Report

Periclinal chimera breeding approach may boost cassava tuberous root yield

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Abstract: Plant periclinal chimera is a genotypic mosaic concentrically organized. Producing it to combine useful traits from different species has been tried but practical results remains to be reached. Cassava, a staple crop feeding millions of poor people needs many characters of the wild to improve its productivity. This article reports a high yielding periclinal chimera-derived from cassava, constituted by cassava epidermis and wild species's internal tissue. It yields large edible roots 7-folds higher than common cassava reaching 14 kg per plant by 12 months-old compared to 2-3 kg of common varieties. These results provide a new approach for cassava breeding based on synthesizing interspecific chimeras. Root enlargement is attributed to epigenetic effect due to growing in diploid and triploid tissues from different species side by side. Since cassava is a food for more than billion poor people, these results may contribute significantly for the world food security.

Key words food security, root size, interspecific epigenetic interaction, starch quality, wild cassava.

Cassava is the main staple of at least one billion people in the tropics and subtropics(1). Improvement programs have focused in simple clonal selection and intercultivar hybridization(2–4). Although wild species have contributed to cassava boost food security(3, 4, \uparrow). Interspecific hybridization to transfer characters is difficult due to barriers among species and the need to break them(5, 6). Furthermore, association of undesirable characters coming from the wild is another drawback, which requires time-consuming work across various generations to get rid off the undesirable "linkage drag"(4,5). Polyploidy has little to offer in genetic enhancement because new traits are not brought by this technique(7). There have been suggestions to produce chimeras to combine useful characters from wild and cultivated forms (e.g. host plant resistance to insects found in epidermal tissue)(4, 8), but practical results are yet to be achieved(9, 10).

Sporadic interspecific chimera have been documented by some authors(8, 11-14). They arose from adventitious shoot formed on a graft union of a scion and rootstock. Very few chimeras have been also produced by tissue culture but they have no economic value(10, 15, 16).

An early article(17) reports the use of a periclinal chimera in cassava between *M. fortalezensis* cassava cultivar UnB 201,which led to producing a high yielding cultigen. On the present article, we report a synthesized interspecific periclinal chimera involving the wild species *M. fortalezensis* and the *M. esculenta* cultivar UnB 032 (**A, Fig. S1**), which differ in ploidy level. We used the clone UnB 032 – known by its good consumption quality and moderate productivity but susceptible to borers and vulnerable to drought. The wild species *Manihot fortalezensis* shows some host plant resistance to borers and adapts well to drought because of its deeply penetrating roots(*18*).

This chimera shows a distinct phenotype than its parents, and was deduced to be periclinal because of the homogeneous characters on the whole plant, and a set of new features evidencing combination of tissues from both parents (Tab.S1). The roots did not, however, resemble either parent because they are long and tuberous at the same time. With respect to root production and starch granules, Chimera 3 produced 14 kg per plant 12 months after planting, while root harvest of cassava UnB 032 at same age weighed 2.1 kg per plant. Chimera 3 reached 4m high, while cassava UnB 032 was about 2m height. Anatomic analysis indicate Chimera 3 roots are tuberous and show starch granule distribution and shape similar to cassava UnB 032; i.e., being round, and smaller than this cassava cultivar in diameter size (small granules with $6.4\pm 2.6 \mu m$, while medium granules in UnB 032 were $15.3 \pm 4.4 \mu m$, Tab. S4)(*19*),

which has a relatively fast starch hydrolysis that is adequate for fine printing paper and biodegradable films(19).

Chimera 3 had a set of new features distinct to both parents. Chimera 3 produced very large and exceptional edible roots, being almost 7-folds higher than cassava, while the parents had predominantly long fibrous root (*M. fortalezensis*) and short tuberous root (cassava cultivar UnB 032)(**A**). We hypothesize that this new phenotype ensues from an epigenetic interaction on the chimera. New phenotypes have been reported in chimera plants that emerged sporadically such as fruit aroma and plant height (12, 14). However the mechanism remains to be elucidated (8, 20). Recently, evidence of stable inheritance of epigenetic alterations due to DNA methylation changes altering heritable complex traits such as plant height has been reported(21). This phenomenon may provide a molecular basis to phenotypic changes , which were once named "graft hybridization". It may also help to clarify heterosis (20, 22).

This result offer method, technique and material to other breeders and researches to combine interspecies traits and investigate deeply interspecies interaction. This method is useful to combine, mainly, species hardly to combine by classical hybridization method, as in infertile triploids. The chimera proven to be a valuable material to study the mecanism under the great root enlargement, in addition to be useful to cassava production.

We have shown a new approach for cassava breeding based on synthesizing interspecific chimeras comprising tissues of cultivars and related wild *Manihot* species, which may significant enhanced the cultigen yield as a result of a significant root enlargement.

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[†] The impact of Interspecific hybrids, breeded by Nagib Nassar, on food security in Africa was reported on media by IDRC(Canadian International Development Research Centre). Available on:<

http://www.idrc.ca/EN/Resources/Publications/Pages/ArticleDetails.aspx?PublicationID=163>

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Chimera 3

Supplementary Materials for

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Materials and Methods

<u>Material</u>

To induce interspecific chimeras the cassava cultivar UnB 032 and wild species and wild species *M. fortalezensis* were used. UnB 032 is a low shrub of 1,5 m, yields 2-3 kg/ year, its chromosome number 2n=36.

M. fortalezensis is native to savanna forest of Ceará (Caatinga), Brazil where drought predominate. This species is an erect woody shrub, ca. 4 m tall with deep fibrous roots (Nassar et al., 2011), its chromosome number is 2n=54. Both are maintained at the living collection University of Brasilia (UnB).

For deduction layer genotype, samples were collected from 9 clone plants replicated by stalks from the chimera plant (arisen from the grafting fusion region of UnB 032 and *M. fortalezensis*) and parental species, UnB 032 and *M. fortalezensis*. All clone plants were planted in the same time and under sun for comparative studies (Tab. S1).

Methods

Chimeral Synthesis

Shoots 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 5 mm scion. The graft unions and remaining graft tissue was covered by cotton receiving 4 drops of 0.01% α -naphthaleneacetic for 7 days (adapted from (*1*)(Fig. S1).

At the end of the growing season, shoots which exhibited distinct morphological characters were propagated vegetatively for tissue constitution reconnaissance. To help layer deduction: morphology of fruits, leaves and roots; meiosis in flower buds and mitosis in root cuttings; transversal petiole sections and longitudinal stem apices sections was analyzed, and root production of chimeras were compared with the parent species.

Morphological characterization

Nine clone plants, mature with 12 to 18 months, were observed to morphological characterization based on distinctive characters of habit, leaves, inflorescence, fruits and root which distinguish cassava from others wild species.

Cytogenetic Analysis

Male buds and closed mature staminate flowers of both parental plants and chimera were collected at 8 a.m.(daylight saving horary), fixed in Carnoy solution preserved in 70% ethanol, smeared and stained with 1% acetocarmin. To assay pollen viability in *M. fortalezensis* all pollens from two flowers had diameters measured to calibrate the viability, once it produce two size pollens indistinguishable on nude view, using only pollens with the same size range of diploid fertile plant. Root tips were collected from germinating chimera cuttings, pre-treated with colchicine 0.25% in distilled water for 2h, fixed in Carnoy solution, hydrolyzed in HCl 5N for 10 min, smeared and air dried before stained with 5% Giemsa (adapted from (2)).

Anatomical assessment

Shoot Apical meristem (SAM), petioles and leaf blades was analyzed to help deduction of layers genotype of chimera considering meristem organization on 3 independent layers: L1(outermost layer), L2(subsequent layer) and L3 (inner layer). It was analyzed SAM longitudinal sections, surface leaf blades and transversal sections from petioles and roots. All material were fixed using FAA 70 - formaldehyde, 70% ethanol and glacial acetic acid- solution (5:5:95, v/v) (*3*) and permanently mounted in synthetic resin (*4*) after stain and sectioning. SAM were embedded in butyl acetate series and paraffin (*5*), 8 μ m thick sectioned on a rotary microtome RM 2145(LEICA, Germany) and double-stained by safranin and fast-green series; while leaf blade surface was released by glacial acetic acid and hidrogen peroxide solution and stained with 1% (5). Petioles were free-hand cut on a microtome, and stained with 1% safranin and 1% aqueous alcian blue. Roots were free-hand sectioned and treated with Lugol's (5). Thichomes highlighted images were provided by a JEOL JSM 700 1-F scanning electron microscope, after gold coatting in a Leica EM SCD 500 metalyzer.

Epidermal features as leaf blade cell shape, trichomes, stomatal length, and petiole ordinary epidermal cell width helped deducing L1layer origin. Al least 4 clone plants were sampled. Stomatal length and trichomes frequency were determined on 4^{th} and 6^{th} leaf, based on nail polish imprints from abaxial leaf surface; while petioles cells width were measured on 6^{th} leaf of fresh leaves. Starch granules size and distribution on roots were observed to evaluate edible potential on 2 clone plants.

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

Statistical analyses

Stomatal lenght and petiole epidermal and cortical cells width were evaluated by ANOVA followed by Tukey's test. The premises of normality and homoscedasticity were tested by Kolmogorov-Smirnov and Bartlett tests, respectively. Measurements was did using Image-Pro Plus version 4.5.0.29 and statistical analysis was did with the software Assistat 7.6 beta. For all analyzes assumed a α 0.05 "

Root production

Root weight was measured to estimate the potential production of the Chimera 3. Two clone plants had all roots weighed when stalks had 12 month planted.

Supplementary Text

Chimera tissue assessment

Stem apical meristem

A plant chimera is a tissue mosaic with genetically different cells existing in the shoot apical meristem (6, 7), Chimera 3's apical meristem, when anatomically analyzed, allows determining layer constitution. Chimera 3 outermost layer (L1) was similar to that of its wild ancestor *M. fortalezensis*, which has the same same ploidy level as the former (Fig.S1).

Determining L1 layer origin through morphological and anatomical research

Fruit wings enabled to determine L1 origin because this "diagnostic trait" belongs to cassava(8)(Tab.S1).

Cell shape, trichomes, stomatal length and petiole cell width were used to determine the L1 constitution (Fig. S2, Tab. S3)). Trichomes found over leaf veins in Chimera 3, are a specific to cassava and derive from a L1 cell(9). The intermediate stomata length and petiole epidermal cell could be attributed to the interaction of both species tissues as noted previously (10, 11).

L2 and L3 layer origin elucidated by cytogenetic analysis

Meiotic chromosome counts allowed determining L2 layer origin because gametes are usually derived from this layer (9, 12). There were 54 chromosomes at meiosis metaphase 1 in Chimera 3 (Fig. S3). The ploidy of the chimera was the same as its wild parent *M. fortalezensis*(Tab. S2). Mitotic chromosome counts enabled determing the origin of L3 layer. Roots originate from the pericycle, which it self derives from L3 (13,

14). Chimera 3 had 54 chromosomes on root tip cells, which confirm the same ploidy as its wild parent*M*. *fortalezensis*.





Fig. S2

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: Stomatal lenght. Stomatal intermediary lenght in Chimera 3. G,H, I: Trichome on leaf adaxial face veins. Trichome presence on Chimera 3, distinghish 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.



Fig. S3

Meiotic metaphase I (upper part) of Chimera 3 and parental species, and mitotic metaphase of Chimera 3. A: *M. fortalezensis*, B: Chimera 3, C: UnB 032. Blue marks: bivalents, black: marks univalents. Bar: 20 µm.



 Table S1. Morphological characterization of the Chimera 3 and parental species (*M. fortalezensis* and UnB 032).

032).	M. fortalezensis (FFF)	Chimera 3(EFF)	Cassava UnB 032		
Plant habit and stems	Erect shrub normally solitary, ca. 8m, 7-10cm 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. 4m, 1-2 central stems from the same base, 2-5cm diameter, semi- decumbent branch, dichotomously and trichotomously branching. Purple young branch. Upper part of stems tetragonal. Enlarged nodes and stipels scars.	(EEE) Erect shrub ca. 2m, 2-3 central stems arising from the same base, 1-5cm 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 acute acuminate acute apex. Petiole length 7-30 cm.		
Inflorescence	Panicle with 2 lateral branches from the same base, with pistilate 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 no winged, except on base, being slender straight wings, with peduncle	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.		
	forming a globe near to the fruit				
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.		
¹ 12 months old	plants.	-	-		

Table S2. L2 and L3 meristem layers deduction based on chromosome counts on L2 derived pollen mother cell and L3 derived roots of Chimera 3 and parental wild species *M. fortalezensis* and cassava cultivar UnB 032.

Material	M. fortalezensis	Chimera 3	UnB 032
Pollen mother cell Methaphasechromosome counts (L2)	2n=54	2n=54	2n=36
Root tips Methaphase chromosome counts (L3)	2n=54	2n=54	2n=36

Table S3. Leaf characterization showing distinct anatomical characters of Chimera 3 compared to parental species: *M. fortalezensis* and cassava cultivar UnB 032, on the 6th node (stomatal length: n=5; petiole cell width: n=4).

	M. fortalezensis (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)	$18.10 \ \mu m \pm 1.643 \ a$	$17.54~\mu m \pm 2.167~ab$	$14.73~\mu m\pm1.103~b$		
Trichomes over the leaf blade veins (adaxial view)	Absent	Absent Low frequency High f			
Petiole epidermis	All species show non-stratified epiderm with thickened cell walls and cuticle. The species differ only in shape and width				
	Tabular to oval cells				
Cell shape	frequently intercalated Oval to isodiametric cells Isodiametric cells by isodiametric cells				
Cell width*	$24~\mu m \pm 4.636~a$	19,57 μ m ± 2.5298 ab	$14{,}96\;\mu m \pm 1.4212\;b$		
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 Cell width*	Oval cell 29.70 μm ± 1.23 a	Oval cell 21.93 µm ±0.46 b	Isodiametric cell 18.60 μm ±0.39 b		
Collenchyma Cell width *	20.37 µm ±4.647 a	13.79 µm ±1.697 b	13.16 µm ±1.7291 b		
Inner parenchyma Cell width* Cell content	32.98 μm ± 2.98 a Low frequency of calcium oxalate druses	30.42 µm ± 3.568 a Frequent calcium oxalate druses	28.95 μm ± 0.9115 a 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> . There were differences in the content of parenchyma phloem cells				
Cell content	Lacking druses	Lacking druses	Druses seldom noted		
Vascular bundle (#)	8	9 to 11	9 and 10		
Xylem	Xylem composed by	vessel of the same diameter, bu	t of varied number of rays		
Xylem vessel rays	7 to 10	6 to 8	5 to 8		
Petiole medulla	Polyhedral cells containin	g starch grains and calcium oxa bundles. Differing only in ca	alate druses near of the ell content.		
Cell content	Starch grains frequent	Starch grains seldom noted	Starch grains seldom noted		
con content	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's range test ($P \le 0.05$).

	Diameter(µm)			
	Mean±standard deviation	Mininum	Maximum	
Quimera 3	6.4 ± 2.6	2.8	11.9	
UnB 032	15.3 ± 4.4	7.6	23.4	

Table S4. Starch granules diameters (mean ± standard deviation, minimum and maximum)of Chimera 3 and cassava cultivar UnB 032on xylem parenchyma cells

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