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Development and Identification of Cassava Clones With Special Starch Characteristics

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ABSTRACT

Cassava (*Manihot esculenta* Crantz) is an important food security crop for many tropical and subtropical countries. It is also acquiring an increasing role in rural development as raw material for different industries. The most important industrial uses of cassava are as a source of energy in the feed industry and for the starch and ethanol industries. To consolidate and expand the industrial uses of cassava, an increased emphasis in the search for value-added traits, while maintaining or enhancing its productivity, has recently been given by the cassava-breeding project at CIAT. Different strategies have been implemented simultaneously. Since most traits offering qualitative changes in starch properties are recessive in nature, these strategies rely heavily in the self-pollination of a wide range of cassava genotypes to expose useful recessive traits. Ongoing research for the production of doubled-haploid lines will reduce the time required to reach full homozygosity. Plants from irradiated seed in a mutation-breeding project have been evaluated in the field and many self-pollinations have been made to implement the TILLING system. Several novel starch types have been identified. A waxy starch mutation has been fully characterized. In addition, several other mutations have been identified and are currently being characterized. Among them a small-granule type which showed (in a preliminary evaluation) higher-than-normal levels of amylose. Another abnormal starch with granules that looked hollow through optical microscopy was also identified. There were several other abnormal types that need further analyses because the preliminary evaluations were made on single plants derived from botanical seed. All these off-types have been cloned and will be harvested again by the end of 2007.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a perennial crop native to tropical America, with the probable center of origin in the southern rim of the Amazon basin of Brazil (Olsen and Schaal, 2001). Cassava is one of the most important sources of food energy in many tropical and subtropical countries. There are an estimated 200 million people who obtain more that 500 cal/day from cassava, (Cock, 1985; Kawano et al., 1998). The crop produces reasonably well under marginal conditions of climate and soil, and is frequently identified as a famine reserve due to its tolerance to drought and infertile soils and its ability to recover from disease and pest attacks. It can also produce competitively in non-marginal areas. Cassava offers the advantage of a flexible harvesting date, allowing farmers to hold the roots in the ground until needed (Iglesias et al., 1997).

In addition to its important role in subsistence farming and food security, cassava is acquiring an increased role in rural development as raw material for many industrial applications. Cassava has had a huge impact particularly in Asia, helping resource-limited farmers to improve their livelihoods (Howeler, 2005; Kawano, 2003). The most important industrial uses of cassava are as a source of energy in animal diets in the feed industry and for the starch and ethanol industries. The main strategy used until now to promote cassava as an industrial raw material has been to increase its productivity and/or reduce production costs, allowing for a competitive price of the roots. However, very little effort has been made to increase the value of cassava roots.

Compared with the many economically useful mutations found, for example, in the maize kernel (sweet corn, pop corn, waxy maize starch, opaque 2, etc.), very little variability has been reported for cassava. It is valid to assume that such variability exists in the crop, but has not been found, at least, because of two main reasons: (a) Starch mutations in the roots are more difficult to detect than in grain kernels (where they can be easily identified by visual inspection without the need for any sophisticated tests). To detect a mutation in the cassava root starch, the breeder has to cut the roots and most likely to conduct a particular test (i.e. iodine test) or biochemical analysis to be able to pick potentially useful variants. It is possible, therefore, that clones with valuable traits had already been grown in breeding nurseries but could not be detected and, not showing an outstanding agronomic performance, they were unfortunately discarded; (b) The known starch mutants are usually recessive. The fact that cassava seldom undergoes inbreeding drastically reduces the chance of (expected) low-frequency recessive alleles from expressing in the phenotype.

The fact that roots are not reproductive or multiplicative organs may offer cassava (and other root crops) an advantage over the true seed-propagated crops. It is valid to assume that cassava roots could withstand mutations that would otherwise be lethal for reproductive organs such as the kernels of cereals.

In spite of the problems mentioned above, the globalization of economies and new technological breakthroughs are offering a unique opportunity for cassava never available to the crop before. Tropical production of maize is facing increasing problems in competition with maize from temperate regions. This situation has prompted government and private sectors of many tropical countries to turn to cassava as a competitive alternative to imported maize. In addition, advances in molecular biology,

genetic engineering, plant-tissue culture protocols and starch technologies provide important tools that will allow bridging the main gaps between cassava and the cereals.

Many of the concepts mentioned in this introduction were first mentioned during the 3rd Conference on Starch Technology in 2005 (Ceballos et al., 2005). This paper however, describes the first results of the approaches taken at CIAT to develop and identify cassava clones with higher value in their roots.

MATERIALS AND METHODS

Most useful mutations are recessive in nature. Therefore, to identify them some degree of inbreeding needs to be involved to allow the recessive alleles to express themselves. Alternatively, molecular approaches can now be incorporated if the target is a particular gene, which has been properly characterized. DNA TILLING (for *Targeted Induced Local Lesions in Genome*) has been successfully used in different plant species (McCallum et al., 2000; Perry et al. 2003; Till et al. 2003) to identify genotypes that carry mutant recessive alleles, even if they are in the heterozygous condition. Results presented in this report come mainly from two different approaches which are briefly described below.

Self-pollination of a wide range of cassava germplasm: The introduction of inbreeding in the genetic improvement of cassava offers several advantages which have been described (Ceballos et al., 2004; Pérez et al., 2005a; 2005b). One of the advantages, which has a direct bearing with the theme of this paper, is that it would allow for the identification of useful recessive traits (such as the starch quality mutants found in different crops, particularly maize), which may lead to the development of value-added genetic stocks.

CIAT, with the support of the Rockefeller Foundation, and in collaboration with several cassava breeding programs in Africa, Asia and Latin America began in 2004 a research project to systematically self-pollinate elite cassava germplasm as well as accessions of landraces held in the Germplasm Collection at CIAT. Large number of botanical seed with varying degrees of inbreeding has been produced (Ceballos et al., 2005). The project also involves the development of an anther culture protocol for the production of doubled haploids. As soon as partially inbred lines are produced, seed is germinated and the resulting plants analyzed in search of novel starch types. The production of doubled haploids in cassava provides an appealing option for feasible introduction of inbreeding in cassava genetic improvement by drastically reducing the time required to produce homozygous parental lines.

Mutagenesis and the "TILLING" System: Breeders have used chemical products or irradiation such as gamma rays to induce mutations and generate genetic variability with relative success, particularly in the decades of the 1950s and 1960s (Maluszynski et al., 2001; Ahloowalia et al., 2004). Mutation breeding has a few drawbacks. Events are totally random, recessive in nature and usually appear as chimeras. Therefore, thousands of genotypes need to be evaluated before a useful mutation in the desired gene can be found. With the advent of molecular biology tools, an interesting system (DNA TILLING) was developed to overcome some of the limitations of mutation breeding. Sexual seeds are mutagenized and, to avoid ambiguities caused by chimeras in the first

generation plants ($\mathbf{M_1}$), they are self-pollinated. The resulting plants ($\mathbf{M_2}$) are then evaluated while DNA is extracted from them. For screening purposes, DNAs are pooled eightfold to maximize the efficiency of mutation detection. (description of the TILLING method adapted from Till et al., 2003).

CIAT is participating in a project led by Universidad Nacional de Colombia, which is supported by the IAEA (International Atomic Energy Agency). About 4,000 seeds from six different cassava clones were irradiated with gamma rays (using a Cobalt 60 source with a dosage level of 200 Gy) or with fast neutrons. Seeds were germinated and transplanted to the field early in 2004. Plants have been carefully evaluated in search of promising mutant forms (although it is recognized that the occurrence of chimeras and the lack of expression of recessive mutations will certainly reduce the probabilities of finding such mutants at the $\mathbf{M_1}$ stage). As soon as plants started to produce viable flowers, they were self-pollinated. As many as 5,000 $\mathbf{M_2}$ seeds, from about 140 different $\mathbf{M_1}$ plants, have been obtained. Several genes related to starch biosynthesis will be targeted for TILLING analysis.

High Capacity Root Quality Laboratory: The approaches described above are specifically targeting the identification of known mutations (i.e. waxy starch using TILLING). However, it is valid to assume that unknown mutations may also be available in cassava. A common need of many of the strategies described in this paper is for the availability of a high capacity root quality analysis laboratory to screen large numbers of samples (>15,000) in search of those genotypes with novel pasting properties of starch or enhanced nutritional value. CIAT is developing jointly with Universidad Nacional de Colombia a laboratory that will be able to generate thousands of amylograms per year using a battery of rapid viscoanalyzer, Brabender, DSC, and other standard equipment and protocols. The use of Near Infrared Analyzers has been reported recently for the quantification of amylose and amylopectin (Bao et al, 2007)

CIAT strategy, therefore, relies on a two-steps approach that aims at solving the two main limiting factors described in the introduction. Inbreeding and mutagenesis aim at inducing genetic changes and allowing them to express. The laboratory is a requisite for the phenotypic identification of any genotype with abnormal starch types.

RESULTS

Waxy starch mutation: An amylose-free starch mutation has been identified in March 2006 (Ceballos et al, 2007) as a result of the systematic self-pollinations to produce thousands of partially inbred germplasm from a wide array of cassava germplasm. In December 2004 several self-pollinations were made on a cultivated cassava genotype as part of the project to introduce inbreeding in cassava genetic enhancement at CIAT. The S_1 family AM 206, one among many S_1 families produced and evaluated, included 79 seeds, which were germinated in a greenhouse on April 2005. Only 40 plants were viable and transplanted to the field in June 2005. Of those, 17 plants survived with a good development after 9 months. In March 2006, roots from the S_1 genotype AM 206-5 showed a unique and distinctive staining when treated with an iodine solution. There was a differential staining with the iodine solution on roots and stems from AM 206-5 compared with those from other genotypes. Roots and stems from AM 206-5 stained

brown-reddish, while roots and stems from other genotypes showed the typical blue-dark staining. The differential staining prompted to carry out other tests on AM 206-5 and their results are presented below.

Upon the discovery of the special characteristics of the single seedling plant representing AM 206-5, up to forty stem cuttings were obtained to clone this genotype and planted at CIAT-Palmira on June 2006. Roots from five random cloned plants were then harvested in April 2007 and analyses made to confirm the properties first identified in the seedling plant in March 2006. Results of the analysis of two from "normal" cassava starches (from clones MCOL 2208 and MPER 247) are also provided to highlight where the starch of AM 206-5 behaves differently. Tables 1 through 4 present the most important results of the comparison of the starch of AM 206-5 versus the "normal" starches.

Table 1 presents results of the proximal analysis of root flour, including dry matter and starch contents of the three genotypes reported. Flour from AM 206-5 was extracted from the five cloned plants and analyzed independently. Results from the two check genotypes were based on non-replicated analyses and are included just as a reference for the reader. Dry matter content for the three genotypes fell within normal ranges for cassava, although that of AM206-5 was slightly lower than those of the reference genotypes. Ash content tended to be higher in AM206-5 than in the other genotypes (Table 1). The starch extraction procedure utilized left only traces of protein. In the case of AM206-5, for example, average protein content of the starch from the five plants sampled was 0.12% with a standard deviation of 0.037.

Table 1. Proximal analysis % (g/100g db) from the three cassava genotypes analyzed. Within parenthesis the standard deviations based on the independent analyses of the roots from five different cloned plants of AM206-5.

Parameters	AM206-5*	MCOL 2208	MPER 247
Dry matter % (g/100g wb)	31.5 (1.3)	34.8	35.7
Ash content (%)	3.0 (0.2)	1.6	2.2
Crude fiber content (%)	4.6 (0.7)	2.6	3.2
Total sugars (%)	1.6 (1.1)	2.9	3.6
Reducing sugars (%)	0.8 (0.8)	0.9	1.3
Starch content (%)	86 (3.9)	88	86

wb= wet basis

* Within parenthesis standard deviation values

Table 2 presents starch physico-chemical properties. Data from AM 206-5 comes from the starches of five random cloned plants harvested in April 2007 and analyzed individually. Data from MCOL 2208 and MPER 247 were based on three independent quantifications of a sample from pooled roots following the standard procedure at the cassava-breeding project of CIAT. Paste clarity was not different in the starch of AM 206-5 compared with the other two genotypes. Average amylose content using the

colorimetric method was 3.4%, compared with the averages of about 20% for the wild types, typical of cassava starch. Amylose content using the DSC indicated total absence of amylose in the starch. Both types of quantifications were statistically significant. The detection of small amount of amylose using the colorimetric method can be due to lack of purity in the commercial potato amylopectin standard used in the analysis. Also, some long-chain amylopectin branches can bind like amylose, acting somewhat like amylose in colorimetric tests (Bertoft, 2004).

Table 2. Starch physico-chemical properties of the starches from the three cassava genotypes analyzed. Within parenthesis the standard deviations based on the analyses of roots from five AM206-5 plants or three aliquots from root samples of MCOL 2208 and MPER 247*.

Parameter	AM 206-5	MCOL 2208	MPER 247
Paste Clarity (%)	57.6 (1.6)	56.2 (0.3)	50.3 (0.6)
Colorimetric amylose content (%)	3.4 (0.2)	20.4 (0.3)	19.7 (0.4)
Amylose content (%)	0.0 (0.0)	19.2 (0.0)	19.0 (0.5)
Gelatinization onset temperature (GT) in °C	63.1 (0.7)	60.4 (0.1)	61.8 (0.1)

* Within parenthesis standard deviation values
J/g = Joules per gram

Table 3. Pasting behavior of amylose-free and normal cassava starch from the three genotypes analyzed. Within parenthesis the standard deviations based on the analyses of roots from five AM206-5 plants or three aliquots from root samples of MCOL 2208 and MPER 247.

Parameter	AM 206-5*	MCOL 2208	MPER 247
Pasting temperature (PT) in °C	68.3 (0.8)	65.4 (0.4)	67.5 (0.2)
Peak viscosity (PV) in cP	890 (38)	577 (19)	746 (20)
Hot paste viscosity (HPV) in cP	399 (18)	329 (12)	456 (16)
Cool paste viscosity (CPV) in cP	490 (17)	416 (16)	580 (18)
Breakdown (BD) in cP	491 (31)	249 (7)	290 (27)
Setback (SB) in cP	-400 (32)	-161 (8)	-166 (24)
Consistency (CS) in cP	91 (3)	88 (6)	124 (6)

* Within parenthesis standard deviation values
cP=centipoise

The most relevant results from the pasting behavior of waxy and normal cassava

starch obtained from the amylograms obtained using the rapid viscoanalyzers is presented in **Table 3**. AM 206-5 showed higher viscosity peak (890 cP) versus those from the other two genotypes (577 to 746 cP). Overall the amylograms show the typical performance of cassava profile: lack of resistance to high temperature and sensitivity to shearing stress. Breakdown was noticeably different in AM 206-5 (491 cP) compared with typical cassava (249 and 290 cP), suggesting a reduced tolerance to shear stress in the mutant. The starch from AM 206-5 also showed a distinctive setback value (-400 cP) compared with the starches from the two checks (-161 and –166 cP). There was no relevant difference for consistency.

Solubility, swelling index and dispersed volume fraction measurements for the starch from AM 206-5 showed contrasting results in comparison with those from the other two "normal" genotypes (**Table 4**). Solubility of the starch from AM206-5 was about half of the values observed for MCOL 2208 and MPER 247. This is to be expected because amylose is more soluble than amylopectin. This behavior further suggests that the starch of AM 206-5 had considerably lower amounts of amylose than normal cassava starches. Swelling index in AM 206-5 was considerably higher (55.7 g g^{-1}) than for the other two starches (30.8 to 32.3 g g^{-1}). Volume fraction of dispersed phase was slightly higher in AM 206-5 (0.5 Φ) compared with the starches from the other two genotypes (0.41 to 0.45 Φ).

Table 4. Solubility and swelling values of waxy and normal cassava starch. Within parenthesis the standard deviations based on the analyses of roots from five AM206-5 plants or three aliquots from root samples of MCOL 2208 and MPER 247.

Genotype	Solubility (%, db)	Swelling index ($g\ g^{-1}$)	Volume fraction of dispersed phase (Φ)
AM206-5	6.0 (0.5)	55.7 (2.3)	0.50 (0.03)
MCOL 2208	14.1 (0.6)	32.3 (0.7)	0.45 (0.00)
MPER 247	13.4 (0.4)	30.8 (0.4)	0.41 (0.01)

db= dry basis

All analyses converged to support the hypothesis that genotype AM206-5 has amylose-free (waxy) starch and based on data from the seedling plant derived from botanical seed (starch extracted and analyzed in 2006) and the five random cloned plants (starch extracted and analyzed in 2007). Differential iodine staining of roots, stems and starch from AM 206-5 was the first indication, which was then supported by the absence of the GBSS enzyme in the SDS-Page electrophoresis (Figure not shown). Functional properties of starch from this genotype were also different and in agreement with the expectations for an amylose-free starch (high viscosity, high swelling index, and low solubility). Colorimetric and DSC results to quantify amylose content finally proved that the starch from AM206-5 has very low levels or absence of amylose, respectively. Granule morphology was not affected by this mutation. The combined results reported in this study produce convincing evidence that AM 206-5 has a naturally occurring mutation on the *Wx* locus which is the one codifying for the GBSS enzyme. Molecular and

traditional genetic analyses are currently underway to further confirm this.

Crosses of AM 206-5 are underway to transfer the mutation to germplasm adapted to the most important cassava growing environments. Because the heterozygous nature of parents used in cassava breeding a traditional back-cross scheme cannot be properly implemented in cassava. Therefore, the strategy relies on making a first cycle of crosses between AM 206-5 and elite germplasm. All the resulting F1 genotypes will be heterozygous (*Wx wx*) for the mutation and are, therefore, expected not to produce amylose-free starch. The F1 plants from the first cycle of crosses will be crossed among themselves to produce a second cycle of crosses. It is expected that about 25% of the segregating progenies will be homozygous (*wx wx*) for the GBSS locus and, therefore, will produce amylose-free starch. Because crosses will have been made among the genetically diverse germplasm from the first cycle of crosses, inbreeding in the second cycle of crosses will be minimized. It should be possible, therefore, to identify vigorous and productive genotypes with waxy starch in the second cycle of crosses.

Small-granule mutation: In March 2006 a second mutation was identified but from an entirely different background. Two M_2 sister plants derived from the same M_1 mutagenized plant showed a very distinctive starch phenotype. **Figure 1** presents an illustration of scanning electron microscope photographs of such a mutation compared with "normal" cassava starch.

The small-granule mutations have only been evaluated in plants originated from botanical seed. Upon their identification the plants were cloned, grown and will be harvested in September 2007. Results presented here are only based on the seedling plants and need further confirmation once the cloned plants are harvested. The amylograms presented in **Figure 2** compare "normal" cassava and starches from the waxy mutation reported above (based on starch from the seedling which was slightly different as to be expected from those of cloned plants) as well as the small-granule mutations. **Table 5** presents additional information on the pasting properties of the small-granule mutation, which should be taken with caution until further confirmation. Very relevant from these results is the high amylose content observed in these mutations, parallel to a very low viscosity in their gels. It is feasible that this mutation is equivalent to the amylose extender (ae_1) mutation found in maize and molecular tests will be conducted to test this hypothesis (Neuffer et al. 1997).

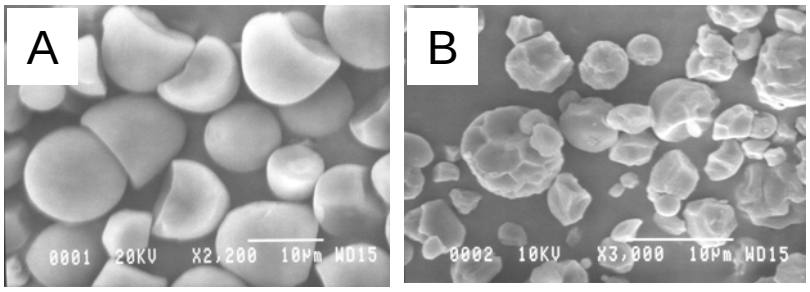

Figure 1. Scanning electron microscope images of normal starch granules (A) and of a mutation that has small, fragmented granules (B).

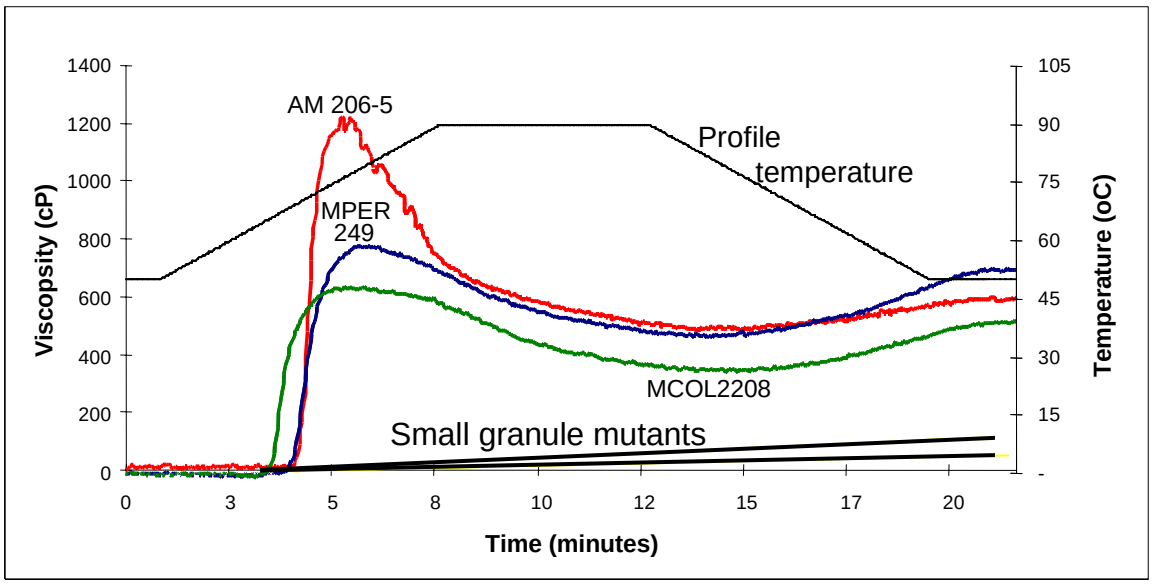

Figure 2. Amylograms from cassava starches with different pasting properties. AM 206-5 is the genotype where the waxy starch was first identified. MPER249 and MCOL 2208 are "normal" cassava starches and close to the horizontal axis the very distinctive behavior of the two small-granule mutations.

Table 5. Proximal analysis comparing pasting and biochemical properties of small granule mutations with those of a "normal" cassava starch.

Parameter	"Normal" cassava	Small Granule-1	Small Granule-2
Dry matter content	88.3	89.7	88.8
Onset gel (°C)	59.2	52.4	52.3
Peak gel (°C)	65.0	58.4	60.3
End gel (°C)	73.7	65.5	75.7
Delta H	14.9	7.4	11.3
End (°C)	80.7	79.3	80.1
Peak (°C)	84.9	83.9	84.5
Onset (°C)	87.2	87.5	87.5
Delta H	-5.8	-10.4	-7.6
Amylose DSC (%)	20.07	36.96	26.53
Water absorption (%)	4.97	4.95	5.03
Water solubility (%)	2.43	16.48	15.44
Swelling power (%)	4.97	4.87	4.95
Clarity	39.5	17.0	26.0
Easy cooking (min.)	5.61	3.90	6.26
Maximum viscosity RVA	71	4	8
Gel instability	17.3	1.5	1.3
Gel index	17.17	1.33	4.17

Other mutations: Different off-types have been observed and identified during the last year in addition to the waxy and the small-granule mutations. These include a group of mutations that do not seem to store starches but rather low dry matter root, which may be related to the mutations reported by Carvalho and co-workers in 2004. Another interesting mutation was observed in a genotype whose roots did not show post-harvest physiological deterioration (PPD) even three weeks after harvest and stored in conditions that induces PPD within 1-3 days. Finally a third abnormal type was observed in a genotype that presented starch granules that looked hollow inside when looked through the light microscope. All these mutations were observed in plants derived from botanical seed. Therefore they come from single-plant observations and need further confirmation in the harvest that will take place in September, 2007.

CONCLUSIONS

Few important conclusions can be drawn from the events that took place since the last Conference on Starch Technology in November 2005:

1. The methodologies used (mutagenesis and inbreeding) have been extremely useful in producing cassava germplasm with special starch characteristics.
2. Mutations similar to those observed in other crops have started to surface in the *Manihot* gene pool.
3. Results clearly indicate that a waxy-starch mutation has been identified in cassava.
4. Convincing evidence of a high-amylose mutation is also gradually being build-up.

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LITERATURE CITED

Ahloowalia, B.S., Maluszynski, M. and Nichterlein, K. 2004. Global impact of mutation-derived varieties. Euphytica 135:187-204.

Bao, J., Y. Wang, Y. Shen. (2007) Determination of apparent amylose content, pasting properties and gel texture of rich starch by near-infrared spectroscopy. Journal of the Science of Food and Agriculture 87: 204 –2048.

Bertoft, E. 2004. Analyzing starch structure. In *Starch in food. Structure, function and application.* Eliasson A.C. Ed.; CRC Press USA and Woodhead Publishing Limited, England, pp 57-96.

Carvalho, L.J.C.B.; de Souza, C.R.B.; Cascardo, J.C.M.; Junior, C.B.; Campos, L. Identification and characterization of a novel cassava (*Manihot esculenta* Crantz) clone with high free sugar content and novel starch. Plant Molecular Biology **2004,** *56,* 643-659.

Ceballos, H., Iglesias, C.A., Pérez, J.C. and Dixon, A.G.O. 2004. Cassava breeding: opportunities and challenges. Plant Mol. Biol. 56:503-515.

Ceballos, H., M. Fregene, Z. Lentini, T. Sánchez, Y.I. Puentes, J.C. Pérez, A. Rosero and A.P. Tofiño (2005). High-Value Cassava Clones for the Starch Industry. Proceedings Starch Update 2005: The 3rd Conference on Starch Technology. Bangkok, Thailand. November 4-5, 2005.

Ceballos, H., T. Sánchez, N. Morante, M. Fregene, D. Dufour, A. M. Smith, K. Denyer, J.C. Pérez, F. Calle, and C. Mestres (2007). Discovery of an Amylose-free Starch mutant in cassava (*Manihot esculenta* Crantz). Journal of Agricultural and Food Chemistry (in press)

Cock, J. 1985. Cassava: new potential for a neglected crop. Westview Press, Boulder, USA.

Howeler, R.H., W. Watananonta, W Wongkasem, K. Klakhaeng, and Ngoan Ngoc Tran. 2005. Working with farmers: the key to achieving adoption of more sustainable cassava production practices on sloping land in Asia. Acta Horticulturae 703:79-87.

Iglesias, C., Mayer J., Chávez A.L., and Calle, F. 1997. Genetic potential and stability of carotene content in cassava roots. Euphytica 94:367-373.

Kawano, K., Narintaraporn, K., Narintaraporn, P., Sarakarn, S., Limsila, A., Limsila, J., Suparhan, D. and Watananonta, W. 1998. Yield improvement in a multistage breeding program for cassava. Crop Sci. 38:325-332.

Kawano, K. 2003. Thirty years of cassava breeding for productivity – biological and social factors for success. Crop Sci. 43:1325-1335.

Maluszynski, M., Szarejko, I., Barriga, P. and Balcerzyk, A. 2001. Heterosis in crop mutant crosses and production of high yielding lines using double haploid systems. Euphytica 120:387-398.

McCallum, C.M., Comai, L., Greene, E.A. and Henikoff, S. 2000. Targeting induced local lesions in genomes (TILLING) for plant functional genomics. Plant Physiol. 123: 439–442.

Neuffer, M.G.; Coe, E.; Wessler, S.R. *Mutants of maize.* Cold Spring Harbor Laboratory Press. Plainview, NY, 1997.

Olsen, K.M. and Schaal, B.A. 2001. Microsatellite variation in cassava (*Manihot esculenta*, Euphorbiaceae) and its wild relatives: further evidence for a southern Amazonian origin of domestication. Amer. J. Bot. 88:131-142.

Pérez, J.C., Ceballos, H., Calle, F., Morante, N., Gaitán, W., Llano, G. and Alvarez, E. 2005a. Within-family genetic variation and epistasis in cassava (*Manihot esculenta* Crantz) adapted to the acid-soils environment. Euphytica 145 (1-2):77-85.

Pérez, J.C., Ceballos, H., Jaramillo, G., Morante, N., Calle, F., Arias, B. and Bellotti, A.C. 2005b. Epistasis in cassava (*Manihot esculenta* Crantz) adapted to mid-altitude valley environment. Crop Sci. 45:1491-1496.

Perry, J.A., Wang, T.L., Welham, T.J., Gardner, S., Pike, J.M., Yoshida, S. and Parniske, M. 2003. A TILLING reverse genetics tool and a web-accessible collection of mutants of the legume *Lotus japonicus*. Plant Physiol. 131.

Till, B.J., Reynolds, S.H., Greene, E.A., Codomo, C.A., Enns, L.C, Johnson, J.E., Burtner, C., Odden, A.R., Young, K., Taylor, N.E., Henikoff, J.G., Comai, L. and Henikoff, S. 2003. Large-scale discovery of induced point mutations with high-throughput TILLING. Genome Res. 13:524–530.