

Improving Carotenoids and Amino-Acids in Cassava

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Received: August 19, 2008; Accepted: September 15, 2008; Revised: October 1, 2008

Abstract: More than 800 million people in tropics and sub tropics use cassava as food. However, its roots are poor in protein content (0.7-2%). Amino acids such as lysine and methionine are also low, and some research reports indicate the absence of methionine in cassava edible roots. By inter-specific hybridization it was possible to increase true protein in cassava roots measured by amino acid contents. The amino acid profiles of a common cassava cultivar and an inter-specific hybrid, namely ICB 300, were determined using the computerized amino acid analyzer Hitachi L-8500. The inter-specific hybrid has 10-fold lysine and 3-fold methionine than common cassava cultivar: lysine content was 0.010 g per 100 g in the common cassava cultivar while it reached 0.098 in the inter-specific hybrid. Methionine in the common cassava cultivar was 0.014 g per 100 g whereas it reached 0.041 g per 100 g in the inter-specific hybrid. Total amino acid content in the common cassava cultivar was 0.254 g per 100 g viz. a viz. 1.664 g per 100 g in the inter-specific hybrid. The genetic variability of the profile and quantity of amino acids indicate the feasibility of selecting inter-specific hybrids that are rich in both crude protein and amino acids. Carotenoid content could be improved in cassava edible roots by selecting cultivars rich in carotenoids. In Brazil, the center of cassava origin, cassava landraces have acquired through their domestication a large diversity in relation to many economic traits such as high content of carotenoids and excellent palatability among other characters. One of these clones, which has been grown by indigenous farmers in Brazil and available at the University of Brasília genebank, showed a high level of lycopene content (5 mg/kg viz. a viz. zero in common cultivars, and 12-20 mg/kg in tomato—a lycopene-rich vegetable). The cassava landrace UnB 400 had a high content of β -carotene (up to 4 mg/kg). This article also discusses relevant patents to the main subject of this research.

Keywords: Biofortification, genetic resources enhancement, lycopene, wild *Manihot* species.

Dedicated to Joachim Voss, a pioneer of Cassava Research Development, and a Visionary Administrator on his retirement from a distinguished service with IDRC and CGIAR.

INTRODUCTION

Cassava is among the most important crops in the tropics and a staple food for more than 800 million people. Cassava is also the principal food for about 60 million people living in northeast Brazil. Cassava roots provide more than 60% of the daily energy intake for the population of northeast Brazil and many countries in Africa.

Cassava protein is comparable to rice protein in digestibility. The biological value (Block and Michell equivalent) of the total protein is 48%. The crude protein content of roots appears to be relatively stable and constant with maturity of the plant. Cassava roots, however, are a poor source of protein, despite that the quality of this protein is fairly good, as is the proportion of amino acids as well [1, 2]. Methionine and lysine are limiting amino-acids in cassava edible roots [3]. Furthermore, the protein of processed cassava includes the highest percentage of glutamic acid and the lowest of methionine (1%) [4]. If cultivars could be developed with a higher quantity of these amino acids, it would enhance the value of cassava as a food and/or feed. Only about 60% of the total nitrogen derives from amino acids, and about 1% of it is in the form of nitrates and

hydrocyanic acid. The remaining of the total nitrogen (38-40%) remains unidentified.

The majority of cassava clones grown and consumed in northeast Brazil are known to be free of carotenoids, which leads to many health problems for inhabitants of this region. One of the interesting aspects is to screen indigenous clones for cultivars rich in carotenoids. This concept is based on the fact that crop landraces have accumulated, in their center of diversity, desirable mutations that were selected by indigenous farmers during their history of cultivation. The nutritive importance of carotenoids is attributed to its conversion to vitamin A, as in the case of β -carotene, and to its antioxidant property and ability to quench singlet oxygen as in the case of lycopene. Lycopene interacts with free radicals eliminating their deleterious effect. Some landraces maintained at the genebank of the Univ. of Brasília may be a source of carotenoids as shown by their red root flesh and yellow color after cooking [5].

Efforts have been made in the past to increase the protein content of cassava roots by interspecific hybridization with a wild species, namely *M. saxicola* and *M. melanobasis*. Over a period of 10 years beginning in 1932 and ending with the Japanese occupation of Java in 1942, Bolhuis [6] carried out a program of cassava breeding for increased protein content in roots. Crosses with *M. saxicola* yielded a few seedlings with as much as 2% protein in the root fresh. In the clones, he propagated from these seedlings protein content fell back

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to typical levels. Moreover, Jennings [7] reported that the roots of the F₁ progeny of *M. esculenta* × *M. melanobasis* possessed approximately twice as much protein as their cassava parent. Such progenies were, however, lost and not grown anywhere, probably because of poor root yield. This article reports the assessment of lycopene content and amino-acid profiles of cassava landraces and an inter-specific hybrid, respectively.

MATERIAL AND METHODS

Evaluation of Carotenoids

The landraces UnB 400 - with yellow color after cooking, and UnB 500 - with red root flesh, which are indigenous cassava clones grown in the Amazon and maintained in the University of Brasília's living *Manihot* species collection were analyzed spectrophotometrically for lycopene content along with the cassava cultivar Pohlii and ICB 300 - an inter-specific hybrid between cassava UNB 01 and *Manihot oligantha*. Root powders were also taken from inter-specific hybrids ICB 300 and Diploide and ICB 300-derived offspring (Progeny 4, Progeny 9 and Progeny 10) for amino-acid profiling.

Lycopene Analysis

Extraction. Ten grams of mature roots was extracted three times with acetone (5 ml per gram). The filtered acetone extract was added in separation funnel containing petroleum ether, distilled water, ethylic ether (100/100/0.3: v/v/v) The aqueous fraction was discarded and the organic fraction was submitted to saponification. Saponification was preferred since it removes accompanying lipids and chlorophylls. In this study, the optimal conditions for mild saponification were achieved with 10% methanolic potassium hydroxide solution (100 ml) overnight at room temperature. After saponification, the aqueous fraction was discarded and the organic fraction was dried with anhydrous sodium sulfate. The organic fraction was evaporated to dryness at 30°C, re-suspended in 1000 µl ethyl acetate and methanol (v/v; 50/50) and submitted to HPLC separation.

Equipment. Carotenoid analyses were performed with a Shimadzu LC-10A HPLC equipped with a photodiode array detector SPD MXA-10 and a Rheodyne injection valve with a 20-µl loop. The separation was carried out in a C18 Vydac 218TP54 column 250 × 4.6 mm i.d. (5-µm particle size) with 100% MeOH as mobile phase at a flow rate of 1 ml/min at a temperature of 15°C. The chromatograms were processed at wavelengths of maximum absorption (450 nm). The identification of carotenoids was achieved by retention time (t_R) comparisons with those of the standard compounds and using the wavelength of maximal absorption (λ_{max}) and the spectrum profile between 300 to 600 nm compared with data available in the literature [8].

Quantification. The calibration curves for lycopene were purchased from Sigma Inc., and purified from tomato, while for trans-β-carotene (purchased from Sigma Inc.), and for α-carotene (purified from alfalfa) were constructed with a minimum of the concentration levels thrice. All curves showed a good relation of area and concentration achieving a coefficient of determination (R²) of 0.96, 1 and 0.98, for lycopene, trans-β-carotene and trans-α-carotene,

respectively. The cis isomer of lycopene was quantified using the calibration curve of lycopene. The vitamin A values were calculated according to the conversion factor given by NAS-NRC, in which 6 mg of trans-β-carotene correspond to 1 mg of retinol equivalent (RE), and the activities are related as follows: 100% for trans-β-carotene, 50% and for trans-α-carotene and cis-β-carotene.

Evaluation of Amino-acid Profiles

Sample extraction. Samples (500 mg) of cassava root powder were extracted with 1 mL 10 mM HCl for 4 h, at 25°C, under agitation at 1,200 rpm in Thermomixer (Eppendorf, Hamburg, Germany). The suspensions were then centrifuged for 4 min at 6,000 rpm in a bench centrifuge. The supernatant (800 µL), called acid extract, and the remaining powder were dried down in a SpeedVac vacuum centrifuge (Savant, NY, USA). The dried powder was extracted in the same way with 1 mL 10 M NH₄OH producing an alkaline extract. The dried acid extract was resuspended with 750 µL 10 mM HCl, washed with an extra 750 µL of the same dilute acid, and added to 750 µL of the alkaline extract. The total extract was exhaustively dialyzed against MilliQ water and vacuum-dried in a SpeedVac centrifuge.

Amino acid analysis. Aliquots of 150 µg of each extract were dissolved in 75 µL 100 mM HCl. Acid hydrolysis of the samples was performed in 6 M HCl under vacuum for 24 h at 109°C. After acid hydrolysis, the hydrolyzed samples were solubilized in 75 µL 100 mM HCl, and 50 µL was injected into an amino acid analyzer (Hitachi L8500, Tokyo, Japan). The analyses for determination of amino acid compositions were performed in triplicate. The total protein contents of the samples were calculated by summing up the amounts of the amino acids. Amino acid compositions from *Manihot* proteins were determined by analyzing sample extracts which were dialyzed with water to remove free amino acids, salts, monosaccharides, and other small molecules. Tryptophan could not be analyzed since it is degraded upon acid hydrolysis. By summing the amounts of the analyzed amino acids, it was possible to determine the protein content for each sample.

RESULTS AND DISCUSSION

Selection of Carotenoid-Rich Cassava Landraces

UNB 400 has a gray stem, which is 1.5 m high, has large raised scars and 2 or 3 branches. Leaves have 5 to 7 lobes, and the leaf lobe is obvater, with the margins slightly sinuous, whereas the medium lobe is 14 to 16 cm in length. The leaf has a green petiole, and the young foliage is reddish. The inflorescence is a 5- to 8-cm glabrous panicle. Bracts and bracteoles are inconspicuous and caduceus. Flowers are monoecious showing pistillate flowers with basal opening, whereas the staminate apical opening occurs 3 weeks later. Fruits are green and winged and the roots are conical, with a rough, pink-brownish surface. The root flesh is creamy but turns yellow after cooking (Fig. 1a). UNB 500 has a gray stem, which is 1.5 m high with large raised scars and 2 or 3 branches. Its leaves are 7-lobed, and the leaf lobe is linear, with the margins slightly sinuous, whereas the medium lobe shows 10 to 12 cm in length. The leaf has a green petiole, and the young foliage is reddish. The

inflorescence is a 3- to 6-cm glabrous panicle. Bracts and bracteoles are inconspicuous and caduceus. Flowers are monoecious showing pistillate flowers with basal opening, whereas the staminate apical opening occurs 3 weeks later. Fruits are green and winged and the roots are conical, with a rough, pink-brownish surface. The root flesh is slightly red and turns dark red after cooking Fig. (1b).

The chromatogram profiles of lycopene isolated from tomato and the cassava clone extract are shown in Fig. (2a & b), respectively. Lycopene was shown to be the major carotenoid, although α -carotene and cis-lycopene were also found. The identification and characterization of the peaks are given in Table 1. Other carotenoids could not be detected in the cassava clone, which showed a concentration of 5 mg lycopene per gram of wet root weight. Fig. (3a & b) shows the spectrogram profile (range 300-600nm) of peak referent to lycopene isolated from tomato and of cassava clone roots, respectively. Carotenoids can be encapsulated through novel proprietary methods such as the one included in the patent by Barenholz *et al.* [9].

Table 1. Quantification (ug/g of Tissue) of Lutein, *trans*- β -Carotene and *cis*- β -Carotene of some *Manihot* Cultivar Organs

	Lutein	<i>trans</i> - β -carotene	<i>cis</i> - β -carotene
Roots			
Pohlii	-	0.16	0.09
UnB 400	236.83	1.24	0.96
ICB 300	-	0.19	0.12
Leaves			
Pohlii	782.15	13.85	2.37
UnB 400	3081.69	24.12	3.28
ICB 300	9108.98	18.02	1.88

The retention time in HPLC system and the similarity of the spectral profile 300-600 nm in photo-diode array of 0.98 confirm the presence of lycopene in this cassava clone. *Trans*- β -carotene reached 27.40 μ g/g in UnB 400, making it

a source for this micro-nutrient among indigent populations who depend on the daily consumption of cassava as an essential food. The root of this clone is creamy colored but turns yellow when cooked. Its palatability is excellent and almost free of fibers.

The retention time in HPLC system, and the similarity of the spectrogram profile 300-600nm in photo-diode array of 0.98 Fig. (3) confirms the presence of lycopene in this cassava clone.

This research provides a means of better understanding cassava domestication and further breeding by indigenous Amazon farmers. The clone UNB-400 is grown in the Amazon, and from there it was brought to the State of São Paulo, where it was further grown by a few farmers. This clone could have originated from a gene mutation that breaks the sequence of β -carotene formation, and then adopted by Amazon farmers who used it probably for rituals or cultural ceremonies. This clone forms few roots compared to other improved cultivars. However, increasing its root yield appears feasible by crossing with another clone possessing high combining ability for root yield. A patent (US200874322873) on a carotenoid ketolase mutant was submitted by Stead *et al.* [10].

Lycopene occurs in tomato, guava, watermelon, and pink grapefruit and its consumption appears to be associated with reduced degenerative diseases. Formulations of lycopene and whey protein are used for the treatment of atherosclerotic disorder [11]. Other potential human health benefits include a possible role in the fight against digestive tract, breast, lung, stomach and prostate cancers [8, 12-15]. King *et al.* indicated the carotenoid and analogs are medically suitable for the treatment of prostate cancer [16]. Epidemiological studies have shown that high intake of vegetables containing lycopene is inversely associated with the incidence of certain types of cancer. For example, regular intake of tomato products, with high lycopene content [17], has been inversely associated with the risk of cancer of the digestive tract. Lycopene is a precursor of β -carotene, whose synthesis includes an enzymatic cycle in the chain-end [18]. The high lycopene level found in this cassava clone may indicate a disruption in the biosynthesis of β -carotene. The lycopene accumulation in this cultivar may therefore be the result of a deficiency in β -carotene synthesis due to a mutation. UnB 400 can be considered a good source for β -carotene in

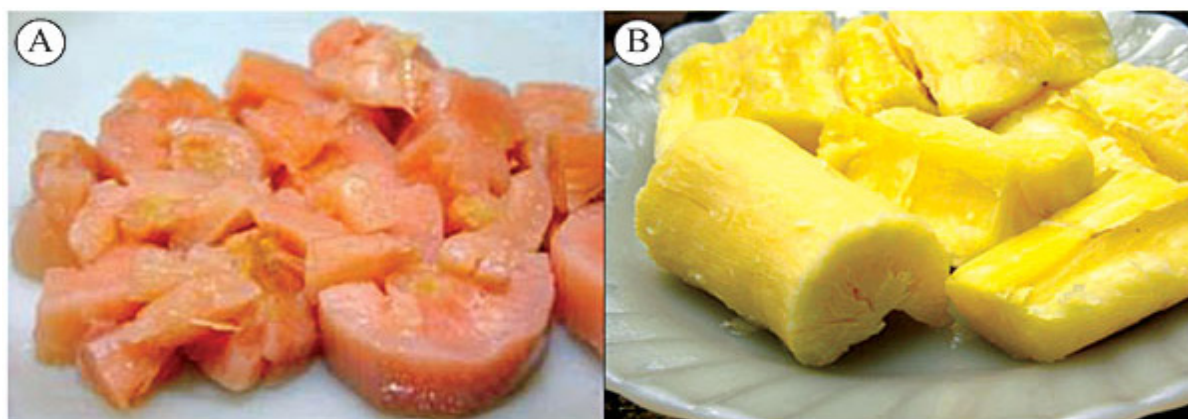


Fig. (1). Cooked roots of clone UnB 400 (A) and UnB 500 (B).

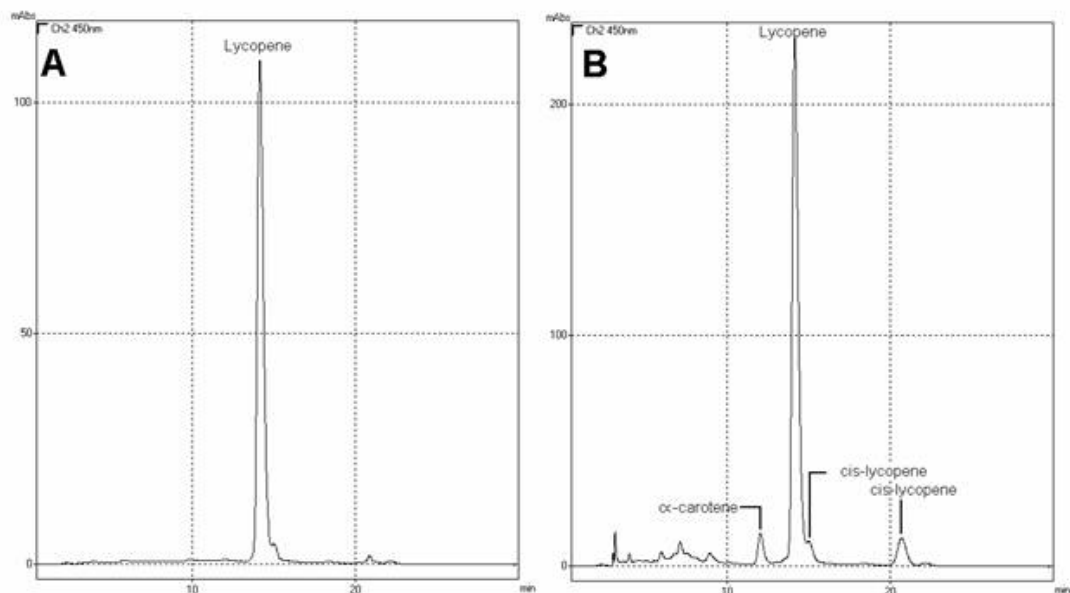


Fig. (2). Chromatogram profile of (A) lycopene and (B) cassava clone, showing peaks of trans- α -carotene, lycopene and cis lycopene. HPLC analysis conditions: RP column C18 Vydac 218TP54 column 250x4.6 mm, mobile phase 100% MeOH, flow 1.0 ml/min.

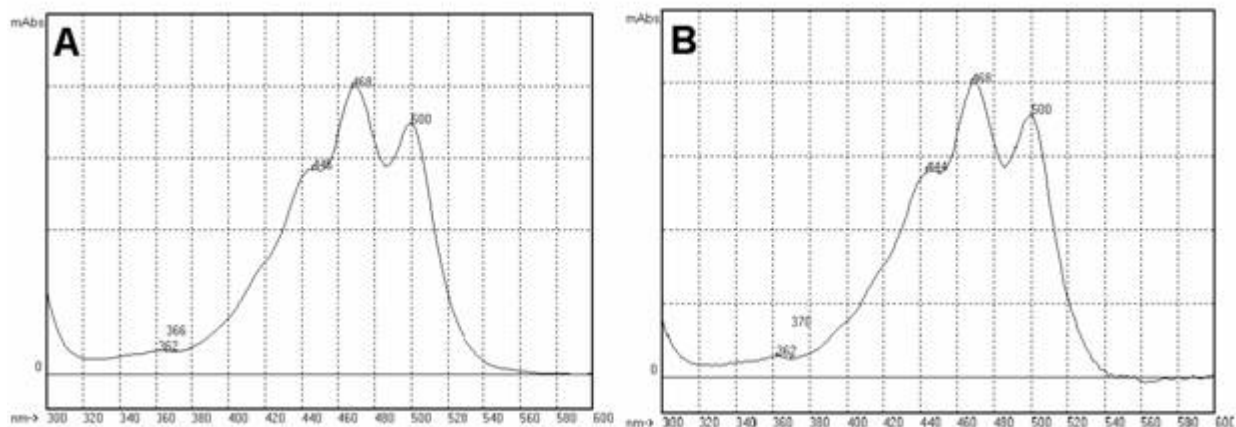


Fig. (3). Spectrogram profile (range 300-600nm) of peak referent to lycopene (A) isolated from tomato and (B) of cassava clone roots, showing a similarity of 0.98. RP column C18 Vydac 218TP54 column 250 x 4.6 mm, mobile phase 100% MeOH, flow 1.0 ml/min.

regions where cassava is the day-to-day principal food. β -carotene-like lycopene is an antioxidant, besides being a precursor of vitamin A, which is important in protecting against eye and skin diseases [19, 20]. Chomczynski has elaborated further on the significance of antioxidant dietary supplement for maintaining healthy skin [21].

AMINO ACID PROFILES IN CASSAVA INTER-SPECIFIC HYBRID AND DERIVED OFFSPRING

ICB 300 derives from the wild species *M. oligantha*, which contains 4% crude protein [22]. This inter-specific hybrid shows a shrub with 2 to 3 m height, has conical roots of 40 to 60 cm length with a 3 to 4 cm neck, and they are abundant (5-10 kg per plant). Root surface color is dark brown, whereas the root flesh is white. This hybrid possesses a brown story, and a 1-1.2 m height story. Scars on stem are moderately raised. Stems are 3-branched. Leaves have 3 lobes, occasionally 1 or 4 lobes. Leaf lobe is obovate, with

simple margins and green reddish petioles. Young foliage at apices is green. Inflorescence is a 10 to 20 cm panicle glabrous, with bracts and bracteoles inconspicuous and caducous. Flowers are monoecious, pistillate, 2 to 3 based, open 3 weeks earlier than staminate ones. Perianth has 5 separate tepals, and the yellow pistillate ovary has a ovary sublobed by a non-lobed yellow disk, 3 carpellate and glabrous. Fruit is winged and smooth surfaced. Seeds are carunculate elongate and brown.

Among the six samples analyzed in this study Table 2, Fig. (4), the sample of the interspecific hybrid ICB 300 showed the highest amount of protein (1.654 g/100 g sample powder), followed by Diploide (1.454 g/100 g) and Progeny 9 (0.922 g/100 g). Progeny 10, Progeny No. 4 and UnB 01 showed poor protein content (0.350 g/100 g). The levels of essential amino acids were also higher in inter-specific hybrid ICB 300 (His, Leu, Lys, Met, Phe, and Val) and Diploide (Ile and Thr), with low or undetectable amounts in

Table 2. Amino Acid (AA) Profile (g per 100g Sample Mass) in Peeled Roots of Cassava Cultivar UnB, its Inter-Specific Hybrid with *Manihot oligantha* ICB 300 (Sample 3 and Diplóide), and ICB 300-Derived Offspring (Progeny 4, Progeny 9 and Progeny 10)

AA	UnB 01	ICB 300 Sample 3	Progeny 10	Progeny 4	Progeny 9	ICB 300 Diplóide
Ala	0.020	0.093	0.017	0.019	0.040	0.098
Arg	0.037	0.261	0.061	0.082	0.320	0.108
Asp	0.016	0.146	0.023	0.033	0.052	0.137
Cys	0.027	0.029	0.026	0.025	0.026	0.025
Glu	0.039	0.222	0.044	0.065	0.151	0.221
Gly	0.012	0.078	0.012	0.015	0.037	0.075
His	0.000	0.038	0.010	0.010	0.027	0.036
Ile	0.008	0.068	0.010	0.010	0.018	0.069
Leu	0.016	0.131	0.013	0.000	0.041	0.127
Lys	0.010	0.098	0.020	0.019	0.034	0.079
Met	0.014	0.041	0.004	0.000	0.019	0.037
Phe	0.016	0.129	0.058	0.000	0.065	0.120
Pro	0.000	0.054	0.000	0.000	0.000	0.066
Ser	0.012	0.088	0.013	0.018	0.033	0.078
Thr	0.008	0.061	0.007	0.013	0.022	0.066
Tyr	0.000	0.000	0.000	0.000	0.000	0.000
Val	0.019	0.115	0.027	0.025	0.039	0.112
Total	0.254	1.654	0.344	0.336	0.922	1.454

the other materials. This results shows that inter-specific hybridizations provides materials that could be more interesting sources for breeding nutritious cassava for human consumption. Furthermore, Progeny 9 showed an equal amount of protein as its inter-specific hybrid parent (ICB 300), i.e., doubling that of common cassava, thereby indicating high heritability of this trait and the possibility of selecting for high-protein cassava.

The results of amino acids profiles of cassava roots agree with those from the available literature. For example, Bailey [23] indicated a deficiency in sulfur-containing amino acids (methionine, cystine and cysteine), whereas Osuntokun et al, [24] pointed out that both cysteine and cystine are involved in cyanide detoxification. Cyanide is produced when the cyanogenic glucoside linamarin, present in cassava, is hydrolyzed by linamarinase or by acid. Cyanide is mainly detoxicated by conversion to thiocyanate, in the process of which it reacts with cysteine and cystine. Excessive detoxification may be responsible for the low concentration of sulfur-containing amino acids [24]. In this regard, cassava with high-nitrogenous content could be bitter cultivars with high-glucoside content [25]. Humidity could also influence protein assessment as total nitrogen in cassava edible roots. Excessive drying of the root powder may increase drastically

the percentage of nitrogen by 3-fold. Hence, it is important to determine protein content as amino acids jointly with its assessment as total nitrogen to ensure right screening of *Manihot* germplasm [26].

CURRENT & FUTURE DEVELOPMENTS

Cassava cultivars UNB 120, UNB 122, UNB 123 and ICB 300 - all with average tuberous root yields above 15 kg plant⁻¹ - are available to farmers in Brazil through cuttings from the Univ. de Brasilia. They show either yellow or orange flesh due to their carotenoid or lycopene content, respectively. Furthermore, the Government of Brazil plans to enact a law that will obligate mixing wheat flour with about 20% of cassava flour for making bread. In this regard, cassava cultivars rich in essential amino acids and carotenoids will be important for enhancing the nutritional quality of this new bread with cassava and wheat flours.

CONFLICT OF INTEREST

The first author acknowledge funding provided by the National Council for Scientific Development-CNPq, Brasilia for undertaking research to enhance nutritional quality of cassava.

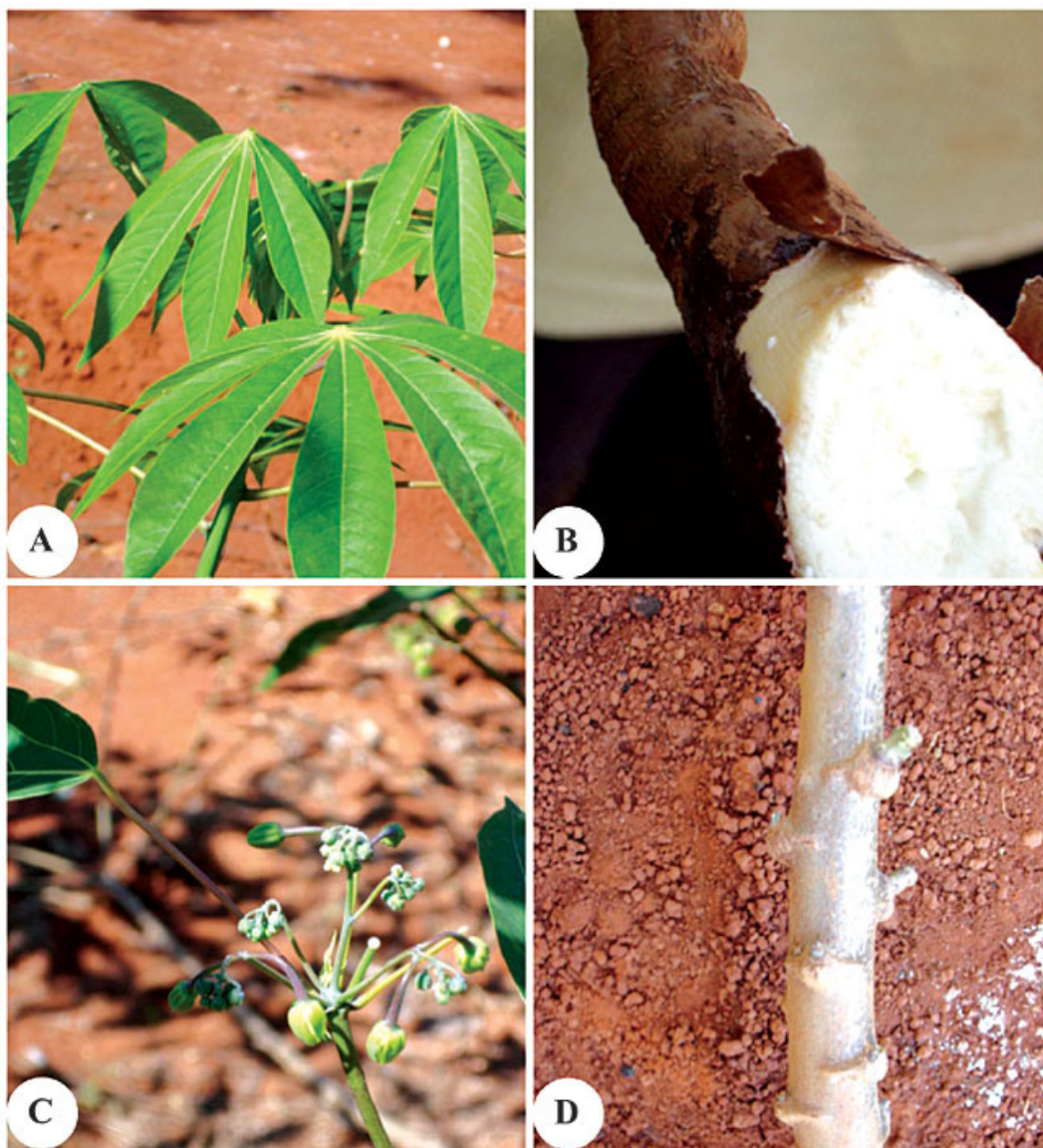


Fig. (4). Morphology of interspecific hybrid ICB 300.

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