

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Anatomical and histochemical aspects of zigotic embryo and leaves in ‘Coqueiro Anão’

Aspectos anatômicos e histoquímicos de embrião zigótico e folhas em ‘Coqueiro Anão’

Marlucia Cruz de Santana¹ Margarete Magalhães Souza² Telma Nair Santana Pereira³
Sílvio Lopes Teixeira³

ABSTRACT

The paper provides information about histochemical staining reactions in leaves and embryos of ‘Coqueiro Anão’ (Cocos nucifera). It was compared in vitro coconut and autotrophic palm leaves. Reactions for insoluble polysaccharides and acidic compounds, protein, extractable lipids, lignin and other classes of compounds were tested using histochemical tests. None sample gave positive reaction for lignin and phenolic compounds. All the samples gave positive reaction for protein, starch and insoluble polysaccharides while acidic compounds were positive only in in vitro leaves. Both in vitro and autotrophic leaves gave positive reaction for lipids showing presence of cuticle even in in vitro leaves. Only autotrophic palm leaves showed idioblasts containing calcium oxalate crystals.

Key words: Cocos nucifera, culture in vitro, polysaccharides, protein, lipids.

INTRODUCTION

The coconut palm (Cocos nucifera L.) is one of the most important lowland tropical crops, providing copra and coconut oil for the home and international markets (SANTAMARÍA et al., 1999). In Brazil, the coconut crop occupies a cultivated area of almost 300 thousand hectares, with a production of 245 thousand tons, principally on sandy lands situated along the coast that extend from Pará to Rio de Janeiro states (FNP, 2002). On the other hand, around the world, the coconut production is diminishing due to decline in the productivity, and this fact is caused by palm aging, natural calamities such as diseases and drought, lack of well adapted varieties and with high productivity, and genetic erosion (SÁENZ et al., 1999). According to THANH-TUYEN & DE GUZMAN (1983), the problems encountered in conventional coconut breeding for high-yielding ability are those associated with its long life-span and heterozygosity, and consequently, the production of inbred lines is a long and difficult process. Thus, unconventional methods have to be evolved to shorten the inbreeding cycle (HORNUNG, 1995).

Coconut in vitro zigotic embryo cultivation was reported by DE GUZMAN & DEL ROSÁRIO (1964)
for the ‘Makapuno’ coconut. Under natural conditions the seed endosperm degraded, depriving the developing embryo of necessary nutrients. Attempts have been made since then to overcome the difficulties in obtaining normal plants and in the acclimatization stage (RILLÓ, 1999). Nowadays tissue culture has been performed in coconut on seedling and mature tissue explants (BRANTON & BLAKE, 1983) including zygotic embryos (SANTAMARÍA et al., 1999), in order to have non-heterozygous, high yielding disease-resistant clonal coconut plantlets that could provide uniform material for marketing mainly for replacement planting and high-quality parental stocks for breeding programs (BRANTON & BLAKE, 1983).

The morphology of plants cultivated in vitro is greatly affected by this environment. Problems appear such as little or no epicuticular wax formation; bad stomata functioning (ZIV, 1995), low chlorophyll content, low percentage of dry matter, few stomata, low photosynthetic ability and incomplete root development and/or low lateral root development (KOSAI, 1991). Anatomical and histochemical studies are important mainly when plants cultivated in vitro are compared to autotrophic plants, in order to evaluate the existence of differences in reserve substance accumulation and presence or absence of other substances that are involved in plant survival mechanisms or that may make them less competitive. Therefore the objective of the present study was to characterize the most adequate embryo for in vitro culture and to compare the anatomical and histochemical characteristics of leaves from vitroplants and from plants cultivated in a greenhouse, and to investigate whether leaves were modified internally as a consequence of development in such a culture environment.

MATERIAL AND METHODS

Coconut (Cocos nucifera L.) belonging to the ecotype ‘Coqueiro Anão’ was used in this study due to its autogamous reproduction, with low heterogeneity among zygotic embryos. Eleven-month-old fruits were collected from the Experimental Field of PESAGRO in Campos dos Goytacazes, RJ, Brazil. Part of these fruits was grown in a greenhouse using washed sand as substrate, and the other part was used for embryo extraction and in vitro culturing. Ten to twelve-month-old embryos and six-month-old leaves after emission were collected and used for histochemical tests.

After sterilization, the embryos were cultivated in Y3 medium (EEUWENS, 1976) supplemented with the MOREL & WETMORE (1951) vitamins, 100mg L⁻¹ casein hydrolysate, 6% sucrose, 100mg L⁻¹ myo-inositol, 1mg L⁻¹ activated charcoal and 6% agar. The pH was adjusted to 5.8 before autoclaving. Excised embryos were cultivated in essay tubes (25mm x 150mm) until 30 cultivation days (DAI), when the plantlets were transferred to culture medium without agar. By 90DAI, sucrose was reduced to 0.3 per cent, and the plantlets were transferred on to 100mL⁻¹ medium in 600 mL⁻¹ culture bottles.

Leaves from in vitro culture and the greenhouse, and embryos were fixed in 2.5% glutaraldehyde and 4% formaldehyde in sodium cacodiol buffer (0.1M, pH 7.2) for 1h at room temperature, then kept at 4°C until use. After rinsing in buffer they were dehydrated in a graded ethanol series (30 to 100%). The material was infiltrated and embedded in Paraplast® using xyol as transition solvent. Ten μm thick sections were obtained with a rotatory microtome. Sections were stretched on glass slides previously treated with Haupt gelatin fixative (HAUPT, 1930) and 8% formalin, and exposed to a xylol-ethanol series to remove the Paraplast®. For anatomical observation, embryos and leaf sections were stained using astra blue and basic fuchsin (ROESER 1972, modified by LUQUE et al., 1996). They were then mounted in Canada balsam. The observations and photographs were performed on an Olympus B202 optical microscope.

Sections were stained for insoluble carbohydrates with 1% periodic acid-Schiff (PAS) and mounted in Canada balsam; starch and some polysaccharides of the cell wall showed red or magenta colors (O’BRIEN & MCCULLY, 1981). For control, the periodic acid was omitted and glass slides were immersed in α-amylase for 2h at 37°C (MCMANUS, 1946). For pectic substances (acidic polysaccharides) localization, sections were stained with ruthenium red, counterstained with methylene blue and mounted in Canada balsam, and the test was considered positive if red color was observed (LANGERON, 1949). Sections were stained for starch with IKI solution and mounted in 50% glycerol; the test was considered positive if a blue-black or dark brown color was observed (JOHANSEN, 1940). For lipids localization, sections were kept in 95% ethanol, immersed in 2% Sudan IV in 95% ethanol for 30min and mounted in 50% glycerol, and the test was considered positive if a red-pink or pink color was observed (GERLACH, 1984). For calcium carbonate localization, 10% glacial acetic acid drops were deposited on sections free of Paraplast®; the test is considered positive when there is elimination of carbonic gas bubbles (CHAMBERLAIN, 1932, modified by KRAUS & ARDUIN, 1997). For calcium oxalate, 10%
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RESULTS AND DISCUSSION

Different morphology was observed in the leaves from plants cultivated in vitro (Figure 1) and from plants cultivated in the greenhouse (Figure 2). Sections of leaves from plants cultivated in the greenhouse showed the layer of palisade cells well-organized and some of these cells underwent periclinal division; well-developed cuticle; presence of idioblasts containing crystals (Figure 3). Sections from plants cultivated in vitro displayed irregularly shaped palisade cells of different sizes and with wavy anticlinal wall and absence of idioblasts containing crystals. Both in plants cultivated in vitro and in the greenhouse, some cells of photosynthetic parenchyma were detected containing substances which showed different staining degree in relation to others, even though on sections from vitroplants these same cells stained less. Only eleven- and twelve-month-old embryos showed two well-developed meristematic zones which resembled the shoot and root poles, as well as a developing cotyledon (or haustorium). It was possible to observe a well-differentiated apex and plumular tissue in twelve-month-old embryos (Figure 4).

The PAS reaction was positive for insoluble carbohydrates stained more intensely in twelve-month-old embryos (embryo axis and cotyledon), and they were considered mature and qualified to be cultured in vitro. According to DE SOUZA (1998), carbohydrates play an important role in biological systems, associating among themselves and forming a macromolecular complex which has an important functional role, not only acting as a metabolite reserve in the cells, but also participating in the phenomenon of cellular recognition. It has been demonstrated that many vital processes such as germination and embryogenesis are influenced by sugar (JANG & SHEEN, 1997).

In palm trees, even when the fruit ripens, the embryo can have little differentiation (CORNER, 1966). The germination irregularity of in vitro coconut embryos may be caused by the embryo immaturity. In this study, about 50% of the embryos obtained from mature seeds were still little developed, with a different structure from that described for the mature embryo, with small and with poorly developed cotyledons.

The coconut embryo is cylindrical and divided into parts by a small constriction in the median region; the plumule-root axe is found in the apical part of the cotyledon (SREEKUMARI KARTHA, 1981). Coconut zygotic embryo development was studied by HACIUS & PHILIP (1979) who concluded that there was not just a bipolar structure with stem and root meristem but there was also a cotyledon meristem. During the embryo development, the cotyledon is the predominant tissue. At the mature stage, the cotyledon increases in volume so that the stem and root premordials are proportionally insignificant. These data corroborate the idea that the 12-month-old embryo described in this study was already at the mature stage. The morphological aspect of the embryo presented in this work is similar to that presented by HORNUNG (1995).

The results of this study for the plantlet leaves cultivated in vitro showed disarrangement in
Figure 1 - Histochemical staining reactions in leaves and embryos of Cocos nucifera. 1: Leaf from plants cultivated in vitro; 2: Leaf from plant cultivated in the greenhouse; 3: Detail of mesophyll showing calcium oxalate crystals (arrow); 4: Embryo tissue stained with PAS reagent for total insoluble polysaccharides with the apex (A) stained intensely; 5: Leaf from plants cultivated in vitro tissue stained with PAS reagent; 6: Leaf from plants cultivated in the greenhouse stained with PAS reagent; 7: Leaf from plants cultivated in vitro tissue showing grains of starch in the palisade (arrow) and chlorophyllain tissues; 8: Leaf from plants cultivated in vitro tissue showing grains of starch in the chlorophyllain tissue; 9: Leaf from plants cultivated in vitro tissue showing positive reaction for lipids demonstrating the cuticle presence; 10: Leaf from plants cultivated in the greenhouse showing presence of cuticle; 11: Leaf from plants cultivated in vitro tissue demonstrating positive reaction for Pectic substances (acidic polysaccharides) on the epidermis and mesophyll; 12: Leaf from plants cultivated in vitro showing only counterstain. Bar = 50mm.
the tissue structure especially in the palisade parenchyma that presented a very irregular architecture. These results are in line with the literature, and several authors have reported anomalies in in vitro cultivated plants (SANTAMARIA et al., 1999, CARVALHO et al., 2001). The modification of internal leaf structure in response to environmental conditions is a known phenomenon (GROOT & ASTON, 1978). No increase was observed in coconut in the number of palisade tissue layers compared to the plants cultivated in a greenhouse, unlike observations by FIDELIS et al. (2000) in Brosimum gaudichaudii Tré. 

Leaves from micropropagated plants in a conventional cultivation system, confined in the hermetically closed recipients, and under low photosynthetic active radiation and high relative humidity, were small, thin and sometimes translucent with little development of the epidermis tissue and the mesophyll (ZIV, 1995). The stomata apparatus of the epidemic tissue of micropropagated plants differed greatly from the plants cultivated in a greenhouse or in the field. In several species studied, the meristem of the leaf cultivated in vitro had poorly formed palisade tissue consisting mainly of spongy parenchyma with large intercellular air spaces. 

The leaf cuts from coconut plants cultivated in the greenhouse corresponded to coconut leaf descriptions reported by several authors, in transversal cuts the smooth upper epidermis was formed by a line of cutinated cells. The adaxial epidermis was uniform, with cells varying from more or less rectangular or cubic longitudinally. The leaf center or mesophyll was occupied by the palisade parenchyma. The lower epidermis is slightly grooved with short, sparse, trichomes (PURSEGLOVE, 1979). Raphides bundles were observed as described for the family Palmae (PRYCHID & RUDALL, 1999).

Differences were observed in starch accumulation. In the leaves of plants cultivated in the greenhouse, starch was observed only in the spongy chlorophyll tissue. However, in those cultivated in vitro, starch was observed in the palisade parenchyma and chlorophyll. Most plants store starch or sucrose as reserve carbohydrates, but others storage fructans that may have functions other than carbon storage; they have been implicated in protecting plants against water loss, in the form of waxes on the leaf epidermis, for example, and are directly involved in the adaptation to drought stress (REPELLIN et al., 1997).

Twelve-month-old embryos (embryo axis and cotyledon) presenting well- differentiated apex and plumular tissue were considered mature and qualified to be cultured in vitro. Sections of leaves from plants cultivated in vitro showed structural disarrangement in the palisade cells, presence of starch in the palisade tissue, acidic polysaccharides located on the epidermis and mesophyll, and epidermis with cuticle denser when compared to plants cultivated in the greenhouse. The differences were observed as a consequence of development in the culture environment.

CONCLUSION

Twelve-month-old embryos (embryo axis and cotyledon) presenting well- differentiated apex and plumular tissue were considered mature and qualified to be cultured in vitro. Sections of leaves from plants cultivated in vitro showed structural disarrangement in the palisade cells, presence of starch in the palisade tissue, acidic polysaccharides located on the epidermis and mesophyll, and epidermis with cuticle denser when compared to plants cultivated in the greenhouse. The differences were observed as a consequence of development in the culture environment.

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