

## Periclinal chimera technique: new plant breeding approach

P.M. Gakpetor<sup>1</sup>, H. Mohammed<sup>1</sup>, D. Moreti<sup>2</sup> and N.M.A. Nassar<sup>3</sup>

<sup>1</sup>Hawassa University, Hawassa, Ethiopia

<sup>2</sup>Empresa Matogrossense de Pesquisa, Assistência e Extensão Rural, Cuiabá, MT, Brasil

<sup>3</sup>Universidade de Brasília, Brasília, DF, Brasil

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

Genet. Mol. Res. 16 (3): gmr16039790

Received August 3, 2017

Accepted August 11, 2017

Published September 21, 2017

DOI <http://dx.doi.org/10.4238/gmr16039790>

Copyright © 2017 The Authors. This is an open-access article distributed under the terms of the Creative Commons Attribution ShareAlike (CC BY-SA) 4.0 License.

**ABSTRACT.** Plant interspecific periclinal chimeras are a mosaic formed by tissues from two species. They are manipulated here as an efficient plant breeding tool for cassava root yields. In this study, plants synthesized from two chimeras, designated as chimera 2 and chimera 4, were characterized morphologically and cytologically to unravel the origin of their tissue layers (L2 and L3). Root yield of the two chimeras was also evaluated. Chimera 2 that was developed from graft union between *Manihot fortalezensis* (F) as scion and *M. esculenta* (E) as rootstock and the same in chimera 4 was developed from grafting triploid cassava cultivar ( $2n = 54$ ) (C) as scion and *M. pohlii* (P) ( $2n = 36$ ) as rootstock. A new method of inducing interspecific chimeras without using hormones was also tested in this study. Five combinations between four cassava cultivars on one side and *M. fortalezensis* and an interspecific hybrid (*M. glaziovii* x *M. esculenta*) on the other side were experimented to determine compatibility between the parents. Wild species always gave L2 and L3, independent of being used as rootstock or scion. L3 is responsible for producing pericycle. Thus, its performance

was different in each chimera due to specific epigenetic interaction. Of 48 grafts, it was obtained one chimera giving a percentage of 2.1% that is little lower than using hormones but much efficient to use. Chimera induction efficiency in this investigation was the same when using hormones. Thus, our new, less labor, and more cost-effective technique is as much efficient as hormones and is much potential to employ as an effective plant breeding method boosting cassava root yield.

**Key words:** Cassava (*Manihot esculenta*); Histogen layers; Periclinal chimera; Root production

## INTRODUCTION

Cassava is the principal food for poor people in the tropics and subtropics. In 2014, it was grown on an area of 23 million hectares producing 268 million ton and feeding one billion individuals all over the world (Fao Ifad, 2014).

Cassava (*Manihot esculenta* Crantz) is a perennial dicotyledonous shrub that belongs to the family Euphorbiaceae (Rogers and Appan, 1973). There are 98 species of *Manihot*, all having  $2n = 36$  chromosomes.

The wide genetic diversity in the *Manihot* species has been utilized by introgressing important traits such as resistance to diseases (cassava mosaic virus disease and bacterial blight), high protein content, apomixis to cassava cultivars through interspecific hybridization (Nassar, 2007; Nassar et al., 2008; Nassar and Ortiz, 2009; Akinbo et al., 2015). However, there are still limitations for breeding cassava to produce high yielding edible roots. The world average yield is only 11.2 ton/ha (Fao Ifad, 2014) compared to the potential of 90 ton/ha (Akinbo, 2010; Akinbo et al., 2015).

Then, we need to look for new approaches to the improvement of this crop productivity. Grafting scion of the vigorous interspecific hybrid of the perennial wild tree *Manihot glaziovii*, onto cassava rootstock has improved root production by 30 to 100% (De Bruijn and Dharmaputra, 1974; Nassar and Ortiz, 2010). However, the instability of this increase due to the necessity of realizing grafting every year was an obstacle of adopting this technique.

Preclinical chimeras were noted by Nassar et al. (2012) to emerge from the graft junctions of such combinations, and it appeared to be a more promising method of developing highly productive cassava varieties and perpetuating this high productivity.

A chimera is a meristem with different genetic tissues in one or more of its layers. The components of plant grafts in a chimera do not lose their integrity but coexist harmoniously (Buder, 1911; Goffreda et al., 1990; Hirata et al., 2000).

Contrary to sectorial chimeras, periclinal ones are relatively stable and can be vegetatively propagated as new varieties. Based on this, the trials were started.

Two interspecific chimeras of cassava have recently been produced from grafts of the wild triploid cassava species, *M. fortalezensis*, on rootstocks of two cassava cultivars at Universidade de Brasília (UnB), Brazil. By using hormones, they gave 3- to 7-fold increase in tuber production relative to donor plants (Nassar and Bomfim, 2013; Bomfim and Nassar, 2014).

The inclusion of other wild species and cassava cultivars in grafting may provide new opportunity to improve productivity and other important traits of the crop. Inducing chimeras without the use of hormones may also serve as a more cost-effective method. This study was,

therefore, designed to test the efficiency of developing cassava chimeras without the use of hormones in graft combinations involving various wild *Manihot* species and cassava cultivars under production and to study the histogenic differentiation of the various tissue layers of these chimeras and to assess their productivity.

## MATERIAL AND METHODS

The experiment was conducted at the experimental station of the UnB, and at Cáceres, Mato Grosso, Brazil, in Latossoil. Two wild *Manihot* species (*M. pohlii* and *M. fortalezensis*) and one interspecific hybrid (*M. glaziovii* x *M. esculenta*) and five *M. esculenta* cultivars (UnB 201, UnB 205, UnB 031, UnB 530 p, and UnB 530-19) were used in this study.

### Synthesizing periclinal chimera

Scions and rootstocks with similar size and vigor were whips grafted in August 2016. Different graft combinations were made using the varieties as mentioned above (UnB 201, UnB 203, UnB 205, and UnB 530 p), one wild species *M. fortalezensis*, and an interspecific hybrid (*M. glaziovii* x *M. esculenta*). The scion was cut using a knife in a slanted position closer to a bud, and the rootstock was cut in the opposite direction. The scion and the rootstock were placed in close contact taking into consideration the juxtaposition of the scion and the rootstock buds. The buds were placed in close contact for interaction. A cello tape was used to fasten and hold them together (Figure 1). All auxiliary shoots and adventitious shoots arising from anywhere except near the graft union were removed as they appeared and this was done to prevent competition with chimera for water and nutrient. Shoot induction and chimera induction rates of the various graft combinations were determined, and compatible combinations of parents were identified.

Plant chimeras were identified based on alterations in stems and leaf morphology. Stem and leaf size and shape were used to differentiate between chimeras and their donor parents using previously established methods (Carlson and Chaleff, 1974; Hirata et al., 2000; Nielsen et al., 2003; Hashimoto-Freitas and Nassar, 2013).

Since the parental species used to synthesize chimeras were of different chromosome number, counting of the chromosomes was done at mitosis and meiosis to identify the origin of every layer.

Two-year-old plants vegetatively reproduced from cuttings of two chimeras designated as chimera 2 and chimera 4, induced in August 2013 and using hormones (Figure 1), were characterized morphologically and cytologically in August 2016 to determine the distinctiveness of the chimeras and to unravel their tissue origin and their productivity. The two chimeras already produced from 2014 grafting, namely chimera 2 and chimera 4, were analyzed morphologically and cytologically by counting chromosome numbers from root tips and anther meiosis. Chimera 2 was developed from graft union between *M. fortalezensis* (2n = 54) as the scion on *M. esculenta* UnB 031 (2n = 36) as the rootstock, and chimera 4 was developed from grafting *M. pohlii* (2n = 36) on *M. esculenta* UnB 530-19 (2n = 54).

Morphological characters of the chimeras were examined for growth habit and stem, leaf, inflorescence, fruit, and root characteristics and compared to those of their donor parents to determine the distinctiveness of the chimeras. Roots of two plants of each chimera were also weighed to determine their good root productivity as compared to the cassava parent.

Meiotic chromosome counts of anthers allow L2 characterization because gametes are usually derived from the L2 layer (Satina et al., 1940; Goffreda et al., 1990), while mitotic chromosome counts on adventitious root tips allow the determination of the L3, since roots originate from the pericycle, which is derived from L3 layer (Medina et al., 2007; Nassar and Bonfim, 2013). Therefore, meiotic chromosome counting of L2-derived anthers and mitotic chromosome counting of L3-derived adventitious root tip cells were used for cytological characterization because of the different chromosome number of the donor plants of both chimera shoots as given above. Twenty plates (cells) were observed for each of the chimeras, and chromosome configurations in metaphase were also observed to judge meiotic regularity.

Tetrads were examined too to judge regularity of meiosis. A total 10 male floral buds with a size between 2-3 mm were observed for this end. The presence of four tetrads of microspores without any micronuclei was considered normal tetrad and the presence of micronuclei with the tetrads, or formation of dyads and triads with or without micronuclei was considered a sign of irregular meiosis.

## RESULTS AND DISCUSSION

### Chimera synthesis

To induce chimera shoots without hormones, graft combinations of cassava and wild *Manihot* species were carried out. Five different kinds of interspecific combinations were used of 223 grafts, and 31 grafts produced shoots giving mean graft compatibility efficiency of 13.9%. Thirty-nine shoots were induced from the 223 graft unions with low shoot induction efficiency of 17.5%. Only one chimera was produced from these 223 grafts (mean of 0.45% chimera induction efficiency).

The most efficient combination was graft combination 1 (Figure 1), with *M. fortalezensis* as the scion and with *M. esculenta* UnB 031 as the rootstock. Eighteen shoots were induced from 48 grafts (37.5% shoot induction efficiency), 16 from separate grafts (one shoot from the scion section of each graft), and two shoots from one graft (one shoot from the rootstock and one from the scion section of the graft). Among these two shoots, one was identified as chimera based on stem and leaf morphology (Figure 1 and Table 1), which was different from that of the two parental species used, *M. esculenta* cv. UnB 031 and *M. fortalezensis* (Figure 2 and Table 2). The percentage of obtaining chimera, in this case, is 2%. The chimera had lighter green, slightly coriaceous (membranous) leaves compared with deep-green leaves of *M. esculenta* cv. UnB 031 and soft glaucous green leaves of *M. fortalezensis*, and the stem had larger nodes as compared to that of parents.

This chimera is similar to chimera 2, induced in 2012 using hormones (*M. fortalezensis* x *M. esculenta* var. UnB 031) and characterized in this study. The four chimeras obtained so far at UnB are, therefore, chimera 1 induced in 2012 from *M. fortalezensis* x *M. esculenta* UnB 201 (Nassar and Bonfim, 2013), chimera 3 induced in 2012 (*M. fortalezensis* x *M. esculenta* UnB 032) (Bonfim and Nassar, 2014), and two others (chimera 2 and chimera 4) induced in August 2013 characterized in this investigation. Graft combination 1 had a chimera induction efficiency of 2.1%; one chimera was produced from 48 grafts that are lower than that the 5% obtained by Nassar and Bonfim (2013) (2 chimeras of 40 grafts) using the cassava cultivar UnB 201 as the rootstock and *M. fortalezensis* as the scion with hormones applied on the graft junction. However, our method is more cost-effective since the cost of hormones was avoided.

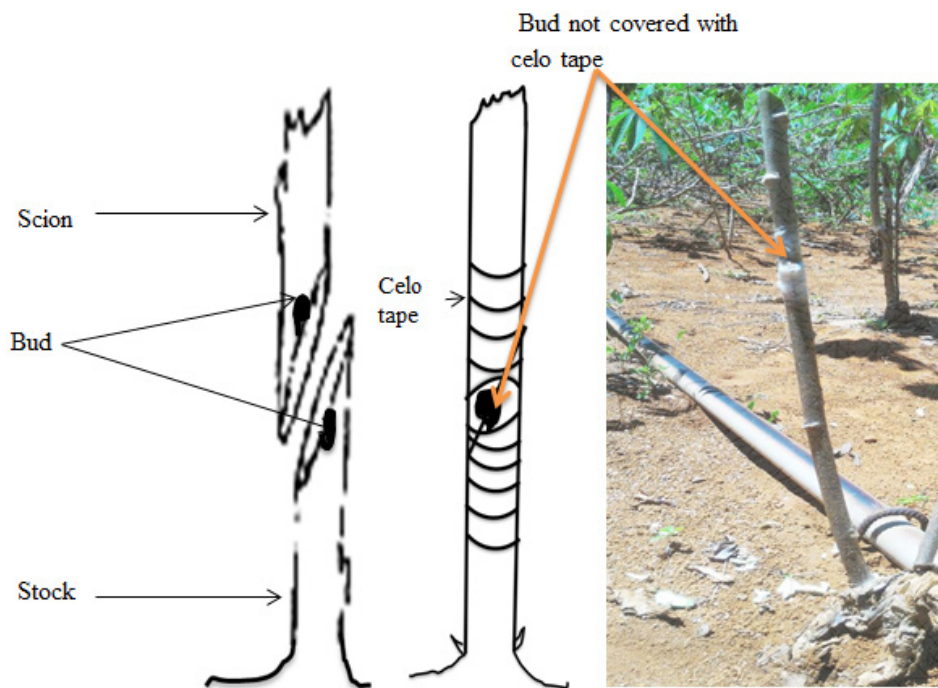


Figure 1. Grafting position for chimera synthesis showing scion bud and rootstock bud in close contact.

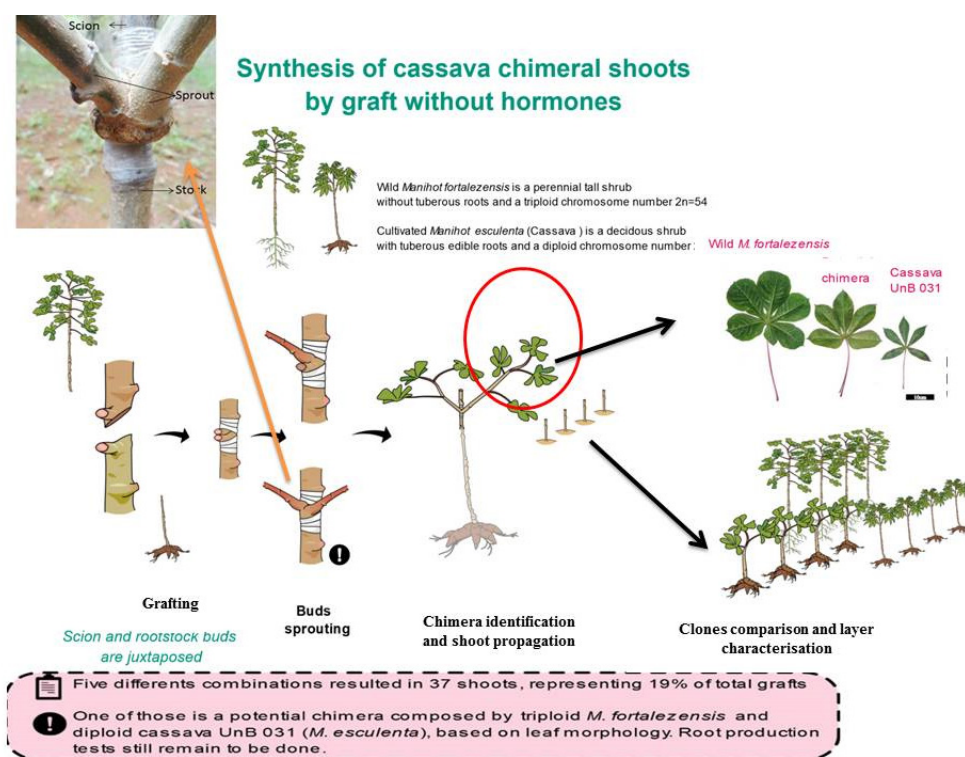
**Table 1.** Graft compatibility efficiency of 5 combinations of cassava cultivars (*Manihot esculenta*) and wild *Manihot* species.

Combination	Rootstock (RS)	Scion (SC)	No. of grafts	Grafts with shoots		GCE %	Total No. of shoots	SHIE (%)	No. of chimeras	CHIE (%)
				SC	RS and SC					
1	<i>M. esculenta</i> UnB 031	<i>M. fortalezensis</i>	48	16	1	35.4	18	37.5	1	2.1
2	<i>M. esculenta</i> UnB 205	<i>M. glaziovii</i> x <i>M. esculenta</i>	54	-	4	7.4	8	14.8	0	0.0
3	<i>M. esculenta</i> UnB 201	<i>M. glaziovii</i> x <i>M. esculenta</i>	51	7	1	15.7	9	17.6	0	0.0
4	<i>M. esculenta</i> UnB 530 p	<i>M. fortalezensis</i>	40	-	1	2.5	2	2.1	0	0.0
5	<i>M. glaziovii</i> x <i>M. esculenta</i>	<i>M. esculenta</i> UnB 201	30	-	1	3.3	2	6.7	0	0.0
	Total		223	23	8	13.9	39	17.5	0	0.45

GCE = graft compatibility efficiency = (grafts with shoots / total grafts) x 100; SHIE = shoot induction efficiency = (total shoots / total grafts) x 100; CHIE = chimera induction efficiency = (No. of chimeras / total grafts) x 100.

Graft combinations 2 [*M. esculenta* UnB 205 x (*M. glaziovii* x *M. esculenta*)] and 3 [*M. esculenta* UnB 201 x (*M. glaziovii* x *M. esculenta*)] also had high shoot induction efficiencies of 14.8 and 17.6%, respectively, but none of these combinations produced chimeras. However, combination 2 had low graft induction efficiency of only 7.4% because only four of the 51 grafts produced shoots (each producing two shoots). The interspecific polyploidized (tetraploid) hybrid *M. glaziovii* x *M. esculenta* seems to be incompatible with these two cultivars [UnB 205 (diploid) and UnB 201 (tetraploid)]. Cassava cultivar UnB 201 also nicked well with *M. fortalezensis*, which is believed to be a spontaneously produced and polyploidized (triploid) version of the cross between *M. glaziovii* x *M. esculenta*, and gave chimera induction efficiency of 5% (Nassar and Bomfim, 2013). *M. fortalezensis* was not compatible with the tetraploid cassava cultivar UnB 530 p. The recent tetraploid interspecific hybrid *M. glaziovii* x *M. esculenta* did not produce any chimera. Graft combinations 3 and 5

had the same two parents as donors, but with the rootstock and scion positions exchanged. Using the cassava cultivar, UnB 201 as the rootstock and the interspecific hybrid *M. glaziovii* x *M. esculenta* as the scion (combination 3), gave shoot induction efficiency of 17.6%; exchanging the parent positions gave shoot induction efficiency of only 6.7%. It seems that care has to be taken even for the same combination when making a decision on which parent to use as rootstock and which to use as the scion.



**Figure 2.** Chimera synthesis showing shoot production and identification of chimera based on stem and leaf morphology. In all combinations with high shoot induction efficiency, the cassava cultivars were used as rootstock.

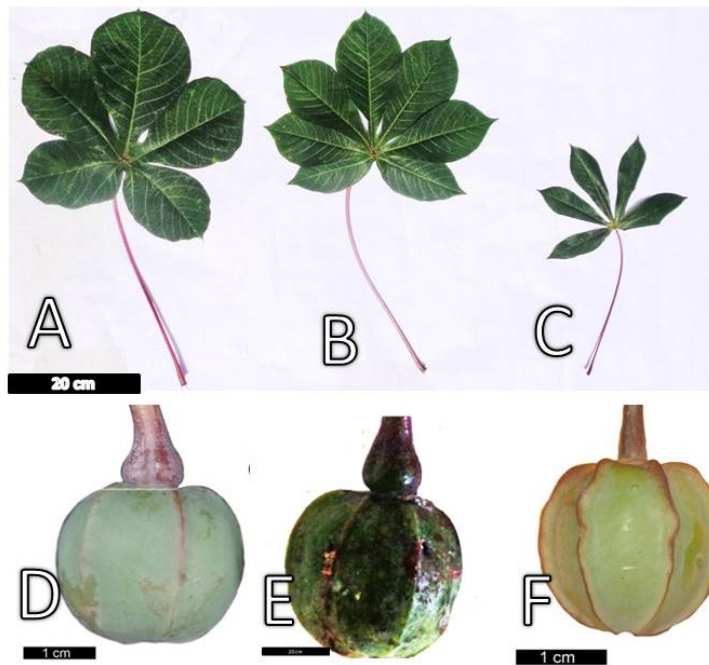
### Chimera 2 morphological characterization (*M. fortalezensis* and *M. esculenta* UnB 031)

Morphological characterization of chimera 2 and donor plants *M. fortalezensis* and *M. esculenta* UnB 031 showed that chimera 2 had many distinct features: It is 8 m tall vs 12 m (*M. fortalezensis*) and 7 m (UnB 031); its panicles have four lateral inflorescences vs 2 and 3 of the parents; its semi-spherical fruits vs spherical fruits of both parents; its fruits had slightly prominent straight wings as compared to no wings on the apex and highly prominent undulating wings of the two parents; its highly enlarged stem nodes vs small nodes of the parents; its flowers are 13 cm long vs 15 and 9 cm of the parents; its leaf diameter is 5 cm vs 2.5 and 4 cm in the parents. Similar distinct features were also observed in branching habit, number and shape of leaf lobes, especially the shape of the central lobe, and other characters (Table 2 and Figures 3 and 4).

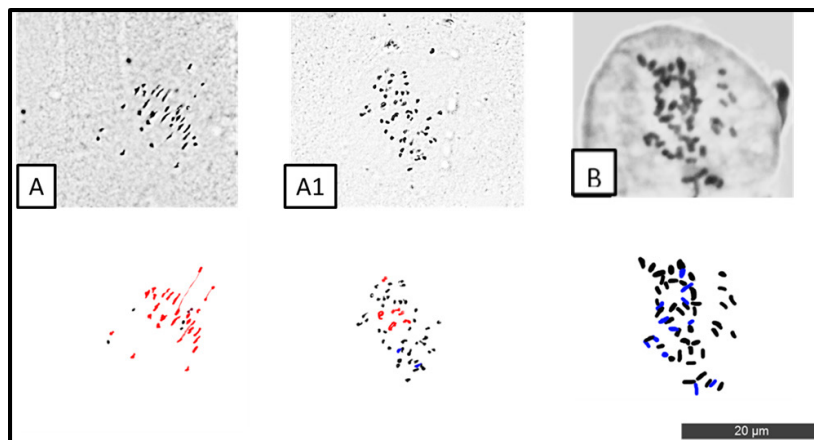
Table 2. Chimera 2 morphological features compared to donor parents.

Characters	<i>M. foetalensis</i>	Chimera 2	UnB 031
Growth habit and stem	Erect shrub, normally solitary, opens ca. 12 m tall, 7-10 cm in diameter; erect branch, dichotomous branching only in the apical part, and young purplish green branch. Small nodes and slightly enlarged stipel scars on the stem and with leaf scars not very apparent.	Semi-erect shrub opens ca. 8 m tall, 7-8 cm in diameter, semi-erect branch, with dichotomous and trichotomous upper branches. Young purplish green branch. Highly enlarged nodes and stipel scars on the stem with leaf scars not very apparent.	Semi-erect shrub normally opens ca. 7 m tall, 3-4 cm in diameter, erect dichotomous branch. Young purple branch, small and protruding nodes with visible stipel scars. Leaf scars not very apparent.
Inflorescence	Panicle with 2 lateral branches from the same base, with pistillate flowers in the central panicle. Cream disc and tepals; flower 15 mm in length. Ovaries not winged.	Panicle with four lateral inflorescences arising from the same base, cream disc with reddish base, cream tepals, and flower 13 mm in length. Ovaries have straight wings.	Panicle with 3 lateral inflorescences arising from the same base, red disc, cream tepals, and flower 9 mm in length. Ovaries have undulating wings.
Fruits	Spherical fruits without wings except at the base, being thin straight wings at base; with peduncle forming a globe near to the fruit.	Semi-spherical fruits with slightly prominent straight wings in whole fruits, peduncle forming a globe at the base.	Spherical fruits with highly prominent undulating wings and thickened erect peduncle near to the fruit.
Leaves	Usually peltate, with 3, 5, or 7 lobes, central leaf obovate with an entire margin and apiculate apex, soft green adaxial surface with a soft glaucous green abaxial surface. Purple petiole of length between 45-50 cm. Petiole inclined horizontally.	Peltate, with 5, 6, or 7 lobes, central leaf obovate-lanceolate with an entire margin and apiculate apex. Membranous green abaxial surface and light green adaxial surface. Red petiole 45-47 cm in length. Petiole inclined horizontally.	Palmate with 6 or 7 lobes, central leaf lanceolate with an entire margin and apiculate apex. Dark green abaxial surface and light green adaxial surface. Red petiole 35-38 cm in length and petiole inclined horizontally.
Roots	150 cm long. Predominantly small fibrous roots and 2.5 cm in diameter.	Cylindrical tuberous roots about 150 cm in length and 5 cm in diameter. Tuber weight 20 kg per plant.	Tuberous conical roots reaching 30-45 cm in length and 4 cm in diameter. Tuber weight 4 kg per plant.

*M. fortalezensis*    Chimera 2    *M. esculenta*  
Unb 031



**Figure 3.** Chimera 2 (middle) morphology compared to donor plants (*M. fortalezensis*, left, and *M. esculenta* UnB 031, right). A. B. C. leaf shape; D. E. F. fruit shape.



**Figure 4.** Chimera 2 metaphase for L2 and L3 characterization based on chromosome counting and maps showing bivalents (red) and univalents (blue and black) in anthers (A and A1). A. Metaphase with 24 bivalents and 6 univalents, resulting in  $2n = 54$ ; A1. Late metaphase showing 10 bivalents and 34 univalents, resulting in  $2n = 54$ . B. Mitotic metaphase of root tips showing a chromosome number of  $2n = 54$ .



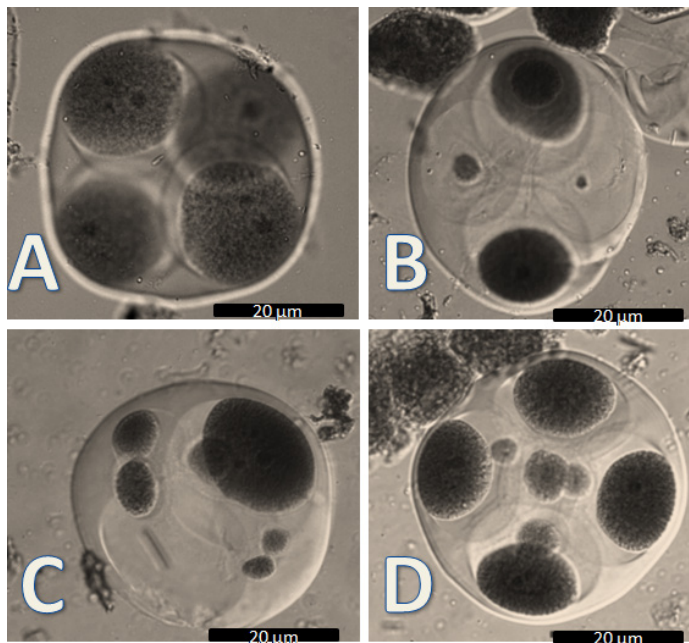
## Cytological characterization

Chimera 2 meiotic metaphase examination of mature staminate flowers (Figure 5A) and mitotic metaphase examination of adventitious root tips (Figure 5B) revealed chromosome number of  $2n = 54$ , similar to the triploid chromosome number of *M. fortalezensis* ( $2n = 54$ ), indicating that L2 and L3 of chimera 2 came from *M. fortalezensis*. With whole fruit having slightly prominent straight wings, it can be established that L1 of chimera 2 is derived from *M. esculenta*, var. UnB 031 which has highly prominent undulating wings (Figure 5 and Table 2). The other parent, *M. fortalezensis*, had thin wings only at the base of the fruit. Chimera 2 can, therefore, be expressed as EFF, “E” designating *esculenta* and “F” designating *fortalezensis*.

Chromosome configurations of meiosis showed bivalents that ranged from 6 to 25 (mean of 13.6) with 50.4% of chromosomes arranged in bivalents. Trivalents were observed in 16 of the 20 (mean 12.8%) and 80% of the cells. Some univalents in a cell varied from 6 to 42 (mean of 19.9 univalents). The noted compatibility of grafting *M. fortalezensis* with cassava might be attributed to its recent evolving from cassava.

## Tetrad normality assessment

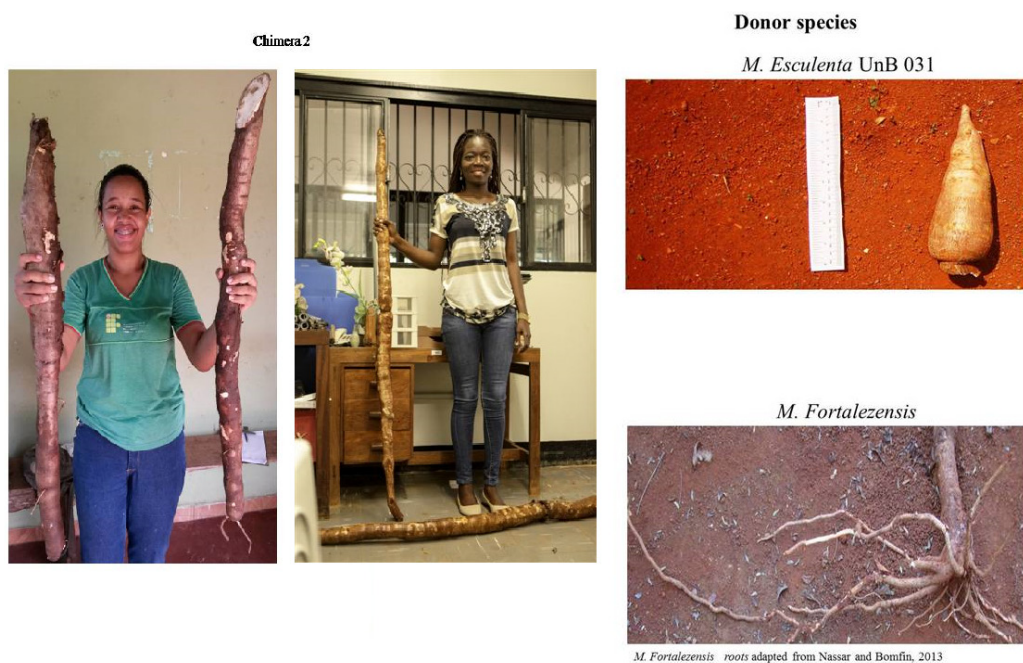
The very low tetrad normality (Figure 5C and D) of 3.1% (45 normal tetrads of a total of 1415 groups) also confirmed the triploid nature the L2 of chimera 2, which is the triploid parent *M. fortalezensis*. The unequal distribution of chromosomes, as observed in irregular anaphase I, explains for the rare quantity of fruits obtained.



**Figure 5.** Chimera 2 with normal and abnormal tetrads. **A.** Normal tetrad. **B.** Abnormal tetrad showing dyads with 2 micronuclei. **C.** Abnormal tetrads showing one microspore (monad) with 5 micronuclei. **D.** Abnormal tetrad with 4 micronuclei.

## Productivity of chimera 2

Transgressive expressions were observed in stem and leaf size and root yield. It had stems thicker, leaves with more lobes, and root yield higher than that of both parents (21.8 vs 4.6 kg/plant or 227.156 vs 47.932 t/ha, at the age of 2 years, as compared to UnB 031) (Figure 6); these roots were much longer (150 vs 30-45 cm) and thicker in diameter (5 vs 4 cm) as compared to that of the cultivated cassava variety UnB 031.



**Figure 6.** Chimera 2 tuberous root mass compared to donor plants.

Under the conditions of Cáceres, Mato Grosso, where it was evaluated by Empresa Matogrossense de Pesquisa, Assistência e Extensão Rural, at the age of 1 year, chimera 2 was more than three times as productive in root yield (88255 vs 24193 kg/ha) and four times as productive in flour (starch) yield (26971 vs 6643 kg/ha), and had higher starch content (31 vs 28%) as compared to *M. esculenta* UnB 220, a cassava variety used by farmers in Cáceres, south center region of the State of Mato Grosso, Brazil. The starch quality of the chimera was not inferior to that of UnB 220 and other varieties under production.

These heterotic phenomena let us call it 'graft hybrid' effects that arise from epigenetic interactions between the two species that coexist together in the same plant, although the specific mechanism remains unknown. Epigenetic interactions that caused complex heritable traits such as plant height have also been reported by Bomfim and Nassar (2014). It was found out that exchange of RNA and DNA to adjacent cells in grafts occurs (Hirata et al., 1999, 2000; Stegemann and Bock, 2009; Nassar and Bomfim, 2013). In plant chimeras obtained by grafting, L1, L2, and L3 origins have also been identified by differential gene expression as well as the presence of different proteins in various histogenic-derived tissues (Filippis et al., 2013).

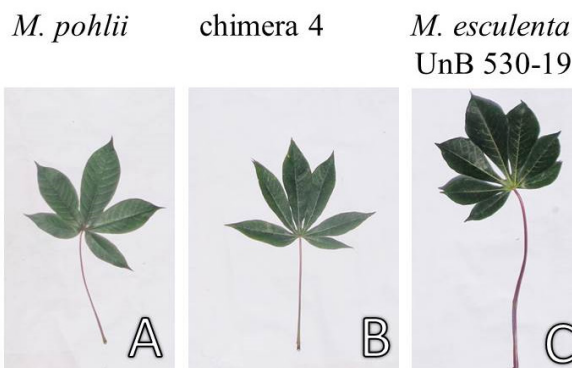
Chimera 1 characterized by Nassar and Bomfim (2013) also gave significantly higher root yield as compared to its parent, UnB 201 (10-12 vs 2-3 kg/plant) but not reached this case. This chimera manifested high parent heterosis not only in root yield but also in plant height. Chimera 3 was also superior in yield to one of its parents, with a 7-fold increase (Bomfim and Nassar, 2014). This chimera was later released as cassava variety UnB 703. It is interesting to note that all three highly productive chimeras induced at UnB so far (chimera 1, chimera 3 and chimera 2, all EFF) involved *M. fortalezensis* ( $2n = 54$ ) as scion and diploid cassava cultivars. The interaction between layers of diploid cassava cultivars used in chimera with the L3 tissue of the triploid wild species *M. fortalezensis* used as scion might have created positive interaction similar to the heterosis phenomenon, and this might have led to high root yield by the chimeras.

### Chimera 4 morphological characterization

Morphological characterization of chimera 4 and donor plants *M. pohlii* and *M. esculenta* UnB 530-19 showed that chimera 4 has some intermediate morphological characters as fruit wings and root shape (Table 3) except in plant height where there was transgressive expression (9 m of the chimera vs 7 and 8 m in *M. pohlii* and UnB 530-19, respectively). For example flower disc color of the chimera was red vs dark orange and wine greenish in the parents; flower sepal color of reddish green vs greenish wine and purple in the parents; semi-spherical fruits vs spherical fruits of the parents; up to 9 leaf lobes vs 3-5 and 7-8 leaf lobes in the parents and acuminate central leaf apex vs apiculate leaf apex of the parents (Figure 7).

**Table 3.** Description of the morphological features of *Manihot pohlii*, chimera 4, and *M. esculenta* UnB 530-19.

Characters	<i>M. pohlii</i>	Chimera 4	<i>M. esculenta</i> UnB 530-19
Growth habit and stem	Decumbent shrub with thick, dichotomous or trichotomous young branches, about 7 m high and 10-15 cm in diameter. One or two stems arising from the central stem. Young dark green stem with small nodes and slightly visible stipule scars.	Erect, thick and open shrub with some young stems creeping on the ground. Dichotomous apical branches; about 9 m high and 10-15 cm in diameter. Two or three stems arising from the central stem; young green stem with protruding nodes and visible stipule scars.	Erect, open shrub, dichotomous or trichotomous branches at the upper part of stems. About 8 m high and 10 cm in diameter. One or three stems arising from woody base, enlarged and highly protruding nodes on the stem. Young greenish purple stems with enlarged stipule and moderate petiole scars.
Inflorescence	Panicle with three inflorescences arising from the same point, dark orange disc with greenish wine tepals. Flower 7 mm in length. Ovaries not winged.	Panicle with four inflorescences arising from the same point, red disc with reddish green tepals. Flower about 8 mm in length. Ovaries winged.	Panicle with 3-4 inflorescences arising from the same base, wine greenish disc with purple pigmentation tepals. Flower about 9 mm in length. Ovaries winged.
Fruits	Spherical fruits without wings, straight lines running along fruits with a thickened peduncle.	Semi-spherical fruits with undulating wings, with the peduncle forming a globe near to the fruit.	Spherical fruits with thin straight wings, peduncle forming a globe near to the fruits.
Leaves	Usually palmate with 3 or 5 lobes; central leaf elliptic with an entire margin and apiculate apex and coriaceous dark green axial and light green adaxial surface; greenish red petiole of length between 21-26 cm and inclined horizontally.	Palmate, with 5, 7, or 9 lobes; central leaf oblanceolate with an entire margin and acuminate apex; yellowish green axial surface and light green hairy adaxial surface, greenish red petiole of length between 29-35 cm and inclined upwards.	Palmate with 7 or 8 lobes; central leaf oblanceolate with an entire margin and apiculate apex with coriaceous dark green hairy adaxial surface, green on abaxial surface, red petiole of length between 38-47 cm (14-29 cm) and inclined upwards.
Roots	Narrow fibrous roots about 60 cm long and 1.5 cm in diameter.	Fibrous roots reaching 10 cm depth with long peduncle and a long tail.	Tuberous conical roots reaching 40-50 cm depth.

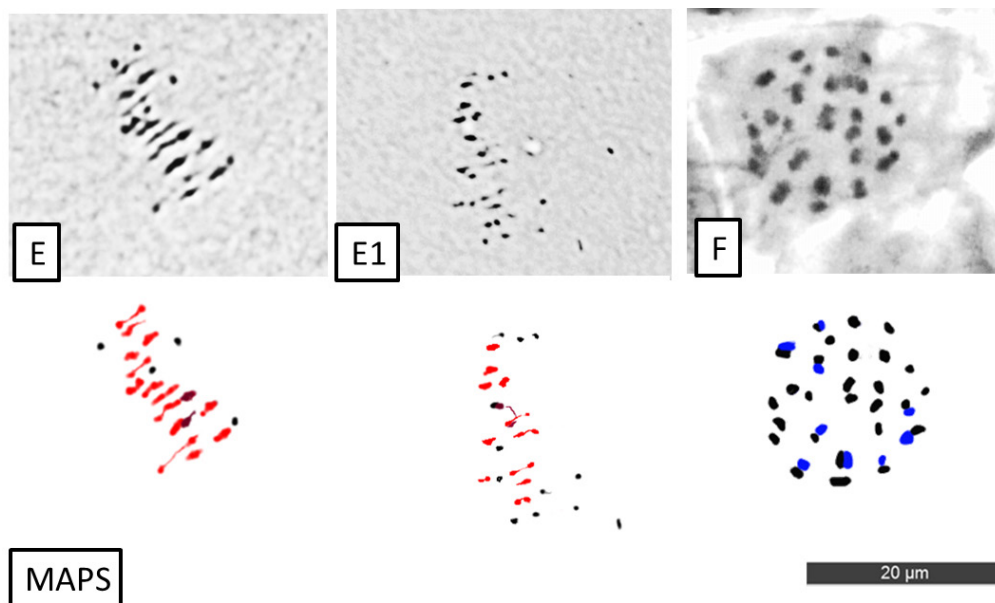


**Figure 7.** Chimera 4 leaves.

### Cytological analysis of chimera 4 L2 and L3.

For chimera 4, meristematic layer characterization, i.e., meiotic metaphase examination of L2-derived PMC (Figure 8E and E1), and mitotic examination of L3-derived adventitious root tips (Figure 8F) revealed a chromosome number of  $2n = 36$ , similar to *M. pohlii* chromosome number ( $2n = 36$ ). The L2 and L3 were derived from *M. pohlii*.

The presence of wings on the ovaries of chimera 4 indicates that L1 of the chimera was from the triploid cassava variety UnB 530-19. Ovaries of *M. pohlii* did not have wings. This chimera can, therefore, be designated as EPP (E designating “*esculenta*” and P representing “*pohlii*” since L1 came from *M. esculenta* variety UnB 530-19 while L2 and L3 came from *M. pohlii*).

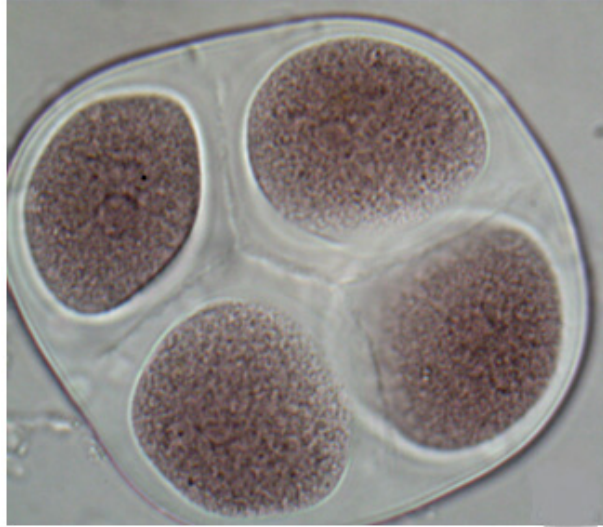


**Figure 8.** Chimera 4 metaphase for L2 and L3 characterization based on chromosome counting and maps showing bivalents (red) and univalents (blue and black). **E.** metaphase I with 16 bivalents and 4 univalents resulting in a chromosome number of  $2n = 36$ . **E1.** Early anaphase showing 12 bivalents and 12 univalents, resulting in  $2n = 36$ . **F.** Mitotic metaphase, also showing a chromosome number of  $2n = 36$ .

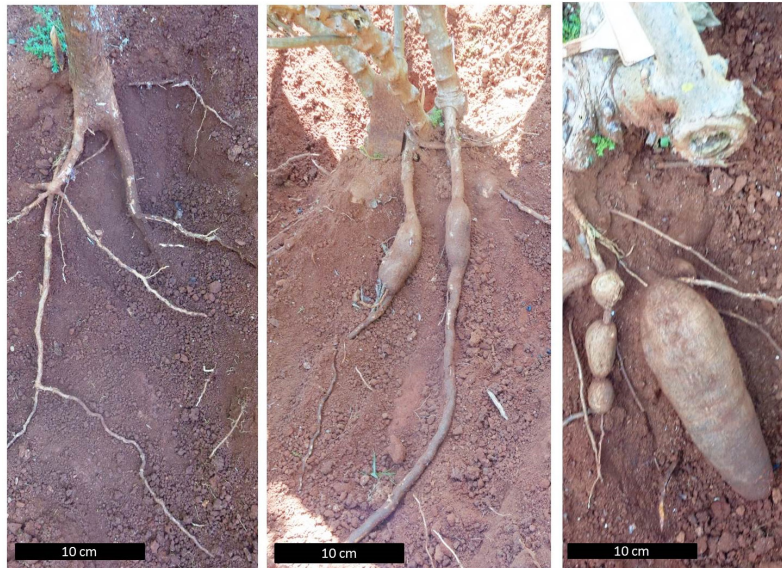
### Tetrad normality

Cytological analysis of the meiotic behavior of PMC revealed tetrad normality of 98.5% in chimera 4. This is coherent with the behavior of diploids such as *M. pohlii* ( $2n = 36$ ), which produce normal tetrads (Figure 9) during micro-gametogenesis.

Chimera 4 produced fibrous roots similar to, but shorter than, those of the wild cassava *M. pohlii*, and was not productive (Figure 10). The combination of the triploid cassava cultivar *M. esculenta* UnB 530-19 and the diploid wild species *M. pohlii* did not have the favorable epigenetic interaction for high root productivity as in chimeras 1, 2, and 3, which had the triploid *M. fortalezensis* in the case of chimera 2.



**Figure 9.** Chimera 4 normal tetrads.



**Figure 10.** Chimera 4 (center) tuberous roots compared to roots of donor plants, *Manihot pohlii* (right) and *Manihot esculenta* UnB 530-19 (right).

It seems that epigenetic effect in chimeras studied here is limited to certain parents with certain genetic structure and ploidy level. The triploid *M. fortalezensis* with diploid cassava was optimum while *M. pohlii* with triploid cassava did not have any epigenetic effect. In both cases, cassava formed L1 while the wild-types *M. pohlii* and *M. fortalezensis* formed L2 and L3. The unique difference in the two cases is that *M. fortalezensis* is a new evolving species that came from interspecific hybridization of *M. glaziovii* and a polyploid cassava.

In classical heterosis studies, it is known as cases of the ability of combination. It is also known that cultivars have this ability with large numbers such as the case of Branca Santa Catarina. We suggest that *M. fortalezensis* may possess this ability concerning epigenetic effect.

## ACKNOWLEDGMENTS

P.M. Gakpetor is the recipient of a scholarship from Fundação Nagib Nassar para Desenvolvimento Científico e Sustentável (FUNAGIB) to whom she extends her gratefulness. Thanks are due to Nayra Bomfim, Welton Reis, and Helena Schuch for their help with laboratory preparations. The above wild cassava living collection was established at Universidade de Brasília with the help of the Canadian International Development Research (IDRC). N.M.A. Nassar is the recipient of a scholarship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) to whom he is thankful.

## REFERENCES

- Akinbo O, Labuschagne MT and Fregene M (2015). Introgression of root protein and yield traits from backcross hybrids between cassava and its wild progenitor (*M. esculenta* ssp *flabellifolia*). *J. Plant Breed. Crop Sci.* 7: 1-8. <https://doi.org/10.5897/JPBCS2013.0407>
- Akinagbe OM (2010). Constraints and strategies towards improving cassava production and processing in Enugu North Agricultural Zone of Enugu State, Nigeria. *Bangladesh J. Agric. Res.* 35: 387-394. <https://doi.org/10.3329/bjar.v35i3.6445>
- Bomfim N and Nassar NMA (2014). Development of cassava periclinal chimera may boost production. *Genet. Mol. Res.* 13: 819-830. <https://doi.org/10.4238/2014.February.10.1>
- Buder J (1911). Studien an Laburnum adami. II. All gemeineanatomische Analyse des Mischlings und seiner Stammpflanzen. *Zschr. ind. Abst. Vererb. Lehre* 5: 209-284.
- Carlson PS and Chaleff RS (1974). Heterogeneous association of cells formed *in vitro*. In: Genetic manipulations with plant materials (Ledoux L, ed.). Plenum, New York, 245-261.
- De Bruijn GH and Dharmaputra TS (1974). The Mukibat system, a high-yielding method of cassava production in Indonesia. *Neth. J. Agric. Sci.* 22: 89-100.
- Fao Ifad WFP (2014). The state of food insecurity in the world (2014): 12.
- Filippis I, Lopez-Cobollo R, Abbott J, Butcher S, et al. (2013). Using a periclinal chimera to unravel layer-specific gene expression in plants. *Plant J.* 75: 1039-1049. <https://doi.org/10.1111/tpj.12250>
- Goffreda JC, Szymkowiak EJ, Sussex IM and Mutschler MA (1990). Chimeric tomato plants show that aphid resistance and triacylglyceride production are epidermal autonomous characters. *Plant Cell* 2: 643-649. <https://doi.org/10.1105/tpc.2.7.643>
- Hashimoto-Freitas DY and Nassar NMA (2013). Cytogenetic and anatomic behavior of cytochimeras and total polyploids in cassava. *Genet. Mol. Res.* 12: 4879-4894. <https://doi.org/10.4238/2013.October.22.7>
- Hirata Y, Xiao QB, Rhaman R, Noguchi T, et al. (1999). Cell to cell interactions in artificially-synthesized chimeras in Cruciferae. *Eucarpia Leafy Vegetable* 257-262.
- Hirata Y, Motegi T, Xiao QB and Noguchi T (2000). Artificially synthesized intergeneric chimera between Brassica oleracea and *Raphanus sativus* by *in vitro* grafting. *Plant Biotechnol.* 17: 195-201. <https://doi.org/10.5511/plantbiotechnology.17.195>
- Medina RD, Faloci MM, Gonzalez AM and Mroginski LA (2007). *In vitro* cultured primary roots derived from stem segments of cassava (*Manihot esculenta*) can behave like storage organs. *Ann. Bot.* 99: 409-423. <https://doi.org/10.1093/aob/mcl272>
- Nassar NMA (2007). Cassava genetic resources and their utilization for breeding of the crop. *Genet. Mol. Res.* 6: 1151-1168.
- Nassar NMA and Ortiz R (2009). Cassava Genetic Resources: Manipulation for Crop Improvement. *Plant Breed. Rev.* 31: 247-275.
- Nassar NMA and Ortiz R (2010). Breeding cassava to feed the poor. *Sci. Am.* 302: 78-82, 84. <https://doi.org/10.1038/scientificamerican0510-78>

- Nassar NMA and Bonfim N (2013). Synthesis of periclinal chimera in cassava. *Genet. Mol. Res.* 12: 610-617. <https://doi.org/10.4238/2013.February.27.10>
- Nassar NMA, Hashimoto DYC and Fernandes SDC (2008). Wild *Manihot* species: botanical aspects, geographic distribution and economic value. *Genet. Mol. Res.* 7: 16-28. <https://doi.org/10.4238/vol7-1gmr389>
- Nassar NMA, Ribeiro DG, Bomfim NN and Gomes PTC (2011). *Manihot fortalezensis*: a new species of *Manihot* from Ceará, Brasil. *Genet. Resour. Crop Evol.* 58: 831-835. <https://doi.org/10.1007/s10722-010-9620-2>
- Nassar NMA, Rayssa GRM, Emanuel R, Danielle Y, et al. (2012). Some interesting cassava cultivars: 12 - UnB 530 - 19. *Geneconserve*: 12(46) - 12:15.
- Nielsen MD, Farestveit B and Andersen AS (2003). Adventitious shoot development from decapitated plants of periclinal chimeric poinsettia plants (*Euphorbia pulcherrima* Willd ex Klotsch). *Eur. J. Hortic. Sci.* 68: 161-168.
- Rogers DJ and Appan SG (1973). *Manihot* and *Manihotoides* (Euphorbiaceae), Flora neotropica, monograph n. 13, Haffner, New York.
- Satina S, Blakeslee AF and Avery AG (1940). Demonstration of the three germ layers in the shoot apex of *Datura* by means of induced polyploidy in periclinal chimeras. *Am. J. Bot.* 27: 895-905. <https://doi.org/10.2307/2436558>
- Stegemann S and Bock R (2009). Exchange of genetic material between cells in plant tissue grafts. *Science* 324: 649-651. <https://doi.org/10.1126/science.1170397>