Development of an *In Vitro* Protocol for the Production of Cassava Doubled-Haploids and its Use in Breeding

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Introduction

Introducing inbreeding in cassava genetic improvement has many advantages, among those are the identification of high-value recessive traits in early generations, the ease application of molecular tools in breeding, and the expedite generation of diversified improved breeding lines. But developing inbred lines through self-pollinations would require in cassava about 9-12 years. Rapid and complete homozygous can be reached by using *in vitro* haploid technology. Androgenesis, the process by which microspores develop to form embryoids and subsequently regenerate into doubled haploids (DH, homozygous) plants has been applied successfully in plant breeding of various crops. The success of *in vitro* production of DH lines by androgenesis is subject to various constraints and it is affected by various factors, including the physiological status of donor plants, special developmental stage of microspore, proper pretreatment of the microspores (stress) prior to *in vitro* culture, and the microspore culture conditions (medium composition, cell density, temperature regime, plant growth regulators, among others). This project aims to develop an *in vitro* protocol for the production of doubled-haploid from anther or microspore culture in cassava.

Materials & Methods

Microsporogenesis in cassava: Histological study was conducted using both light and electron microscopy. Flower buds and anthers were fixed, embedded and sectioned in slides of 800 to 1000 nm thick using an ultra-microtome with diamond knives. Standard procedures were used for light and electron microscopy analysis.

Microspore isolation and culture: Flower buds selection was conducted on ice. Selected buds in groups of 300-500 buds were placed in a baby food jar and surface sterilized using 5% NaClO solution for 8-10 min and washed with H2O for 3 times, 5 min each and then blended at low speed for 20 sec in a blender (Warring, USA) with 40 ml of culture liquid medium. After passing sequentially through two meshes (150μm and 105μm pore size), the filtrates were passed through mesh 88μm or 41μm. Microspores on top of each mesh (88μm or 41μm) were re-collected, washed with the same medium 3 times and resuspended with 1 ml fresh medium. The microspore stage of development and yields of the suspension were recorded. Microspores were cultured in 30×15mm Petri dishes at 26-28℃ in the dark. Different pretreatments and various culture conditions were used to induce cell division toward androgenesis. Each experiment was repeated between 3 to 10 times.

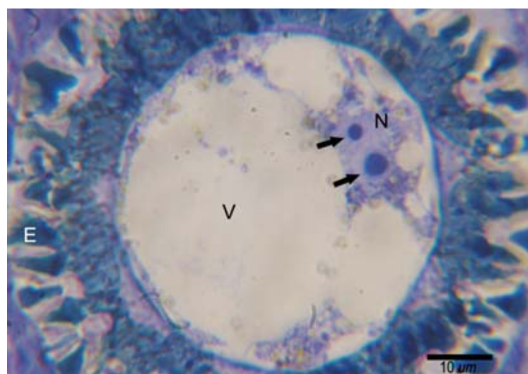

Fig.1.- Detail of a microspore at the late uninucleate to early binucleate stage, showing the vacuole (V), nucleus (N), nucleolus (arrow) and exine (E).

Results

❖ The thick and auto-fluorescent exine wall of cassava microspore hinder the use of fluorescence staining for developmental stage examination and subsequent cell division monitoring.

❖ In cassava, the most responsive microspore stage for androgenesis in model systems (i.e. the late uninucleate to early binucleate), is characterized by i) an oppressed significantly reduced cytoplasm, harbouring the nucleus/nuclei with one or two nucleoli, ii) one or several large vacuoles, accounting for more than 95% of the cell in volume, and iii) well developed exine wall with strong auto-fluorescence (Fig. 1). In HMC-1 and TAI-8 cassava clones, this stage of microspore is contained in flower buds of 2.5-2.6mm in diameter collected from field grown plants during rainy season.

❖Standard conditions and protocols for optimal flower bud selection, harvest and storage until processing or shipment; cell viability monitoring; methods for isolating and culturing microspore suspension and identification of few factors for the induction of first cellular division in some cases are now available.

❖Isolation and culture of a high-quality microspore suspension indicate that some microspore increased its volume in about 4 fold (enlarged microspore, EM) respect to those "un-induced" microspores. EM it is usually associated with the acquisition of embryogenic potential in model systems. Optimal treatments reproducibly induced 20% of EM. Multi-cellular structures, embryo-like structures and micro-callus were observed indicating more advanced structures than EM (Fig. 2).

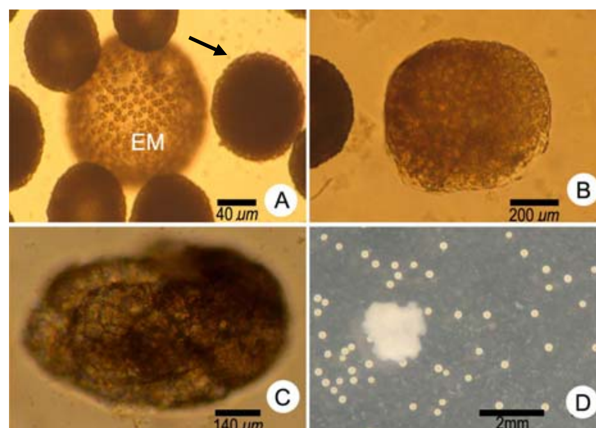

Fig.2.- Structures towards androgenesis in cassava. A) Enlarged microspore (EM) showing increased volume about 4 fold) and the exine pattern, and un-induced microspores (arrows). B) Multi-cellular structure (MCS) derived from microspore cultured *in vitro*. C) Embryo-like structure (ELS). D) Microcallus formation.

Ongoing Activities

Various parameters are being analyzed to increase the androgenesis response for the regeneration of doubled haploid plants using selected commercial clones and lines with increased tolerance to inbreeding depression generated from the breeding program.

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