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Microsporogenesis and pollen formation in cassava

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Abstract

This article describes the complete microsporogenesis and pollen formation in cassava (*Manihot esculenta* Crantz) at the various developmental stages (pollen mother cell, meiosis, tetrads, early free spore, mid uninucleate, late uninucleate, binucleate and mature pollen grain). Light microscopy, transmission electron microscopy and confocal laser scanning microscopy were used for the study. Floral bud size and other inflorescence characteristics were correlated with specific stages of the microspore development. This association allows a rapid selection of floral buds with similar microspore developmental stages, useful when a large number of homogeneous cells are needed for analysis and for *in vitro* induction of androgenesis. This article also compares methods for digestion the exine wall in microspores.

Additional key words: CLSM, FISH, exine digestion, *Manihot esculenta*, ultrastructure.

Introduction

The life cycle of flowering plants involves the alternations of a diploid sporophytic and haploid gametophytic phase (Li *et al*. 2009, Taskin *et al*. 2009). Microsporogenesis occurs at the end of the sporophytic phase and undergoes a clearly defined cell division program resulting in a gametophyte (pollen grain) comprising the generative and vegetative cells. The generative cell divides giving rise to two sperm cells (McCormick 2004). The developmental events of microsporogenesis and pollen formation are exquisitely timed and choreographed, occurring in a precise chronological order that correlates with the floral bud size (Koltunow *et al.* 1990, Scott *et al.* 1991). Efforts have frequently been made to describe microsporogenesis and pollen formation from the cytological, molecular and genetic perspectives largely in model plant species (Goldberg *et al.* 1993, McCormick 2004, Scott *et al.* 2004, Chen *et al*. 2005, Blackmore *et al.* 2007). However, these processes are poorly understood in other plant species such as cassava. Cassava is a perennial shrub, vegetatively propagated and grown throughout lowland tropics. The heterozygous nature of the progenitors complicates traditional breeding as well as genetic and molecular studies. Production of homozygous lines *via* doubled-haploids through the *in vitro* culture of microspores (androgenesis) would facilitate and accelerate the development of advanced germplasm and genetic analysis (Ceballos *et al.* 2004, 2007). The

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Abbreviations: CLSM - confocal laser scanning microscope; FISH - fluorescent *in situ* hybridization; DAPI - 4'-6-diamidino-2-phenylindole; PMC - pollen mother cell; EFS - early free spore; T - tapetum; M - middle layer; EN - endothecium; E - anther epidermis.
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induction of androgenesis requires the culture of microspores at specific developmental stages. Thus the understanding of microsporogenesis and pollen formation in cassava is an important pre-requisite for the development and application of this technology.

This article provides a comprehensive description of the *in vivo* microsporogenesis and pollen formation processes in cassava, the identification and association of microspore developmental stages with specific inflorescence and floral bud characteristics, as well as the application for the development of protocols for *in vitro* production of doubled haploids.

Materials and methods

Cassava (*Manihot esculenta* Crantz) clones, HMC-1 and TAI-8 were planted in the field at Corpoica Experimental Station in Palmira, Colombia. Vegetative cuttings were planted and after sprouting, conventional cultural practices were applied during the entire growing cycle through maturity (Ospina and Ceballos 2002). Inflorescences were collected at 08:00 - 10:00 from healthy and vigorous plants with profuse flowering, and of similar morphology and developmental stage. After collection floral buds were immediately stored in a polystyrene box with refrigerant gel (*Pelton Shepherd Industries*, Stockton, CA**,** USA) to avoid physiological deterioration during transportation.

For the analysis of mother pollen cells, flower buds were fixed in Carnoy solution for 24 h and then preserved in ethanol (75 %). Anthers were extracted from flower buds, squashed in aceto-carmine (2 %) for 5 min and then analyzed with the *Leica* microscope (*Leica Microsystems*, Heidelberg, Germany). Microphotographs were taken using a photo automat camera (model *MPS45*, Heerbrugg, Switzerland). The developing exine structure of mature tetrads was photographed using contrast phase optics with a *Leica* microscope (*Laborlux*, Heidelberg, Germany).

Additionally, intact fresh buds and anthers were first fixed in 2.5 % glutaraldehyde (in 0.1 M phosphate buffer, pH 7.2) for 24 h at 4 °C and post-fixed in aqueous osmium tetroxide (1 %) during 1 h. Samples were then washed three times with distilled water and dehydrated in a ethanol series (25, 50 and 75 %). Specimens were immersed in 2 % uranyl acetate (in 75 % ethanol) for 12 h at room temperature, followed by immersion 1 × in 90 % ethanol and 3 × in 100 % ethanol. Samples were rinsed with pure acetone for 20 min (three times) and then gradually embedded in acetone-Spurr epoxy resin (1:1). Anthers were later infiltrated in pure Spurr epoxy resin for 1 h. Finally, samples were polymerized at 60 °C for 16 h. Embedded anthers were sectioned on an ultra microtome (*MT 6000*, *Sorvall Instruments*, DuPont, Delaware, USA) for semi-thin or ultra-thin sections. The semi-thin sections were stained with toluidine blue-O and analyzed with the *Leica* microscope. The ultra-thin samples were cut with a diamond knife at 60 nm, collected in copper grids and stained with uranyl acetate and lead citrate following Reynolds (1963) procedure. The ultra-thin sections were examined with a transmission electron microscope *Jeol TM1010* (Tokyo, Japan). The images were captured and analyzed using *AnalySIS 3.0* software (*Soft Imaging* System, Münster, Germany).

In addition to the light and electron microscopy evaluations, samples were also analyzed under a fluorescence confocal scanning microscope (*Leica TCS SP2 Confocal*). Because of the exine thickness and auto-fluorescence, the anthers were first fixed in a FAA (ethanol: glacial acetic acid: formalin: H_2O; 50: 5: 10: 35) solution for at least 24 h and then treated with 10 % sodium hypochlorite for 2 min to digest the exine. Microspores with partially digested exines were washed three times in deionized distilled water and stained with 0.05 mg cm^{-3} of acridine orange (AO). The images were captured by a *Leica Confocal* software and processed using *Imaris 3D* software version *4.2.0* (*Bitplane AG*, Zurich, Switzerland).

In order to use effectively confocal laser scanning microscope (CLSM), it was necessary first to treat the microspores to degrade exine wall. Different alternatives of the standard procedure reported in the literature (Rowley *et al.* 1999) based on $KMnO_4$ were evaluated. Microspores were treated with 0.5, 1.0, 2.0, 4.0, 8.0 and 16.0 % $KMnO_4$ solutions for time periods ranging from 1 min up to 24 h. Microspores were also treated with 10 % NaOCl to remove the exine and eliminate the source of the microspore auto-fluorescence, therefore allowing the penetration of the laser beam across the microspore and use of the corresponding fluorescent staining.

Developed pollen grains were fixed in a solution of 96 % ethanol : glacial acetic acid (3:1; v/v) for 10 - 15 min, washed with 70 % ethanol centrifugation at 150 *g* for a 2 min and stained in 4'-6-diamidino-2-phenylindole (DAPI; *Partec*, Münster, Germany) and observed under the fluorescent microscope.

For light microscopy analysis, about 200 pollen mother cells or tetrads (about 2000 microspores) from 10 - 20 male buds were processed at each specific bud size. For the electron microscopy studies, five male buds were processed for each size. CLSM and exine digestion analyses were based on microspores isolated from ten buds for each of the following sizes: 2.4, 2.5, 2.6 and 2.7 mm. Buds were taken from different plants**.**