Embryo sac ontogenesis of different ploidy levels in cassava (*Manihot esculenta* Crantz)

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Abstract

Viability of seeds is coordinated in sexual species by embryo formation and its development. Asexual pathways take place when deviations of sexual sequence of development occur, as a mechanism brought by natural selection for species survival. In cassava it has a special importance because vegetative propagation by apomixis avoids pathogens contamination and provides hybrid vigor. The aim of this research was to determine cassava embryo sac ontogeny specific stages in which deviations from sexual development occur. A comparative analysis was carried out on diploid and cytochimeric types, using clearing and sectioning techniques to describe the stages in megasporogenesis and megagametogenesis. Our data show the Polygonum type for sexual embryo sac of both diploid and chimeric individuals. Polyploidy of L2 in chimeric plant resulted in enlargement of embryo sacs. Moreover, it increased apomixis frequency. Apomictic structures were found to coexist with sexual sacs. This is the first report dealing with chimeric cassava embryo sac ontogeny.

Keywords: Apomixis, cytochimera, polyembryo sac, pro-embryo.

Introduction

Manihot esculenta Crantz (cassava) is the only cultivated species among 98 included in *Manihot* genus (Rogers and Appan 1973). It occupies the fourth position of the most consumed sources of human energy behind rice, maize and sugarcane (FAO 2008). All these factors corroborate to its recent title of crop of the century (FAO 2013). This species belongs to family Euphorbiaceae and tribe Crotonoideae (Webster 1994). Some genera within its family such as *Ricinus*, *Hevea*, *Jatropha* and *Euphorbia* have been well investigated in embryological terms, and embryo sac formation elucidated to vary, ranging from 16-nucleate tetrasporic embryo sacs in *Acalypha*, to tetrasporic Penae and monosporic Polygonum in *Euphorbia* (Kapil and Bhatnagar 1994). More recently, a demonstration of floral development in cassava provided a basic understanding of the female gametophyte (Perera et al. 2012). However, information regarding embryo sac formation is lacking in cassava.

Apomixis has been considered as an important tool for improving crops (Barcaccia and Albertini 2013), especially cassava (Nassar and Ortiz 2010; Nassar et al. 2011). Through this process, total polyploids and partial ones – namely cytochimeras – have been produced (Marcotrigiano and Gradziel 1997; Nassar 2003). Total polyploids are well known by their relation to apomixis (Carman 1997; Nassar et al. 2010); however, apomixis in cytochimeric plants has not been reported yet. Scarce data on reproductive ontogenesis of cassava and its relation to apomixis mechanisms motivated this research, whose main aim was to find cassava embryo sac ontogeny specific stages in which deviations from sexual development occur.

Material and methods

Plant material

A diploid cassava cultivar from indigenous origin, namely UnB 530, and UnB 530p (Nassar et al. 2012), and the chimeric 4n-4n-2n plant, which was obtained by colchicine application on lateral buds of UnB 530, were the sources of female flowers. These plants can be found in the Living Collection of *Manihot* at Experimental Station of University of Brasilia, Brasilia, Brazil.

Fig. 1 Female flowers of *Manihot esculenta* at sequential developmental stages (numbers).

Clearing technique

Female flowers with an ovary length ranging from 0.5 to 7 mm (Fig. 1) were collected from UnB 530 and UnB 530p for sequential observations. They were fixed in Carnoy I for 14 days, conserved in 70% ethanol, dissected, cleared in methyl salicylate (Young et al. 1979), and observed under interferential contrast microscopy. Embryo sac organization and nuclei number were observed.

Sectioning technique

Ovules from diploid and chimeric samples within the same range of ovary length as cited above were included in Spurr's resin (Spurr 1969) and the stain was toluidine blue. Some were also included in paraffin (Kraus and Arduin 1997), stained with safranin/fast green and mounted in synthetic resin (Paiva et al. 2006). Ovules were longitudinally sectioned in Leica RM 2145 microtome within 3 to 5 µm. Apomictic embryo sacs previously screened by the clearing technique were included in paraffin through a series of acetone (Young et al. 1979), sectioned and stained as described above.

All images were taken in light optical microscope Leica DM 2500 and Zeiss Axioscop 2 and processed with Leica Application Suite 4.0 or Photoshop CS4. Composed images were edited for a view of the complete embryo sac structure.

Results

Cleared (Fig. 2) and sectioned (Fig. 3) ovules showed the same results in terms of developmental pathway (Fig. 4) within 1361 diploid and chimeric *M. esculenta* ovules. Stages were numbered from 1 to 10, corresponding to developmental sequence from megaspore mother cell (MMC) to pro-embryo. UnB 530 revealed the initial cell and tetrads in "T" shape" in ovaries at stages 1, 2 and 3(Fig. 2a-c, Fig. 3a-c). At stage 4 of closed buds (Fig. 2d, Fig. 3d), megaspore selection and triads degeneration were noted, and nucellus and internal and external integuments were present and already differentiated. At next stage (Fig. 2e, Fig. 3e), two nuclei were seen, which indicated the timing of first mitotic division. At stage 6

(Fig. 2f, Fig. 3f), second mitotic division was observed. From this point, when the ovary was 4 mm length (Fig. 2g, Fig. 3g), buds were collected all from opened flowers. The embryo sac was already mature, showing the egg cell, two synergids and two polar nuclei. Antipodals were occasionally seen in this stage. Polar nuclei were often found near the egg apparatus. Endosperm presence was observed from this point to older stages, with free nuclei organized in networks along the embryo sac peripheral extension. At stage 8 (Fig. 2h, Fig. 3h) we found pro-embryo and endosperm formation in fertilized sacs. When the size was 6 to 7 mm (Fig. 2i, Fig. 3i), embryo continuous development was observed, and also endosperm densification in sacs with embryo formation. Starch grains were noted along initial mitosis until mature embryo sac stage.

The chimeric type, UnB 530p, showed MMC at stage 1 (Fig. 2j). External and internal integuments as well nucellus were present. At stage 2 (Fig. 2k), dyad phase was characterized by anticlinal division, at stage 3 (Fig. 2l) was possible to find sometimes linear, other times four "T" shaped cells generated through meiosis II. At stage 4 (Fig. 2m), the selected megaspore was evidenced. Stage 5 (Fig. 2n) was marked by two cells presence with prominent nuclei. At stage 6 (Fig. 2o) we saw four nuclei generated by second mitotic division. From 5 mm size (Fig. 2p), buds were collected in opened stage. and the egg cell, two synergids, two polar nuclei and eventually three antipodals were observed. When the ovary was 6 to 7 mm length (Fig. 2q-r), pro-embryo and endosperm formation were seen. The endosperm development followed the pattern observed in diploid type, with free nuclei in network organization and lashing on embryo sac wall. The pro-embryo in both samples had a slender shape with at least one initial anticlinal division. The nuclei number seen at this early embryo development varied from 2 to 4. Pollen tube was not observed in the range of ovules studied in this work.

Polyembryo sacs were found in UnB 530p, among ovules of 6 mm of ovary length, and consequently having open flowers. Two embryo sacs were seen inside these ovules (frequency = 1.3%). Sacs at chalazal location had four nuclei, while mycropilar ones had six nuclei and some degree of degeneration (Fig. 5).

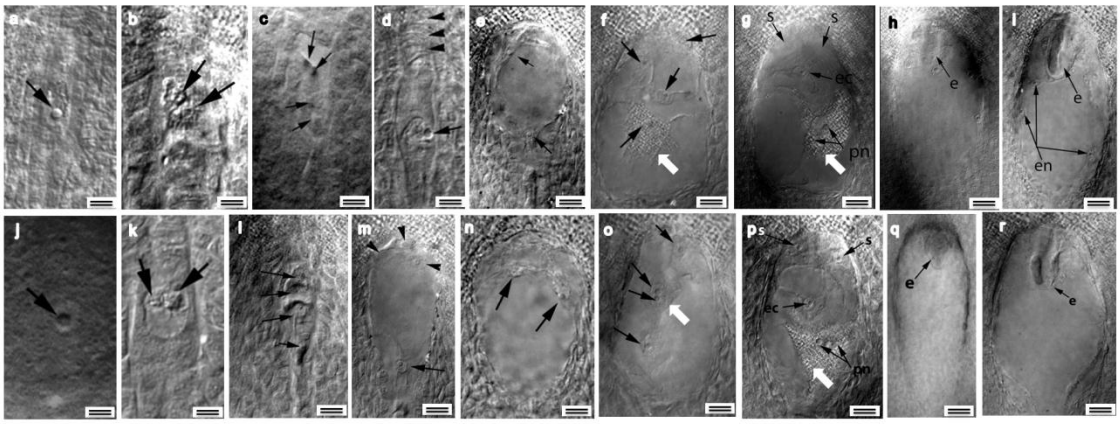

Fig. 2 Embryo sac development in diploid UnB 530 (**a-i**) and chimeric UnB 530p (**j-r**) in images by clearing technique of *Manihot esculenta* types. **a. j.** MMC (arrow). **b. k**. Dyad stage. **c. l**. Tetrad stage. **d. m.** Selected megaspore (arrow) and degenerating triads (arrow heads). **e. n**. Two nuclei (arrows) after first mitosis. **f. o**. Four nuclei (arrows) after second mitosis. **g. p**. Mature embryo sac with two sinergyds (s), egg cell (ec) and two polar nuclei (pn). **h. q**. Uninucleated pro-embryo. **i.** Binucleated pro-embryo. **r**. Tetranucleated proembryo. White arrows point starch grains. Micropylar pole at the top. e= pro-embryo. en= endosperm. Bar 25 µm (**a-d** and **j-l**); 50 µm (**e-g** and **m-p**); 100 µm (**h-i** and **q-r**).

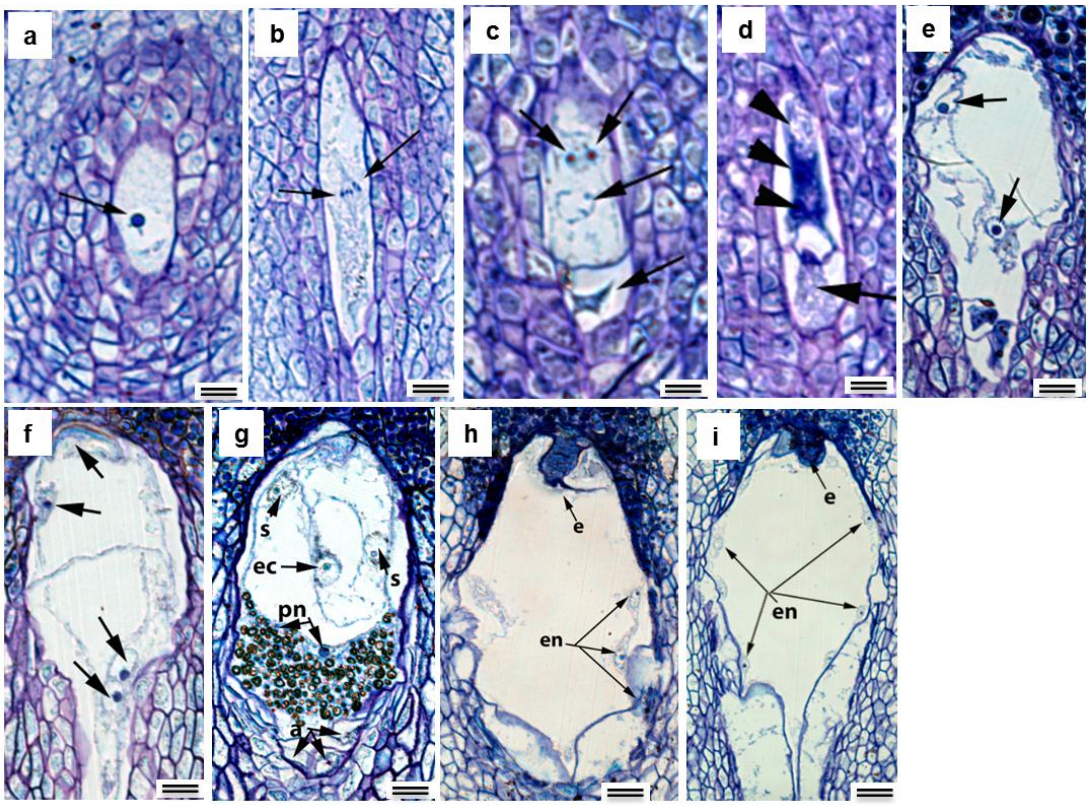

Fig. 3 Sections of embryo sac showing development in *Manihot esculenta* ovules. **a.** MMC (arrow). **b.** Dyad stage. **c.** Tetrad stage. **d.** Selected megaspore (arrow) and degenerating triads (arrow heads). **e.** Two nuclei (arrows) after first mitosis. **f.** Four nuclei (arrows) after second mitosis. **g.** Mature embryo sac with two sinergyds (s), egg cell (ec), two polar nuclei (pn) and three antipodals (a). **h. i.** Pro-embryo. Micropylar pole at the top. e= pro-embryo. en= endosperm. Bar 25 µm (**a-d**); 50 µm (**e-h**); 100 µm (**i-j**).

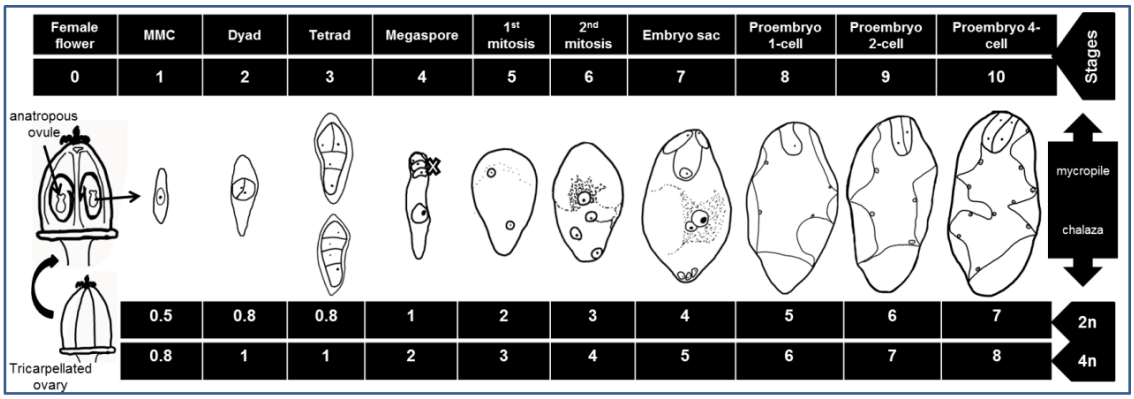

Fig. 4 Scheme of embryo sac development in *Manihot esculenta*. Note linear and "T" shape of tetrads at stage 3, and progressive increase in embryo sac volume. Starch grains occurrence from stage 5-7. Values at the bottom refer to ovary length in mm of diploid (2n) and chimeric (4n) *M. esculenta* types.

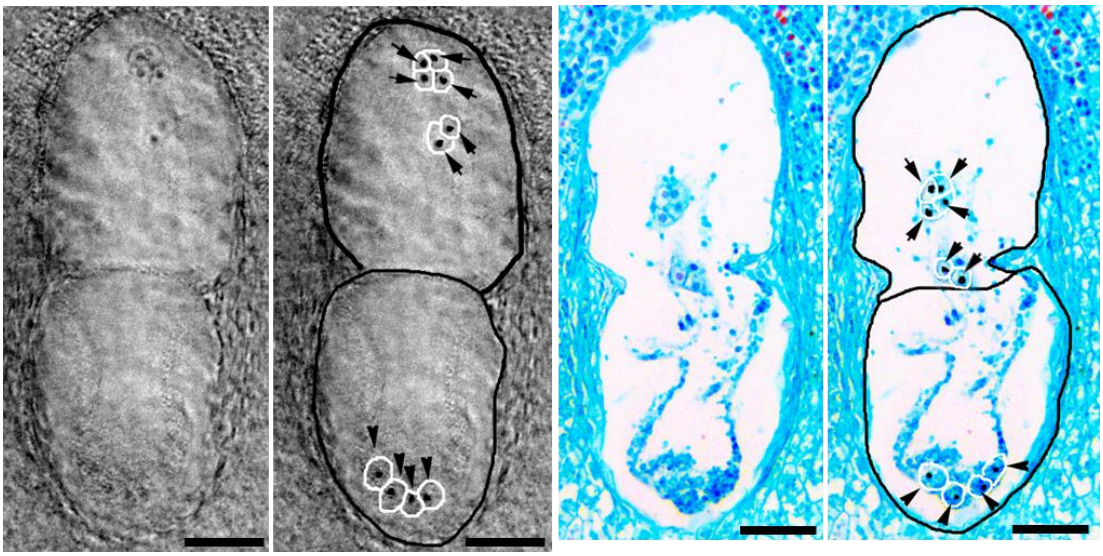

Fig. 5 Ovule of *Manihot esculenta* chimeric type (UnB 530p) in clearing technique (**a-b**) and sections (**c-d**) showing two adjacent embryo sacs. **b** and **d** represent drawings of **a** and **c**, respectively. Note six nuclei (arrows) at micropylar sac and four degenerating (arrow heads) at chalazal one. Bar 25 µm.

Discussion

Megasporogenesis and megagametogenesis

Embryo sac development in *M. esculenta* is monosporic, evidenced by the occurrence of a single selected megaspore among tetrads at stage 4, which went through mitotic divisions till the 8-nucleate sac formation. The mature embryo sac, both in diploid and chimeric *M. esculenta*, is composed by eight nuclei, similarly reported in other cultivated plants of this species (Rao and Rao 1976; Perera et al. 2012).

Megasporogenesis follows through dyad stage with cells side-by-side horizontally, and tetrads have configuration in linear or "T" shapes. Latter arrangement had been cited previously for *M. esculenta* (Rao and Rao 1976). Megaspore closest to chalaza enlarges before mitosis and the three nonfunctional

spores degenerate, as already noted in another genera, such as *Zea* (Wu et al 2011) and *Ambrosia* (Chen et al. 2013).

Three mitotic divisions and migration in megagametogenesis were noted, as found by Kägi and Gross-Hardt (2007) in *Arabidopsis*. After the first mitosis, each of the two nuclei migrates to micropylar and chalazal poles, while two groups of four nuclei are generated in each pole after the second mitotic division, and after the third mitotic division, one nucleus of each pole migrates to central region of the sac. During megagametophyte development, a polarized axis mycropile-chalaza is formed, and cellular differentiation leads to specialization of egg cell and synergids at the micropylar pole, and antipodals at the chalazal pole (Yadegari and Drews 2004). Thus, it is possible to note along embryo sac maturation, the formation of two synergids, one egg cell, three antipodals and one central cell composed by two nuclei, with one of them very prominent. As in other dicots, *M. esculenta* axis may be potentially generated by an auxine gradient (Zhang and Laux 2011).

Starch grains are observed from mitotic divisions to mature embryo sac, and often associated to polar nuclei. A higher concentration of grains in the central cell was also reported for *Torenia* (Wallwork and Sedgley 2000). Carbohydrate reserves are essential for embryo survival, although endosperm is the main site for this accumulation (Lopes and Larkins 1993). Their occurrence inside the embryo sac is a common phenomenon in plant families such as Cactaceae, Tiliaceae, Asclepiadaceae and Crassulaceae (Maheshwari 1950; López-Férnandez and Maldonado 2013).

Antipodals were occasionally seen until mature embryo sac stage of *M. esculenta*, predicting their degeneration prior fertilization. In some species, such as *Arabidopsis*, antipodals undergo cell death immediately before fertilization, while in others like *Zea* they proliferate after fertilization (Maheshwari 1950; Drews *et al*. 1998). In Euphorbiaceae, antipodals are reported to persist after its formation as described for *Euphorbia preslii* (Carmichael and Selbo 1999).

Synergids were present in mature embryo sacs of *M. esculenta*, as noted for antipodals, and their disappearance is related to embryo formation. They have an important role in fertilization pathway promoting guidance of the pollen tube to the egg cell (Reiser and Fisher 1993). For *M. esculenta* plants, pollen tube neither its trace were detected. Similar observation was reported for *E. esula* (Carmichael and Selbo 1999). It seems pollen tube pathway through synergids is quick and ephemeral, leaving no clues of its pass after fertilization.

Polar nuclei noted in diploid and chimeric samples were significantly bigger than the other nuclei of the embryo sac. This distinction of the polar nuclei dimensions was also observed in *E. esula*, in which they kept separated until fertilization (Carmichael and Selbo 1999). Polar nuclei often near the egg apparatus corroborates observations of their fusion in this location (Kapil and Bhatnagar 1994).

Nuclear endosperm formation was observed to begin at the mature embryo sac stage through the embryo formation. *M. esculenta* diploid and chimeric plants have presumably a triploid and a pentaploid endosperm, respectively. The triploid tissue exhibits nuclear development in earlier stages as generally described by Euphorbiaceae (Kapil and Bhatnagar 1994) and commented in *E. esula* (Carmichael and Selbo 1999). Pentaploid endosperm rises from a 4-nucleate central cell fertilized by one spermatic (Voigt-Zielinski et al 2012). These four polar nuclei could have their origin by meiotic and mitotic abnormalities, which can lead to apomixis (Koltunow 1993). Diploid and chimeric *M. esculenta* samples showed the same free nuclear organization pattern in earlier stages, and thereafter developed to a cellular type (Perera et al. 2012).

The pro-embryo had a finger shape in both samples, apparently following the slender configuration of the egg cell. Classification of embryo types usually considers first division as periclinal, followed by sequential divisions of basal and terminal cells (Maheshwari 1950). In Euphorbiaceae, Onagrad type is common; however, Asterad type was described for *E. esula* (Carmichael and Selbo 1999). It seems *M. esculenta* displays a different pattern of divisions at very early stages of embryo development, in which zygote divides vertically. This kind of division was classified as abnormal (Maheshwari 1950), and reported in some species of Loranthaceae (Kuijt 2012), but not for *Manihot*.

Embryo sac development, polyploidy and apomixis

Cassava cytochimeras with polyploidized L2 produced giga effect in reproductive organs, issue also observed in total polyploids (Fukuhara 2000; Karcz *et al.* 2000; Fortescue and Turner 2005; Hegarty et al. 2013). Ovule enlargement was accompanied by a slowdown of the embryo sac maturation. Thereby, when the embryo sac of diploid *M. esculenta* was at selected megaspore stage with an ovary having a 1 mm length, the chimeric plant was at megagametophyte differentiation; i.e., in an early step of development.

Ontogenetic research reveals that ovules are derived from the L2 apical shoot (Kwiatkowska 2008; Wang et al. 2011). L1 may be also involved (Koltunow and Grossniklaus 2003). Hence, L2

polyploidization of total polyploids or chimeras may influence gametic structures. Moreover, epigenetic interactions involving embryo sac precursor cells have led to reproductive shift to apomixis (Rodrigues and Koltunow 2005; Grimanelli 2012; Springer 2013).

Polyploidy increases apomixis frequencies in angiosperms (Carman, 1997), which was confirmed by Nassar (2006) and Nassar et al. (2010) in *M. esculenta*. Aposporic and somatic (Nassar et al 2011) types, and its facultative occurrence (Freitas and Nassar 2013) are evidence of apomixis in this species. Sexual and apomictic mechanisms are not excluding each other, and their coexistence within the same ovule has been noted in other plant families (Nogler 1984; Quarin et al. 2001; Bicknell and Koltunow 2004). However, apospory occurrence generates a competition were apomictic embryo usually prevails such as in *Paspalum* (Hojsgaard et al. 2013).

Feom very low frequencies of polyembryo sacs we may conclude that cytochimeric structure was not enough to trigger apomixis increase in cassava partial polyploids.

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