

Development of cassava periclinal chimera may boost production

N. Bomfim¹ and N.M.A. Nassar²

¹Programa de Pós-Graduação em Botânica, Universidade de Brasília, Brasília, DF, Brasil

²Departamento de Genética e Morfologia da Universidade de Brasília, Brasília, DF, Brasil

Corresponding author: N.M.A. Nassar
E-mail: nagibnassar@geneconserve.pro.br

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ABSTRACT. Plant periclinal chimeras are genotypic mosaics arranged concentrically. Trials to produce them to combine different species have been done, but practical results have not been achieved. We report for the second time the development of a very productive interspecific periclinal chimera in cassava. It has very large edible roots up to 14 kg per plant at one year old compared to 2-3 kg in common varieties. The epidermal tissue formed was from *Manihot esculenta* cultivar UnB 032, and the subepidermal and internal tissue from the wild species, *Manihot fortalezensis*. We determined the origin of tissues by meiotic and mitotic chromosome counts, plant anatomy and morphology. Epidermal features displayed useful traits to deduce tissue origin: cell shape and size, trichome density and stomatal length. Chimera roots had a wholly tuberous and edible constitution with smaller starch granule size and similar distribution compared to cassava. Root size enlargement might have been due to an epigenetic effect. These results suggest a new line of improved crop based on the development of interspecific chimeras composed of different combinations of wild and cultivated species. It promises boosting cassava production through

exceptional root enlargement.

Key words: Wild *Manihot*; *Manihot fortalezensis*; Starch quality; Stem apical meristem; Root size; Interspecies epigenetic interaction

INTRODUCTION

Cassava is the principal food for more than 800 million people in the tropics and subtropics (FAO, 2013). Improvement programs have concentrated on simple clonal selection and inter-cultivar hybridization (Ceballos et al., 2004; Nassar and Ortiz, 2009). Interspecific hybridization to transfer characters is difficult due to species barriers and the need to break them (Nassar et al., 1996; Rieseberg and Carney, 1998; Ulukan, 2009). Furthermore, the introduction of undesirable characters from the wild form is another drawback, which may need a number of generations and decades to get rid of this undesirable linkage-drag. (Rieseberg and Carney, 1998; Ulukan, 2009). Polyploidy has little to offer genetic enhancement because new traits were not brought by this technique (Sreekumari et al., 1999; Aversano et al., 2013). There have been suggestions to produce chimeras to combine useful characters from wild and cultivated forms such as resistance to insects found in epidermal tissue (e.g., host plant resistance to insects found in epidermal tissue), but practical results are yet to be achieved (Goffreda et al., 1990; Marcotrigiano, 1997b; Lindsay et al., 1995; Nassar and Ortiz, 2010).

Sporadic interspecific chimeras have been documented by some authors (Stewart et al., 1972; Burge et al., 2002; Zhou et al., 2002; Chen et al., 2006; Zhang et al., 2007). They arose from adventitious shoot formed on a graft union of a scion and rootstock, on field or tissue culture conditions, for different purposes, but not for formation of advantageous varieties, under plant breeding view, and with no significative economic value (Marcotrigiano and Gouin, 1984; Goffreda et al., 1990; Kaddoura and Mantell, 1991; Marcotrigiano, 1997b; Burge et al., 2002; Chen et al., 2006).

There are sporadic reports of interspecific chimeras that had arisen from an adventitious shoot formed on a graft union of a scion and rootstock (Stewart et al., 1972; Burge et al., 2002; Zhou et al., 2002; Chen et al., 2006; Zhang et al., 2007). A few chimeras have also been produced by tissue culture, but they have no economic value (Marcotrigiano and Gouin, 1984; Kaddoura and Mantell, 1991; Chen et al., 2006).

In our earlier study, we reported the first periclinal chimera in cassava between *Manihot fortalezensis* and cassava cultivar UnB 201, which was highly productive (Nassar and Bomfim, 2012, 2013). In the present paper, we report a second interspecific periclinal chimera involving the *Manihot* species *M. fortalezensis* and another cultivated *Manihot esculenta* cultivar UnB 032, which also differ in chromosome number and ploidy level. We used the UnB 032 clone, which is known for its good food quality and moderate productivity, but it is susceptible to borers and vulnerable to drought, and the wild *M. fortalezensis*, that has the same resistance to borers and is well adapted to drought because of its deep-penetrating roots (Nassar et al., 2010).

MATERIAL AND METHODS

Plant material

The cassava cultivar UnB 032 and wild species *M. fortalezensis* were used to induce

interspecific chimeras. UnB 032 is a low shrub of 1.5 m and yields 2-3 kg/year, and is diploid with $2n = 36$, while *M. fortalezensis* is an erect woody shrub, ca. 4 m tall with deep fibrous roots, native to savanna forests of Ceará (Caatinga), Brazil, where drought predominates (Nassar et al., 2010), and is triploid with $2n = 54$. Both are maintained at the living collection of the Universidade de Brasília (UnB).

Samples for determining layer genotype were collected from 9 clone plants replicated with stalks from the chimera plant (arising from the graft union region of UnB 032 and *M. fortalezensis*) and parental species, UnB 032 and *M. fortalezensis*. All clone plants were planted at the same time and under sunlight for comparative studies.

Chimera development

Stems of *M. fortalezensis* were whip grafted onto 40 rootstocks of UnB 032. Two months later, a cut parallel to the graft union was made leaving on a 5-mm scion. The graft unions and remaining graft tissue were covered by cotton, moistened with 4 drops 0.01% α -naphthaleneacetic acid, and repeated for 7 days (adapted from Kaddoura and Mantell, 1991) (Figure 1).

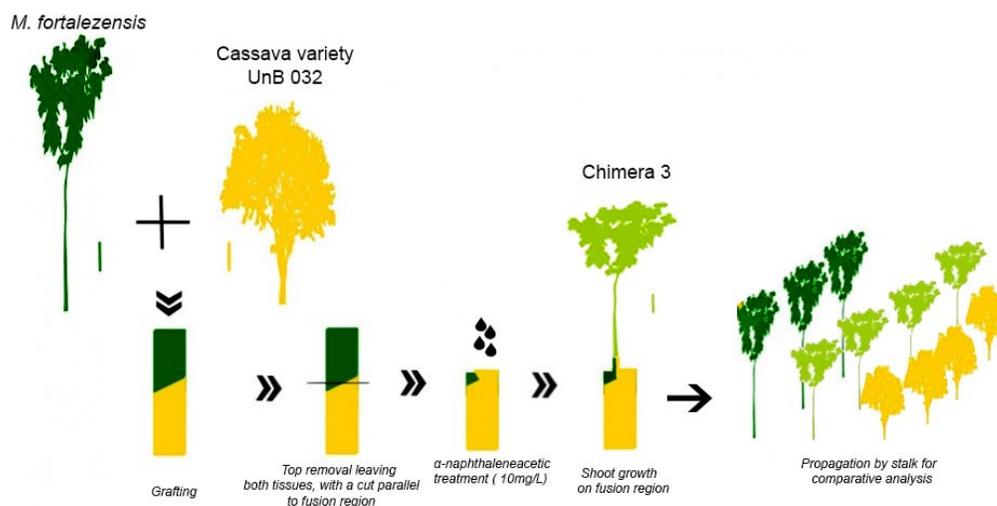


Figure 1. Chimera development by grafting.

At the end of the growing season, shoots that exhibited distinct morphological characters were propagated vegetatively for determination of tissue constitution. To characterize the layers, we analyzed the morphology of fruits, leaves and roots, meiosis in flower buds, mitosis in root cuttings, transversal petiole sections, longitudinal stem apex sections, and root production of chimera were compared with those of the parent species.

Morphological characterization

Nine mature clone plants of 12 months were examined for morphological character-

ization based on distinctive characters of habit, leaves, inflorescence, fruits and roots, which distinguish cassava from other wild species (Rogers and Appan, 1973; Nassar et al., 1996).

Cytogenetic analysis

Male buds and closed mature staminate flowers of both parental plants and chimera were collected at 8:00 a.m. (daylight saving time), fixed in Carnoy solution, fixed in 70% ethanol, smeared and stained with 1% acetocarmin. Root tips were collected from germinating chimera cuttings, pre-treated with 0.25% colchicine in distilled water for 2 h, fixed in Carnoy solution, hydrolyzed in 5 N HCl for 10 min, smeared, and air-dried before being stained with 5% Giemsa (adapted from de Carvalho and Guerra, 2002).

Anatomical investigation

Shoot apical meristems (SAMs), petioles and leaf blades were analyzed to help determine the layer genotype of the chimera considering meristem organization in 3 independent layers: L1 (outermost layer), L2 (next layer) and L3 (inner layer) (Marcotrigiano, 1997a). SAMs longitudinal sections, leaf blade surface and transversal sections of petioles and roots were analyzed. All materials were fixed using FAA solution (formaldehyde, 70% ethanol and glacial acetic acid; 5:5:90, v/v) (Johansen, 1940) and permanently mounted in synthetic resin (Paiva et al., 2006). SAMs were embedded in butyl acetate series and paraffin (Kraus and Arduin, 1997), and 8- μ m thick sections were obtained with a rotary microtome RM 2145 (LEICA, Germany) and double-stained with safranin and fast-green series (Kraus and Arduin, 1997). Leaf blade surface was released by glacial acetic acid and hydrogen peroxide solution and stained with 1% safranin (Kraus and Arduin, 1997). Petioles were free-hand cut on a microtome, and stained with 1% safranin and 1% aqueous alcian blue (Kraus and Arduin, 1997). Roots were free-hand sectioned and treated with Lugol's solution (Johansen, 1940). Images highlighting trichomes were provided by a JEOL JSM 700 1-F scanning electron microscope, after gold coating in a Leica EM SCD 500 metalizer.

Epidermal features as leaf blade cell shape, trichomes, stomatal length, and petiole ordinary epidermal cell width helped determine L1 layer origin, once they are derived from it (Goffreda et al., 1990; Burge et al., 2002). At least 4 clone plants were sampled. Stomatal length and trichome frequency were determined on the 4th and 6th leaves, based on nail polish imprints from the abaxial leaf surface; while petiole cell width was measured on fresh 6th leaf. Starch granule size and distribution on 3-cm diameter roots were observed to evaluate edible potential in 2 clone plants.

Photographs were taken under a light microscope (Leica DM2500, Germany). Shoot apices were photographed using interferential contrast.

Root production evaluation

Root weight was measured to estimate the potential production of Chimera 3. Two clone plants had all roots weighed 12 months after planting stalks.

Statistical analyses

Measurements were made using Image-Pro Plus version 4.5.0.29 and statistical analy-

sis was performed with the Assistat 7.6 beta software. Variations on stomatal length, petiole epidermal and cortical cell width were evaluated by analysis of variance (ANOVA) followed by Tukey's test ($P = 0.05$) to compare means.

RESULTS

Morphological characterization

Chimera 3 showed homogeneous characters on the whole plant, which were very distinctive characters when compared to parents (Table 1): semi-erect shrub ca. 4 m with semi-decumbent branches versus erect shrubs ca. 8 m in *M. fortalezensis* and ca. 2.5 m in UnB 032 (Figure 2 A-C); brevipeltate leaves with obovate lobes versus peltate obovate leaves in *M. fortalezensis* and emarginated oblong-lanceolate leaves in UnB 032, and tuberous cylindrical roots reaching 90 cm versus fibrous roots reaching 1.5 m in *M. fortalezensis* and tuberous conical roots of 30 cm in UnB 032 (Figure 2D-F).

Table 1. Morphological characterization of the Chimera 3 and parental species (*Manihot fortalezensis* and UnB 032).

	<i>M. fortalezensis</i> (FFF)	Chimera 3(EFF)	Cassava UnB 032 (EEE)
Plant habit and stems	Erect shrub normally solitary, ca. 8 m, 7-10 cm diameter, erect branch, dichotomously branching only in the apical part. Red young branch. Slightly enlarged nodes, and not enlarged stipels scars on stem.	Semi-erect shrub ca. 4 m, 1-2 central stems from the same base, 2-5 cm diameter, semi-decumbent branch, dichotomously and trichotomously branching. Purple young branch. Upper part of stems tetragonal. Enlarged nodes and stipels scars.	Erect shrub ca. 2 m, 2-3 central stems arising from the same base, 1-5 cm diameter, erect branch, dichotomously and trichotomously branching. Green reddish young branch. Enlarged and small nodes and stipels scars.
Leaf	3, 5 ou 7 lobes, normally peltate, soft green adaxial face, and glauca green abaxial face. Central lobes broadly obovate with apiculate apex. Petiole length 10-25 cm.	Palmately leaf with 1 to 7 lobes, normally 5 lobes, brevi-peltate, deep green adaxial and abaxial face. Central lobes obovate with apiculate apex. Petiole length 10-40 cm.	1-5 lobes, emarginate, deep green adaxial and abaxial face. Central lobes oblong-lanceolate with acuminate acute apex. Petiole length 7-30 cm.
Inflorescence	Panicle with 2 lateral branches from the same base, with pistillate flowers in the central panicle. Flowers length 15 mm. Ovaries no winged.	Inflorescence in panicle with 2-3 lateral branches from the same base. Flowers length 14 mm. Ovaries winged.	Inflorescence in panicle with 2-3 lateral branches arising from the same base. Flowers length 9 mm. Ovaries with red wings.
Fruit	Globose fruits not winged, except on base, being slender straight wings, with peduncle forming a globe near to the fruit.	Globose fruits with slender straight wings in whole fruit, with peduncle forming a globe near to the fruit.	Semi-esferic fruits with ondulate wings in whole fruit, with a thickened peduncle.
Root ¹	1.5 m. Predominantly narrow fibrous roots and rare 2.5 cm diameters roots.	Tuberous cylindrical roots reaching 90 cm. Predominantly tuberous roots with 5 cm diameter. Light cream periderm.	Tuberous conical roots reaching 30 cm. Predominantly tuberous root with 7 cm diameter (base). Light cream periderm.

¹Twelve-month-old plants. The three letters (FFF, EFF or EEE) represent L1, L2 and L3 layers, respectively. F = *M. fortalezensis*; E = *Manihot esculenta*.

Cytogenetic characterization

Meiotic metaphase I and mitotic metaphase analyses showed $2n = 54$ in Chimera 3 (Figure 3), demonstrating the same chromosome number as *M. fortalezensis* in inner tissues of the chimera.

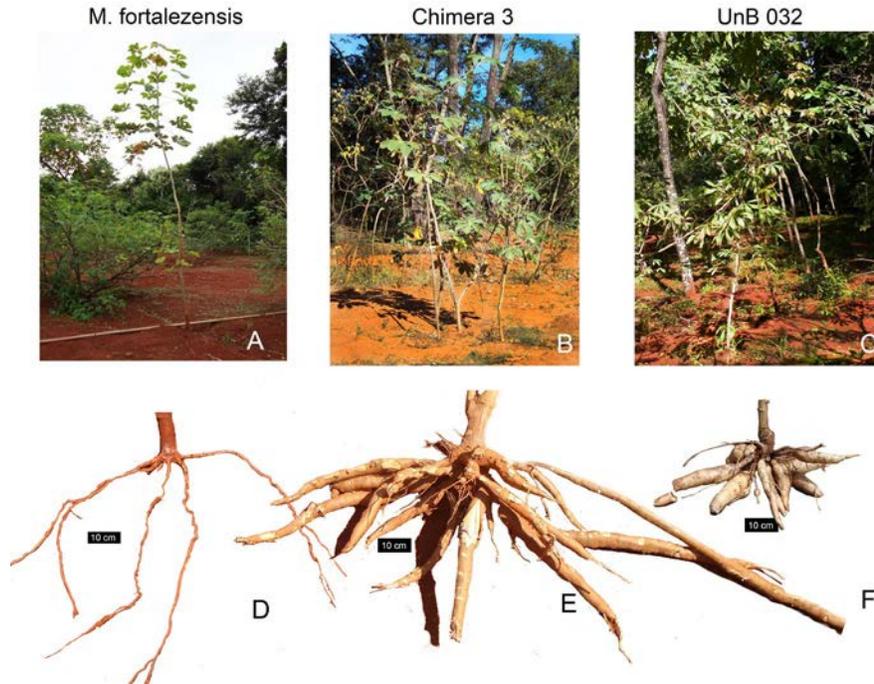


Figure 2. Whole plant and roots of Chimera 3 (centre) compared to parental species *Manihot fortalezensis* (left) and *Manihot esculenta* (cassava variety UnB 032) (right): **A.** erect shrub normally solitary ca. 8 m. **B.** Chimera 3: semi-erect shrub ca. 4 m, with branches. **C.** Cassava variety UnB 032: erect shrub with branches ca. 2 m.

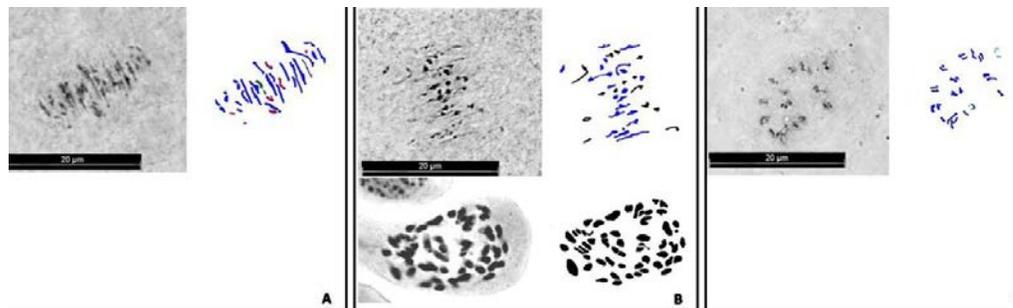


Figure 3. Meiotic metaphase I (upper part) of Chimera 3 and parental species, and mitotic metaphase of Chimera 3. **A.** *Manihot fortalezensis*; **B.** Chimera 3; **C.** UnB 032. Colored marks represent bivalents and univalents on meiotic metaphase I (blue = bivalents; black = univalents). On mitotic metaphase all black marks represent a chromosome. Bar = 20 µm.

Anatomical investigation

Stem apical meristem, leaf blade and petiole

In the stem apical meristem, the three species showed no size difference between their

own three outermost meristem cell layers, but it was possible to note a larger cell size in Chimera 3 and *M. fortalezensis* when compared to UnB 032 cells (Figure 4A-C).

Leaf epidermis of Chimera 3 resembled that of cassava variety UnB 032 and differed from that of *M. fortalezensis*: it showed regular ordinary cell shape (Figure 4D-F) and tector trichomes over adaxial leaf veins, which are cassava-specific features (Figure 4G-I). Stomatal length and petiole epidermal cell width were intermediate when compared to parents (Table 2 and Figure 4D-F, J-L). Furthermore, Chimera 3 could be distinguished by cell content of inner petiole tissue, showing fewer starch granules but more calcium oxalate druses than in parents, in general (Table 2).

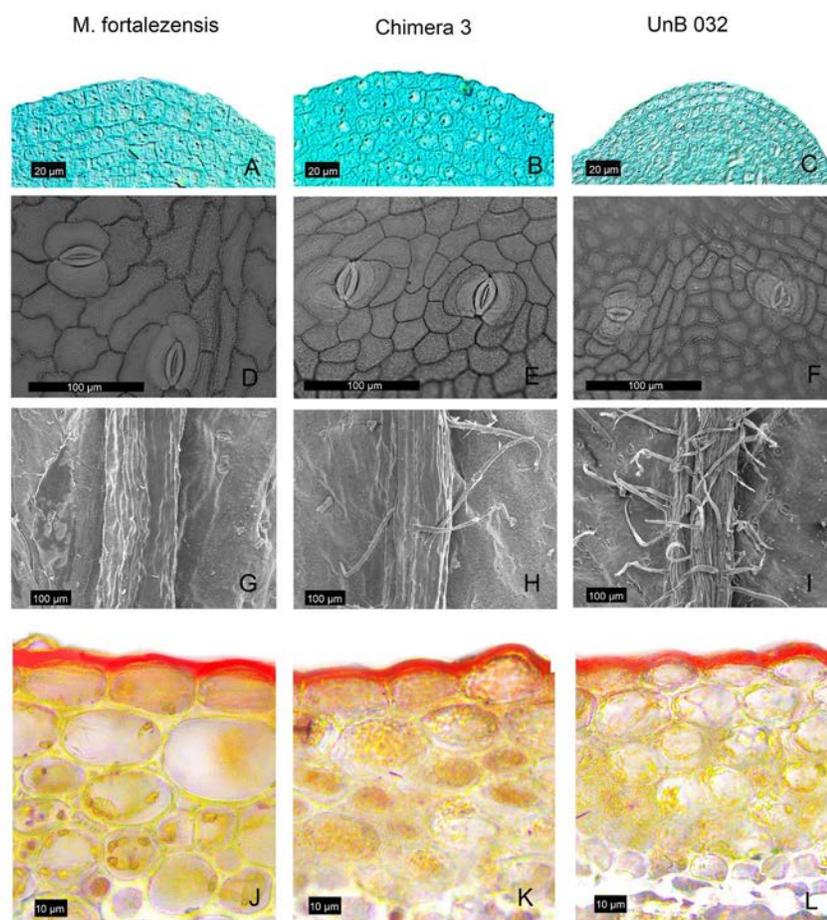


Figure 4. Anatomical characterization of Chimera 3 compared to parents: *Manihot fortalezensis* and cassava cultivar UnB 032. **A. B. C.** Cell layers on stem apical meristem. No different cell size between layers. **D. E. F.** Ordinary cell shape and stomatal length. Regular ordinary cell shape in Chimera 3, as in cassava variety UnB 032. Stomatal intermediary length in Chimera 3. **G. H. I.** Trichome on leaf adaxial face veins. Trichome presence on Chimera 3, distinguish it from *M. fortalezensis*. **J. K. L.** Petiole epidermal and cortical cells. Intermediary width Chimera 3 epidermal cells and similar to UnB 032 cortical cells. Thick cuticle (red stained) on all plants.

Table 2. Leaf characterization showing distinct anatomical characters of Chimera 3 compared to parental species: *Manihot fortalezensis* and cassava cultivar UnB 032, on the 6th node.

	<i>M. fortalezensis</i> (FFF)	Chimera 3 (EFF)	Cassava UnB 032 (EEE)
Leaf epidermal cells size and shape (adaxial view)	Larger cells with sinuous anticlinal walls	Medium cells with regular anticlinal walls	Small cells with regular anticlinal walls
Stomatal length (abaxial view)	18.10 ± 1.643 ^a μm	17.54 ± 2.167 ^{ab} μm	14.73 ± 1.103 ^b μm
Trichomes over the leaf blade veins (adaxial view)	Absent	Low frequency	High frequency
Petiole epidermis	All species show non-stratified epiderm with thickened cell walls and cuticle. The species differ only in shape and width		
Cell shape	Tabular to oval cells frequently intercalated by isodiametric cells	Oval to isodiametric cells	Isodiametric cells
Cell width*	24 ± 4.636 ^a μm	19.57 ± 2.5298 ^{ab} μm	14.96 ± 1.4212 ^b μm
Petiole cortex	The three species has a parenchymatous tissue intercalated by lamelar collenchyma tissue, showing the same number of cell layers in the three (4-7 at outermost parenchyma, 3-5 at collenchyma and inner parenchyma). The difference between the species is noted in the cell size, shape of the outermost parenchyma, and content of the inner parenchyma. The three species has calcium oxalate druses at the inner parenchyma, but <i>M. fortalezensis</i> show a lower frequency of this salt		
Outermost parenchyma			
Cell shape	Oval cell	Oval cell	Isodiametric cell
Cell width*	29.70 ± 1.23 ^a μm	21.93 ± 0.46 ^b μm	18.60 ± 0.39 ^b μm
Collenchyma			
Cell width*	20.37 ± 4.647 ^a μm	13.79 ± 1.697 ^b μm	13.16 ± 1.7291 ^b μm
Inner parenchyma			
Cell width*	32.98 ± 2.98 ^a μm	30.42 ± 3.568 ^a μm	28.95 ± 0.9115 ^a μm
Cell content	Low frequency of calcium oxalate druses	Frequent calcium oxalate druses	Frequent calcium oxalate druses
Endodermis	There is a different starch grains and calcium oxalate druses frequency between the species.		
Cell content	Starch grains and calcium oxalate druses very frequent	Starch grains infrequent and calcium oxalate druses frequent	Starch grains and calcium oxalate druses infrequent
Pericyclic fibers	Poligonal fibers often gelatinous		
Petiole vascular bundles	Bicolateral bundles in ring, having a phloem with some laticifers, easily to notice in <i>M. fortalezensis</i>		
Cell content	There were differences in the content of parenchyma phloem cells		
Vascular bundle (#)	Lacking druses	Lacking druses	Druses seldom noted
Xylem	8	9 to 11	9 and 10
Xylem vessel rays	Xylem composed by vessel of the same diameter, but of varied number of rays		
Petiole medulla	7 to 10	6 to 8	5 to 8
Cell content	Polyhedral cells containing starch grains and calcium oxalate druses near of the bundles		
	Differing only in cell content.		
	Starch grains frequent	Starch grains seldom noted	Starch grains seldom noted
	Druses seldom found	Druses often noted	Lack druses

*Mean and standard deviation. Different letters in the same line indicate significant differences among means according to the Tukey range test ($P \leq 0.05$). Stomatal length: N = 5; petiole cell width: N = 4. # = number of vascular bundles on petiole transversal section.

Root production and starch granules size and distribution

Chimera 3 production was 14 kg per plant, in a 12 month-old plant, while cassava variety UnB 032 with the same age produced 2.1 kg per plant, denoting vigorous plant growth. Chimera 3 reached 4 m high, while cassava variety UnB 032 was 2 m tall under the same conditions.

Anatomic analysis indicated the Chimera 3 starch granules were similar as in cassava with round shape but differed in diameter (Table 3) compared to cassava UnB 032. Starch granules were present in whole xylem, except vessel elements, axial parenchyma and fibers of primary xylem, while in cassava there were granules in axial parenchyma and a lesser portion of primary xylem without starch granules. Phloem also displayed parenchyma and fiber cells completely filled with starch granules, except sieve tubes, companion cells and laticifers. Chimera 3 starch granules were small, while cassava granules were medium sized, following Lindeboom et al. (2004) size classification.

Table 3. Starch granules diameters (means \pm standard deviation, minimum and maximum) of Chimera 3 and cassava cultivar UnB 032 on xylem parenchyma cells.

	Diameter (μm)		
	Means \pm standard deviation	Minimum	Maximum
Quimera 3	6.4 \pm 2.6	2.8	11.9
UnB 032	15.3 \pm 4.4	7.6	23.4

DISCUSSION

Chimera production potential

Chimera 3 showed a set of features distinct from that of the two parental species (Table 1), which can be considered new features, because they are not exhibited by the parents: height, branching, leaf, fruit and root shape. It showed to be more vigorous than both parental species, once it produced very large and quite edible roots, which were almost 7-fold larger than cassava (UnB 032), while the wild species *M. fortalezensis* did not yield tuberous roots. Chimera 3 root phenotype was long, tuberous and weighted 14 kg per plant, while cassava parent roots, although tuberous, were short and yielded only 2.1 kg per plant with the same age. Apparently, an interaction of the genotypes resulted in a novel feature. We hypothesize that this new phenotype ensues from an epigenetic interaction between the two interspecies coexisting in the chimera. Recently, epigenetic alterations causing heritable complex traits such as plant height have been reported (Johannes et al., 2009). Epigenetic alterations, on specific locus, were also noted in plants, where two species coexisted on the same individual, being attributed to the interaction between species (Wu et al. 2013). This phenomenon was cited as a possible molecular basis for phenotype changes as noted in grafts, called graft hybridization, and heterosis (Tsafaris et al., 2008; Johannes et al., 2009; Wu et al., 2013).

Chimeric roots proved to be edible according to transversal sections showing parenchyma cells filled with round starch granules. Starch granule distribution of this chimera denotes an edible root very similar to that of cassava. They also showed small granules, which allows faster starch hydrolysis, suitable for the manufacture of fine printing paper and biodegradable films (Lindeboom et al., 2004).

Chimeric tissue identification

Stem apical meristem

Considering that a chimeric plant is a mosaic with genetically different cells existing in the SAM (Marcotrigiano, 1997a), Chimera 3 apical meristem, when anatomically analyzed, did not showed size difference between layers. That is coherent when ploidy level difference is evaluated (only one set of chromosomes, i.e., 1n). All layers showed the same size, which were similar to *M. fortalezensis*. This is expected, once cell size control of L1 (the outermost layer) is not autonomous, meaning that it depends on adjacent cell layer size (Savaldi-Goldstein et al., 2007; Marcotrigiano, 2010).

L1 tissue constitution by morphological and anatomical epidermal cell assessment

The presence of wing on the fruits allows to determine L1 constitution (Tabela 1), because this “diagnostic trait” belongs to cassava (Rogers and Appan 1973; Nassar et al., 1996). Additionally, epidermal traits, such as ordinary cell shape and width, trichomes, and stomatal length and petiole cell width, were used to determine L1 constitution, once they are derived from it (Table 2 and Figure 4) (Satina et al., 1940; Goffreda et al., 1990; Evert, 2007; Marcotrigiano, 2010).

Ordinary epidermal cell shape was identical to cassava variety shape and different from that of *M. fortalezensis* (Figure 4), indicating the cassava variety UnB 032 on the epidermis.

Trichomes were found only on Cassava variety UnB 032 and Chimera 3 (Figure 4G-D), denoting a cassava presence on the epidermis. However, trichomes were present in a lower frequency on Chimera 3, which probably demonstrate an interaction between cassava pubescent epidermis and *M. fortalezensis* glabrous tissues (inner tissues).

Stomatal length and petiole epidermal cell width demonstrated intermediary cell size to parental species *M. fortalezensis* and *M. esculenta* (cassava variety UnB 032), which is in accordance to the stem apical meristematic cells, since it was not noted cell size difference between layers (Figure 4A-C) and epidermal cell size is controlled by internal tissue development (Savaldi-Goldstein et al., 2007; Marcotrigiano, 2010).

L2 and L3 layer constitution by cytogenetic analysis

Meiotic chromosome counts allowed L2 layer characterization, because gametes usually derive from the L2 layer (Satina et al., 1940; Goffreda et al., 1990). The meiotic metaphase 1 in pollen mother cells showed 54 chromosomes on the Chimera 3 as in *M. fortalezensis* (Figure 3), while in cassava (UnB 032), it was $2n = 36$. Therefore, is deduced that L2 layers were constituted by *M. fortalezensis*.

Chromosome counts on adventitious root tips allowed determine the innermost layer constitution, because this roots originate in pericycle, which is derived from L3 layer (Evert, 2007; Medina et al., 2007; Bomfim et al., 2011). On this counting, 54 chromosomes were noted, which confirms the same ploidy of parental species *M. fortalezensis*.

Those analyses demonstrated the Chimera 3 constitution as EFF, which represents L1, L2 and L3 layers, respectively, being “E” to *M. esculenta* UnB 032, and “F” to *M. fortalezensis*. Judging by the uniformity of characters on whole plant, it was deduced that Chimera 3 has a periclinal arrangement, where whole epidermis is constituted by *M. esculenta* (cassava variety UnB 032).

CONCLUSIONS

Chimera development offers an important tool for crop improvement with a great potential to boost production, especially in cassava, which has the advantage of vegetative propagation.

This method is useful, specially, to combine species that by conventional methods, as hybridization, are hardly to combine triploid species. The chimera seemed to be a valuable material to study the mechanism under the great increase of roots, furthermore to be quite

useful to cassava yield. We demonstrate a new approach to plant breeding based on chimera interpecific synthesis having two species tissues.

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