Anais da Academia Brasileira de Ciências (2018) 90(4): 3853-3869
(Annals of the Brazilian Academy of Sciences)
Printed version ISSN 0001-3765 / Online version ISSN 1678-2690
http://dx.doi.org/10.1590/0001-3765201820180278
www.scielo.br/aabc | www.fb.com/aabcjournal

Molecular marker assisted selection for increasing inbreeding in S_1 populations of cassava

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Manuscript received on March 15, 2018; accepted for publication on July 2, 2018

ABSTRACT

The objective of the present work was to use MAS in self-pollinated cassava populations for obtaining individuals with high inbreeding (*f*) aimed at rapid development of partial inbred lines. Three progenies (F0222, F1378 and F1662) were self-pollinated, generating a total of 233 S_1 individuals. The progenies and the S_1 individuals were evaluated in the seedlings and clonal evaluation trials (CETs). In the CET, plants were evaluated for the following traits: plant height (PH), root dry matter content (DMC), above ground yield (AGY), root (RY) and starch yield (SY). Twenty-seven microsatellites and five minisatellites were used to determine the level of inbreeding of the S_1 individuals. Inbreeding (*f*) values varied from 0.15 to 0.89 within progenies, whereas most of the S_1 individuals presented *f* values above 0.50. In average, 25% of the S_1 individuals were selected, which resulted in a 38% inbreeding increase in the cassava progenies. In contrast, phenotypic selection showed no differences in inbreeding increase. Furthermore, there was no correlation between the level of inbreeding and agronomic traits. MAS was efficient for the identification and selection of cassava S_1 individuals, with higher inbreeding values contributing to the decrease in the breeding cycles necessary to obtain new cassava inbred lines.

Key words: partial inbred lines, *Manihot esculenta* Crantz, microsatellites, self-pollination.

INTRODUCTION

Cassava is one of the main sources of carbohydrates for millions of people, mainly in Africa, Asia and Latin America, being the second most important source of starch in the world, just behind maize (Ceballos et al. 2015). The production of cassava is also strongly linked to family farming in developing countries (Halsey et al. 2008) and has great industrial potential (Oliveira et al. 2012a) due to the diverse applications in pharmaceuticals, beauty products, food, and the textile industry as well as in paper production and biofuels, among others (Saengchan et al. 2015).

Cassava cultivars for industrial purposes should contain specific characteristics, especially high root yield and dry matter content, as well as resistance to diseases (Ceballos et al. 2012, 2016, Oliveira et al. 2015). However, even though there is great market demand, few improved genotypes are available that meet the growing demands for varieties with high agronomic performance. This

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possibly occurs due to the relatively short period of the cassava domestication for use as large-scale commercial crops as well as to the most diverse environmental constraints to which the crop is submitted, which means that there is a selection of varieties adapted to specific environments or traits. The task of combining different characteristics into a single variety is still overwhelming for cassava breeders. However, cassava breeding programs are relatively recent (beginning in the 1960's), and although there are reports of root yield gains, there is a constant demand for the development of new and highly productive varieties associated with different starch characteristics.

In breeding programs, an alternative for the development of cultivars with higher yield is heterosis (hybrid vigor) obtained through crossings of inbred lines (Prado et al. 2013). This strategy has been successfully used for many years in maize (Li et al. 2014) and has recently been used in autogamous species (Goff and Zhang 2013) and species that have undergone recent genetic improvement such as papaya (Vale et al. 2016). Even in completely different species, the results show a significant yield increase of hybrids obtained in these crops. In contrast, for cassavas, the exploration of heterosis has not been carried out routinely, mainly due to the fact that there is little investment in the development of inbred lines (Ceballos et al. 2012).

One of the main breeding methods used for selecting segregating populations derived from crosses between highly heterozygous elite genotypes is the phenotypic recurrent selection, which allows researchers to explore heterosis effectively (Ceballos et al. 2004, 2012), whereas the exploitation of the effects of heterosis (nonadditive effects) without the use of inbred lines belonging to different heterotic groups can be quite slow (Ceballos et al. 2015). Therefore, efforts should be made to explore the effects of dominance that are prevalent in several productive traits in cassava, such as root yield and harvest index (Wolfe et al. 2016).

Self-pollination has already been used as a strategy to obtain segregating populations with the goal of developing cassava partial inbred lines (Freitas et al. 2016, Rojas et al. 2009). However, factors such as high crop heterozygosity, which results in progenies with a high segregation rate, difficulties in flowering and a long crop cycle, also make it difficult to obtain endogamic materials (Ceballos et al. 2017, Halsey et al. 2008). According to Oliveira et al. (2010), conventional breeding methods based on phenotypic selection in segregating populations to obtain inbred lines are costly, time consuming, space intensive and highly influenced by environmental conditions.

Another approach for the development of inbred lines would be the induction of haploids and subsequent chromosomal duplications through double haploid technology. However, the protocols for obtaining cassava haploids are limited and inefficient due to the development of a large number of abnormal individuals (Perera et al. 2014). Therefore, a feasible strategy to improve the efficiency of the selection process of homozygous individuals would be the use of integrated biotechnology tools in conventional breeding methods, such as molecular markers. Among the several molecular markers available, microsatellites (SSR) are especially important because they are codominant, multi-allelic, highly polymorphic, and highly reproducible (Oliveira et al. 2006), which makes them suitable for inferences about kinship and inbreeding. SSRs have been the most used molecular markers in cassava, especially for genetic diversity analysis (Fregene et al. 2003), paternity tests (Mohan et al. 2013) and molecular marker assisted selection (MAS) (Lokko et al. 2005).

MAS has been used in plant breeding with the aim of selecting genotypes with desired characteristics directly at the DNA level (Idrees and Irshad 2014). This technique has several benefits

when associated with conventional breeding methods, such as low cost, rapid generation advancement and high selection efficiency (Xu and Crouch 2008). The great advantage of MAS in comparison with conventional breeding methods is the reduction of the time to obtain the desired genotype, since it allows the selection of individuals in the early stages of development as well as the early elimination of undesirable genotypes (Morris et al. 2003). This technique has been used for several purposes, such as assisted backcrossing, quantitative trait loci (QTL) mapping, heterosis studies (Collard and Mackill 2008, Kobayashi et al. 2013) and for identifying individuals with a higher homozygosity level in segregating populations (Oliveira et al. 2010, 2012b). In cassava, the most successful application of MAS has been in the selection for resistance to cassava mosaic virus (CMD) (Okogbenin et al. 2007, Carmo et al. 2015).

The use of MAS to obtain inbred lines in segregating populations is aimed at quantifying the inbreeding level of the individuals within progenies and to select the most homozygous for new self-pollination cycles (Oliveira et al. 2010). However, there are no reports of the use of MAS to obtain inbred lines in cassava. Therefore, this is an innovative study with the goal of identifying individuals with a higher level of inbreeding in cassava segregant populations via MAS, as well as its association with agronomic traits, in order to reduce the number of self-pollination generations for obtaining partial inbred lines.

MATERIALS AND METHODS

OBTAINMENT OF SELF-POLLINATED PROGENIES

In order to obtain the S_1 segregant populations, three cassava accessions (BGM0222 = "Vermelhinha Branca", BGM1378 = "Macaxeira Branca" and BGM1662 = "IAC-14"), belonging to the Cassava Germplasm Bank (CGB) at Embrapa Mandioca e Fruticultura (Cruz das Almas, Bahia, Brazil), were selected as parents (S_0). These accessions were selected based on their flowering rate for performing crosses and high dry matter content in the roots. For each accession, the self-pollinations were performed manually in the experimental field at Embrapa Mandioca e Fruticultura in 2014. After ripening, the fruits were collected and stored in paper bags at 8 ºC. The S_1 seeds of each progeny were placed to germinate in plastic tubes containing commercial substrate, coconut fiber and vermiculite in the proportion of 2:1:1, being kept under greenhouse conditions (75% humidity, 29 ºC ± 3 ºC). At 50 days after planting (± 30 cm in height), the seedlings were transplanted to the field without any experimental design, in a step called seedlings evaluation trials (SET).

In SET, the number of S_1 individuals for progenies F1662, F0222 and F1378 was 82, 91 and 101, respectively. The experiment was set up in 2014 at the experimental area of the Federal University of Recôncavo da Bahia (UFRB), in Cruz das Almas (Bahia, Brazil), located at 12º66'17"S latitude, 39º08'28"W longitude, and 225 m of altitude. At 12 months after planting, the individuals were selected and collected individually for the establishment of the clonal evaluation trial (CET). The only criterion used to select the individuals to compose CET was the plants' ability to produce at least five stakes of 20 cm in length.

The CET was set up at an experimental area of a private partner of Embrapa (Bahiamido) in Santo Antônio de Jesus (Bahia, Brazil), located at 13°10'56''S latitude and 39°25'30''W longitude, and 203 m of altitude. A total of 233 individuals were selected from the CET, being 76, 78 and 79 from progenies F1662, F0222 and F1378, respectively. The S_1 individuals, along with the S_0 parents, were evaluated in an augmented block design, with 233 individuals as regular treatments, distributed in four blocks and with plots of five plants. The parents (BGM0222, BGM1378, and BGM1662) and 15 clones/varieties (9783-13,

98150-06, BRS Caipira, BRS Dourada , BRS Formosa, BRS Gema de Ovo, BRS Kiriris, BRS Poti Branca, BRS Verdinha, IAC90, Cigana Preta, Correntão, Corrente, Eucalipto, and Vassoura Preta), were used as common treatments (controls). The spacing used was 0.8 m between plants and 0.9 m between rows.

DNA EXTRACTION

Young leaf tissue samples were collected from the upper third part of the plants of the three S_0 parents and S_1 individuals (274 S_1 genotypes at the SET). The leaf samples were identified and stored in an ultra-freezer at -80 °C. The genomic DNA was extracted using the CTAB method described by Doyle and Doyle (1990) with some modifications. DNA quality and quantity evaluation was performed on 0.8% agarose gels, after staining with ethidium bromide. DNA quantification was estimated by comparing the band intensity with aliquots of standard amounts of the known concentration (Lambda-Sigma DNA). The samples were diluted in Tris-EDTA (TE) and standardized to 10 ng μL^{-1}.

AMPLIFICATION OF MICRO- AND MINISATELLITE MARKERS

PCR reactions were performed in a final volume of 15 µl containing 30 ng of genomic DNA, 0.2 mM of each primer (IDT, Integrated DNA Technologies Inc, CA, USA), 1 U of Taq DNA Polymerase (Pluthero 1993), 2 mM $MgCl_2$, 0.2 mM of dNTP and 1X of Tris KCl (Promega Corporation, WI, USA). Amplifications were performed on the Veritti® 96-well thermal cycler (Applied Biosystems, CA, USA) under the following conditions: denaturation at 94 °C for 5 minutes, followed by 29 cycles of denaturation at 94 °C per minute, annealing temperature (AT) specific for each primer for 60s at 72 °C per minute (Table I) and a final extension of 7 minutes at 72 °C. Electrophoresis of the amplified fragments was performed on 3% agarose gel (Invitrogen, Carlsbad, CA, USA) at 120 v for 4 hours, stained with ethidium bromide (15 $mg.mL^{-1}$ solution) in 0.5X Tris/Borate/EDTA buffer. The fragments were visualized under UV light and photographed with the Gel Logic 212 Pro photo documentation system (Carestream Molecular Imaging, New Haven, USA). Afterwards, the fragments were analyzed in comparison to a known base pair size marker (DNA ladder 100pb) (Fermentas, Glen Burnie, MD, USA) for the determination of the fragment size.

MOLECULAR MARKER POLYMORPHISM

Initially, 55 microsatellite primers (SSRY and EME series) and six minisatellites were tested for inbreeding analysis in S_1 cassava individuals. Microsatellites and minisatellites were previously amplified in the F0222, F1378 and F1662 parents. Primers that presented heterozygosity in the parents, with good amplification pattern and absence of nonspecific bands, were selected to be amplified in all S_1 individuals. After an initial evaluation, a total of 27 microsatellite loci (SSRY and EME) and five minisatellites, were used to check the polymorphism in each progeny. Sixteen microsatellites were polymorphic for progeny F1662, and 18 microsatellites were polymorphic for progenies F0222 and F1378 (Table I). Five minisatellites were used, with 4, 3 and 2 being polymorphic for progenies F022, F1378 and F1662, respectively.

AGRONOMIC EVALUATIONS

Twelve months after planting, the CET was evaluated for agronomic traits using five plants from each plot. The main traits evaluated were: plant height (PH – meter); above ground yield (AGY – $t.ha^{-1}$); root yield (RY – $t.ha^{-1}$); starch yield (SY – $t.ha^{-1}$); and dry matter content (DMC – %, measured using the root's specific gravity, according to Kawano et al. (1987)).

DATA ANALYSIS

Molecular data analyses were performed using the Powermarker 3.25 software (Liu and Muse

TABLE I
Microsatellite (SSRY and EME series) and minisatellites markers (VNTR) were used to evaluate the inbreeding coefficient in S_1 cassava individuals in self-pollination progenies from BGM0222 (F0222), BGM1378 (F1378) and BGM1662 (F1662) cassava accessions.

Markers	Reference	Forward	Reverse	bp	AT (°C)	Markers per S_1 progeny		
						F0222	F1378	F1662
SSRY101	Mba et al. (2001)	ggagaataccaccgacagga	acagcagcaatcaccatttc	213	55		X	
SSRY103	Mba et al. (2001)	tgagaaggaaactgcttgcac	cagcaagaccatcaccagttt	272	55		X	X
SSRY105	Mba et al. (2001)	caaacatctgcacttttggc	tcgagtggcttctggtcttc	225	55	X		
SSRY106	Mba et al. (2001)	ggaaactgcttgcacaaaga	cagcaagaccatcaccagttt	270	55		X	X
SSRY13	Mba et al. (2001)	gcaagaattccaccaggaag	caatgatggtaagatggtgcag	234	55	X		
SSRY143	Mba et al. (2001)	gctcatgaactgagccttca	agcagatccaaatcactgaaa	153	55	X		
SSRY165	Mba et al. (2001)	aaatgagttgcaaaggccaa	ggtaaacaaatgatgtggtgttc	243	55			X
SSRY168	Mba et al. (2001)	acagccacacttgttctcca	ctgcaatctccaacagcaac	277	45	X	X	
SSRY170	Mba et al. (2001)	tctcgatttggtttggttca	tcatccttgttgcagcgtta	299	55	X		X
SSRY175	Mba et al. (2001)	tgactagcagacacggtttca	gctaacagtccaataacgataagg	136	55		X	
SSRY179	Mba et al. (2001)	caggctcaggtgaagtaaagg	gcgaaagtaagtctacaacttttctaa	226	55	X	X	X
SSRY182	Mba et al. (2001)	ggaattctttgcttatgatgcc	ttcctttacaattctggacgc	253	55		X	
SSRY28	Mba et al. (2001)	ttgacatgagtgatattttcttgag	gctgcgtgcaaaactaaaat	180	55		X	
SSRY30	Mba et al. (2001)	ccatccactagaaactttaaaagca	caactcagcggagctttttc	220	55		X	
SSRY49	Mba et al. (2001)	tgaaaatctcactggcattattt	tgcaaccatagtgccaagc	300	55	X	X	X
SSRY68	Mba et al. (2001)	gctgcagaatttgaaagatgg	cagctggaggaccaaaaatg	287	55		X	X
SSRY8	Mba et al. (2001)	agtggtttgagaagactggtga	tttccaaaatggaacttcaaa	288	45	X		X
SSRY81	Mba et al. (2001)	ggcgatttcatgtcatgctt	tgattttctgcgtgatgagc	204	55	X		
SSRY82	Mba et al. (2001)	tgtgacaattttcagatagcttca	caccatcggcattaaactttg	211	55			X
SSRY83	Mba et al. (2001)	tggctagatggtgattattgctt	tgcttactctttgattccacg	239	55	X		
SSRY93	Mba et al. (2001)	tttgttgctcacatgaaaacg	cagatttcttgtggtgcgtg	289	55			X
SSRY94	Mba et al. (2001)	aggatggacttggagatgga	ggtggaagtaaggctgttagtg	268	55		X	X

Markers	Reference	Forward	Reverse	bp	AT (ºC)	Markers per S_1 progeny		
						F0222	F1378	F1662
EME189	Kunkeaw et al. (2011)	cagagcacatccagaaattgtt	gaaatagatcaagtgccccatc	180	60	X	X	
EME205	Kunkeaw et al. (2011)	ccagagcgtataactggaac	tgcaggagtgtggatatggtt	250	55	X		X
EME260	Kunkeaw et al. (2011)	gttggagttgtagttgctgc	catgggctgtgaaaatgaact	160	58	X	X	X
EME395	Kunkeaw et al. (2011)	tcaaaggtatcggggaggtag	gtttaccccactaacatcgcat	200	58	X	X	
EME425	Kunkeaw et al. (2011)	cctccacaaccttatcaatca	cggtagccatagccataaca	140	55			X
VNTR4	C.D. Carmo, Unpublished	aatcatatcaggggctggtg	cgagggaaatgctgacctt	220	58	X	X	
VNTR5	C.D. Carmo, Unpublished	ttgcttccaatcttctcaca	gatcaaaacgggcttgaaat	233	58		X	X
VNTR71	C.D. Carmo, Unpublished	tgcagataaactccaaaagtaagaa	gcttcatggttgaggctctt	589	60	X	X	X
VNTR72	C.D. Carmo, Unpublished	gcttaggcgggaagaaaatg	tgttcaactgcctctctttgc	828	58	X		
VNTR90	C.D. Carmo, Unpublished	gaaccgtgaacagtaaccgata	cccggctgtttcaataaaat	835	60	X		

bp: size in base pairs; AT: annealing temperature; X: Primer selected for inbreeding analysis in each progeny.

2005) to determine the expected heterozygosity (He), observed heterozygosity (Ho) and inbreeding coefficient (*f*) in all S_1 individuals and the three S_o parents. The inbreeding coefficient was determined following the method proposed by Wright et al. (1965) in which .

Population structure within selfings was investigated using the Euclidean distance matrix, which shows the extent of diversity between the individuals. Then, the principal coordinate (PCO) analysis was performed using the R function dudi.pco (R Core Team 2017). The PCO analysis indicated no visible population structure (Figure 1), and therefore it was not necessary to calculate the kinship matrix as a variance-covariance matrix to correct for random genotypic effects.

The analysis of the phenotypic data was carried out using the linear mixed model, $y = Xf + Zg + Wb + e$ with the following model, whereas: y – is the phenotypic data; f – is the vector of means of the individuals (fixed effects); g – is the vector of the genotypic effects of the individuals (random effects); b – is the vector of the environmental block effects (random effects); and e – is the vector of residue (random effect). The incidence matrices of the random effects are represented by uppercase letters.

Pearson's correlation was estimated using the *corrgram* package of the R software (R Core Team 2017), based on the best linear unbiased prediction (BLUP) and inbreeding coefficient values of the S_1 individual. The selection of the S_1 individuals based on BLUP analysis of the phenotypic traits was performed using the following selection index: *SI= (PH*5)+(AGY*5)+(RY*10)+(SY*10)+(DMC*10).*

RESULTS

GENETIC PARAMETERS AND INBREEDING ANALYSIS OF S_1 INDIVIDUALS

The distribution of inbreeding coefficients (*f*) was very similar among the three progenies. The highest proportion of S_1 individuals presented *f* between

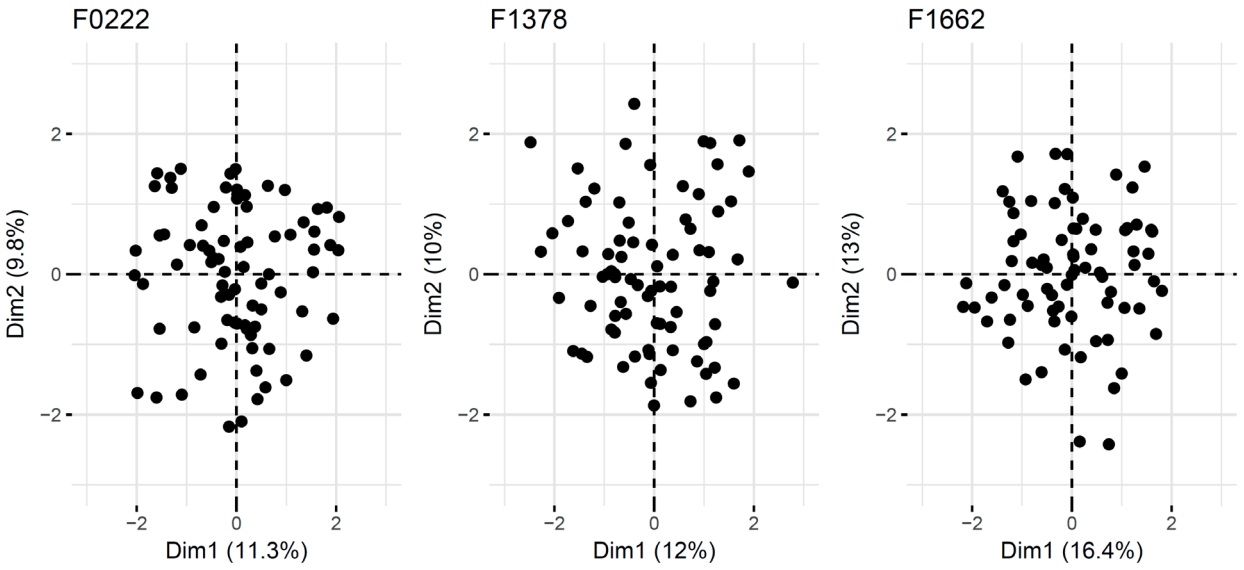

Figure 1 - Principle coordinate analysis for 78, 79 and 76 S_1 progenies derived from parent BGM0222 (F0222), BGM1378 (F1378), and BGM1662 (F1662), respectively, based on mini and microsatellites markers.

0.40 and 0.60, representing 50%, 56% and 41% of the total individuals from progenies F0222, F1378 and F1662, respectively (Figure 2). The distribution of S_1 individuals with *f* ranging from 0.60 to 0.79 (high inbreeding coefficient) was 24% (F0222), 20% (F1378) and 27% (F1662). In contrast, only 1.25% of the individuals from progenies F0222 and F1378, presented *f* estimates above 0.80.

The observed heterozygosity (Ho) of the S_1 individuals presented similar distribution to the inbreeding coefficient for the three progenies, possibly because it is an important component on the *f* formula. Approximately, 50%, 45% and 41% of the individuals in progenies F0222, F1378 and F1662, respectively, presented individuals with Ho between 0.40 and 0.60 (Figure 3). The distribution of individuals with high Ho (> 0.60) ranged from 24% to 32% in progenies F0222 and F1378, respectively. In contrast, the lowest Ho values were 0.17, 0.11 and 0.25 in S_1 individuals of progenies F0222, F1378 and F1662, respectively (Table II).

Regarding the He and Ho analyses, there was very small variation in He values in all progenies (ranging from 0.46 to 0.50 and mean of 0.49), while Ho variation was larger (ranging from 0.35 to 0.65) (Table III). The lowest Ho values were observed for markers SSRY83 (F0222), VNTR71 (F1378) and SSRY170 (F1662) (0.35, 0.37 and 0.38, respectively), while the loci with the highest Ho were SSR08 (0.61) in progeny F0222; as well as SSRY103, SSRY106, SSR168, SSRY175, SSRY28, SSRY49 and VNTR05 (0.60 to 0.65) in progeny F1378 and SSRY49 (0.58) in progeny F1662. Although the F1378 progeny had the highest number of loci with Ho above 0.60, the Ho mean was similar among the progenies, i.e., 0.51 (F0222), 0.51 (F1378) and 0.52 (F1662).

Considering the chromosomal location of the mini- and microsatellite markers, in general, the distribution of the inbreeding coefficient and Ho were more similar in F1662 (*f* and Ho ranging from 0.40 to 0.62 and 0.38 to 0.60, respectively), in comparison with the other progenies (Figure 4). In contrast, the parameters' variation in F0222 (*f* = 0.39 to 0.65 and Ho = 0.35 to 0.61, respectively) and F1378 (*f* = 0.36 to 0.63 and Ho = 0.37 to 0.64, respectively) progenies was not high enough to raise the hypothesis of unbalanced homozygosity in different chromosomes. Although a small number of molecular markers was used, results indicate that

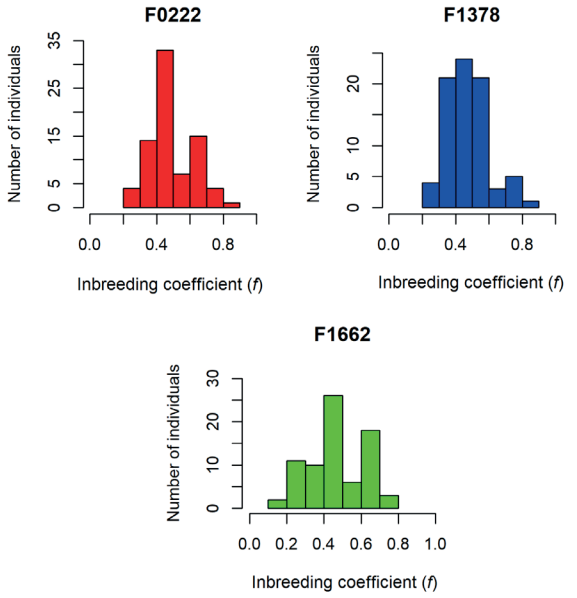

Figure 2 - Distribution of the inbreeding coefficient (*f*) of the S_1 cassava individuals from the self-pollination of the accession BGM0222 (F0222), BGM1378 (F1378) and BGM1662 (F1662).

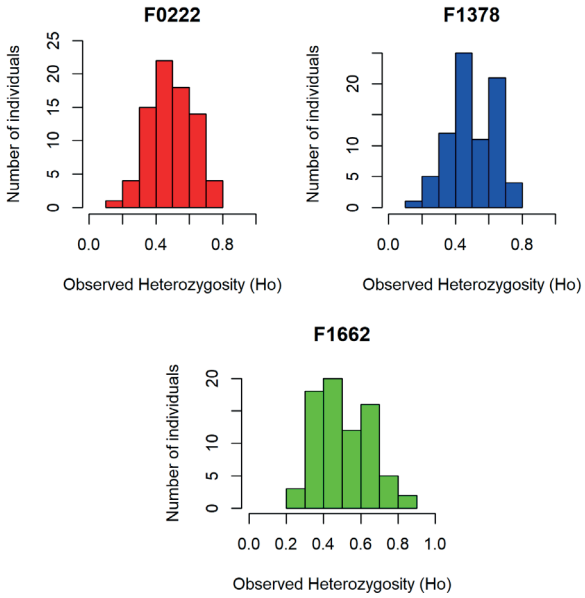

Figure 3 - Distribution of the observed heterozygosity (Ho) of the S_1 cassava individuals from the self-pollination of the accessions BGM0222 (F0222), BGM1378 (F1378) and BGM1662 (F1662).

TABLE II
Minimum, maximum and average values of observed heterozygosity (Ho) and inbreeding coefficient (*f*) in S_1 individuals from different self-pollinated progenies.

Progeny	Parameter	Minimum	Maximum	Average
F0222	Ho	0.17	0.78	0.51
	f	0.22	0.83	0.49
F1378	Ho	0.11	0.72	0.51
	f	0.25	0.89	0.49
F1662	Ho	0.25	0.81	0.52
	f	0.15	0.74	0.49

the genotyping with the mini- and microsatellites was well distributed in the different cassava chromosomes (11 of the 18 chromosomes of *M. esculenta*), which contributes to a better coverage of the level of homozygosity in this species.

ANALYSIS OF INBREEDING AND AGRONOMIC PERFORMANCE BASED ON DIFFERENT SELECTION CRITERIA

Considering the genotypic information of the molecular markers, S_1 individuals with the highest level of inbreeding coefficient ($f \geq 0.60$) within progeny were identified and selected for the next self-pollination cycles. The selection intensity applied was 25%, 21% and 27% in progenies F0222, F1378 and F1662, thus making it possible to select 20, 17 and 21 S_1 individuals, respectively, with higher endogamy.

The comparison between the means of the agronomic traits (plant height, above ground yield, roots and starch yield and root dry matter content) and the S_1 individuals selected based on the inbreeding coefficient ($f \geq 0.60$), showed that there was no significant difference in the means for most of these traits, except for above ground yield (AGY) in progeny F1378, in which the S_1 individuals selected for presenting high *f* also showed high means (Figure 5). Therefore, it is possible to select S_1 individuals in cassava progenies with high inbreeding level without significant losses in AGY. In contrast, as for all selective processes, the selection of the S_1 individuals with the highest inbreeding coefficient resulted in a reduction in phenotypic variation for plant height (F1378 and

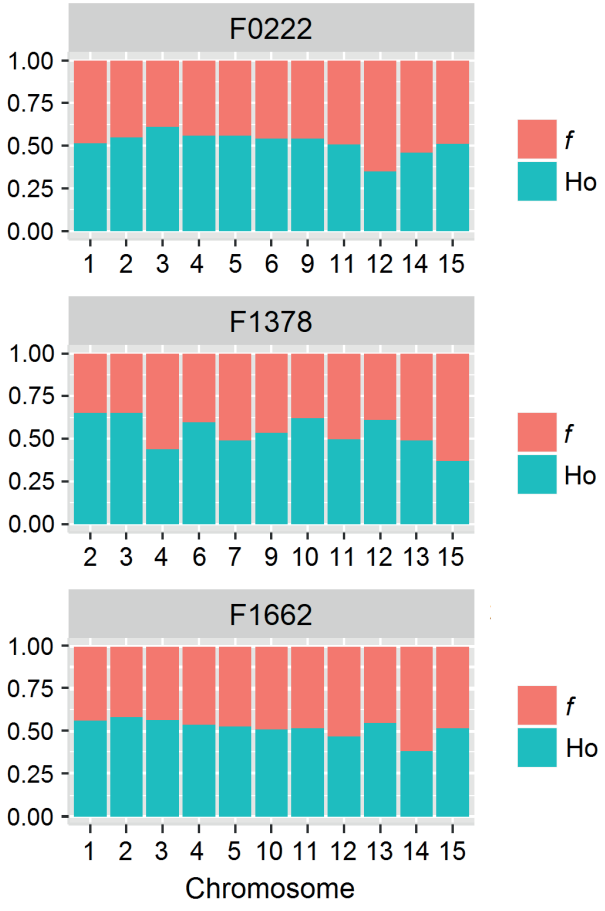

Figure 4 - Distribution of observed heterozygosity (Ho) and inbreeding coefficient (*f*) of the S_1 cassava individuals from the self-pollination of accession BGM0222 (F0222), BGM1378 (F1378) and BGM1662 (F1662), considering the chromosomal location of the molecular markers used for genotyping.

F1662), above ground yield (F0222 and F1662) and root and starch yield (F1662) (Figure 5).

If the selection of S_1 individuals was performed based on the selection index (agronomic performance) rather than the inbreeding coefficient, as is traditionally done in breeding programs, an increase in the trait's means would be expected, especially for dry matter content (F1378 and F1662), above ground yield and root yield (F1378) and starch yield (F0222 and F1378) (Figure 6). In this case, the performance for most of the agronomic traits in the three progenies would be preserved, but there would be a reduction in the inbreeding coefficient in the F0222 and F1662 progenies, which would certainly contribute to an increase in the number of self-pollinations necessary to obtain inbred lines.

RELATIONSHIP BETWEEN INBREEDING AND AGRONOMIC TRAITS

Considering the distribution of the inbreeding coefficient in three different groups, Group 1 ($0.10 < f < 0.39$), Group 2 ($0.40 < f < 0.59$) and Group 3 (> 0.60), there was great phenotypic difference between S_1 progenies within this inbreeding classification (Figure 7). There was no linear relationship between inbreeding and agronomic performance, since, in some cases, the average of Group 2 was lower (dry matter content in the roots, above ground yield and root yield - F1378) or higher (root dry matter content, root and above ground yield in the F1662 progeny and root and above ground yield in the progeny F0222) in comparison to the other groups. In addition, the variations in the agronomic traits were very similar between the different inbreeding groups, indicating a lack of association between the agronomic performance of the S_1 progenies and their inbreeding level, based on molecular analysis.

Except for the correlation between PH and DMC and between AGY and DMC that did not differ from zero, the other correlation estimates were positive and different from zero (Figure 8). There was a strong positive correlation between RY and SY (0.99). In contrast, there was a positive correlation of moderate magnitude between AGY and RY (0.53); AGY and SY (0.52); PH and AGY (0.48); PH and RY (0.30); PH and SY (0.30); DMC and RY (0.21); and DMC and SY (0.28).

DISCUSSION

MOLECULAR MARKERS USED TO SELECT PARTIAL INBRED LINES

The determination of the inbreeding coefficient by the Ho and He analysis of different molecular

TABLE III
Expected (He) and observed heterozygosity (Ho) of microsatellite loci in S_1 cassava individuals originated from self-pollination of accessions BGM0222 (F0222), BGM1378 (F1378) and BGM1662 (F1662).

F0222				F1378				F1662			
Primer	Ho	He	Cr	Primer	Ho	He	Cr	Primer	Ho	He	Cr
EME189	0.54	0.49	9	EME189	0.56	0.50	9	EME205	0.56	0.50	1
EME205	0.49	0.50	1	EME260	0.50	0.48	11	EME260	0.51	0.49	11
EME260	0.51	0.50	11	EME395	0.56	0.49	6	EME425	0.57	0.49	5
EME395	0.54	0.50	6	SSRY101	0.49	0.49	7	SSRY103	0.47	0.49	12
SSRY08	0.61	0.50	3	SSRY103	0.60	0.50	12	SSRY106	0.47	0.49	12
SSRY105	0.44	0.50	14	SSRY106	0.62	0.49	12	SSRY165	0.55	0.50	13
SSRY13	0.53	0.50	2	SSRY168	0.63	0.50	6	SSRY170	0.38	0.49	14
SSRY143	0.51	0.50	11	SSRY175	0.65	0.49	3	SSRY179	0.51	0.49	4
SSRY170	0.48	0.50	14	SSRY179	0.44	0.50	4	SSRY49	0.58	0.50	2
SSRY179	0.56	0.50	4	SSRY182	0.51	0.48	9	SSRY68	0.57	0.50	4
SSRY49	0.57	0.50	2	SSRY28	0.61	0.50	12	SSRY8	0.56	0.50	3
SSRY68	0.56	0.49	4	SSRY30	0.49	0.48	13	SSRY82	0.49	0.49	5
SSRY81	0.56	0.46	5	SSRY49	0.65	0.50	2	SSRY93	0.57	0.49	3
SSRY83	0.35	0.50	12	SSRY68	0.44	0.50	4	SSRY94	0.53	0.50	11
VNRT04	0.52	0.49	11	SSRY94	0.56	0.49	11	VNTR05	0.51	0.49	10
VNRT71	0.51	0.50	15	VNTR04	0.43	0.48	11	VNTR71	0.52	0.50	15
VNTR72	0.54	0.50	1	VNTR05	0.62	0.50	10				
VNTR90	0.49	0.50	11	VNTR71	0.37	0.47	15				
Average	0.51	0.49		Average	0.51	0.49		Average	0.52	0.49	

Cr: chromosomal region of the molecular markers.

markers is an efficient way of determining inbreeding, especially in self-pollination, full- and half-sibling populations (Goudet and Keller 2002). The inbreeding coefficient in the S_1 cassava progenies varied from 0.00 to 0.89 in the different self-pollinated populations. However, most S_1 individuals presented inbreeding values ranging from 0.40 to 0.60. On average, inbreeding was very close to 0.50 (0.48 for F2222, 0.49 for F1378 and F1662). These results are in agreement with the expected average reduction of 50% of heterosis and, therefore, the heterozygosity of the loci (Wu et al. 2016).

Considering only one self-pollination cycle, it was possible to identify S_1 individuals with inbreeding coefficients above the expected mean ($f > 0.70$). This result is of utmost importance to accelerate the development of cassava partial inbred lines, since, after using only a few molecular markers, the results demonstrated the efficiency of MAS for identifying the most homozygous individuals within S_1 segregant populations.

The use of MAS to obtain inbred lines is widely disseminated and has been successfully used in several crops, such as papaya (Oliveira et al. 2010, 2012b), soybean (Song et al. 2014), wheat (Vinod et al. 2015), rice (Hasan et al. 2015), and castor bean (Machado et al. 2016). Most of these studies indicated the possibility of applying MAS to select transgressive individuals in different species and breeding populations. The early selection of the most homozygous individuals certainly will have a positive impact on reducing the time required for the development of these partial inbred lines in comparison to the phenotypic selection used in conventional breeding.

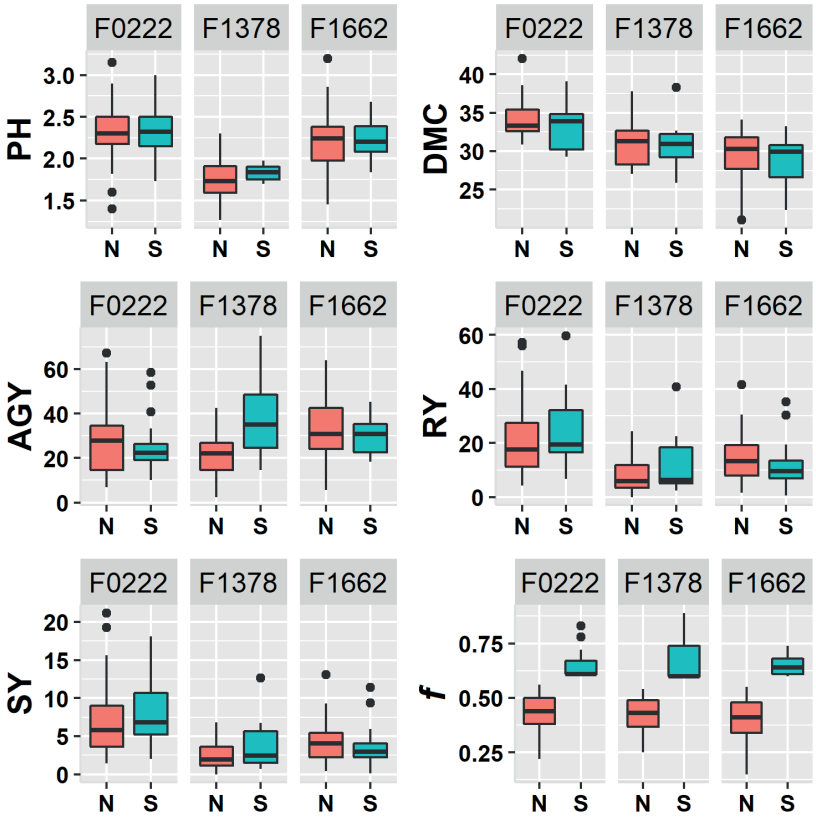

Figure 5 - Boxplot of the selection of S_1 cassava individuals from the self-pollination of the accessions BGM0222 (F0222), BGM1378 (F1378) and BGM1662 (F1662), based on the inbreeding coefficient (*f*). N: group of unselected individuals with $f < 0.60$. S: group of individuals selected with $f \geq 0.60$. PH: plant height (m); DMC: root dry matter content (%); AGY: above ground yield ($t.ha^{-1}$); RY: root yield ($t.ha^{-1}$); SY: starch yield ($t.ha^{-1}$).

Despite the efforts and investments made in the development of molecular markers for establishment of efficient MAS in cassava breeding, few practical results have been reported for selection of the desirable individuals (Oliveira et al. 2012a). Nonetheless, some examples of successful use of MAS in cassava breeding refer to the introduction of the CMD resistance gene in African germplasm using resistance sources from Latin America (Okogbenin et al. 2007). In other crops such as rice, MAS implementation has contributed for increasing the genetic gain in comparison to phenotypic methods, since it has reduced, on average, 40% of the costs for the development of superior rust-resistant genotypes (Kuchel et al. 2005). Additionally, Morris et al. (2003) concluded that MAS did not present an effective cost reduction compared to conventional selection methods in maize, but it was more efficient for reducing the time required to obtain inbred lines.

In our work, selection based on the phenotypic data did not result in significant differences in the mean of the selected and unselected individuals for most of the traits, except for RY and SY in the F0222 progeny, and AGY, RY and SY in the F1378 progeny (Figure 6). By contrast, selection based on individuals with $f \geq 0.60$ resulted in an average increase of approximately 38% in the inbreeding mean of the progenies, without

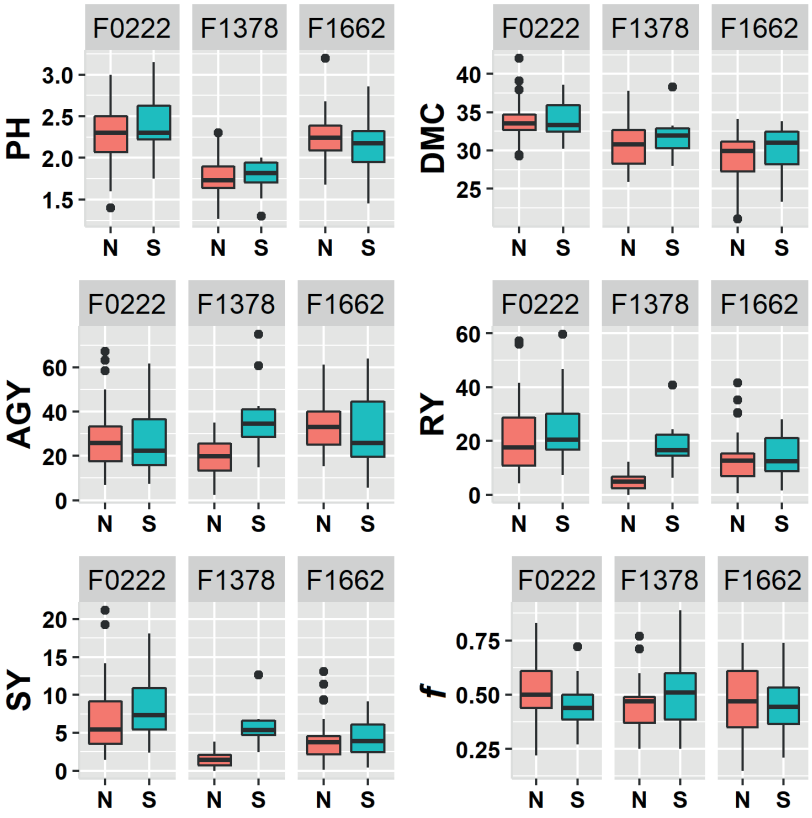

Figure 6 - Boxplot of the selection of S_1 cassava individuals from the self-pollination of accessions BGM0222 (F0222), BGM1378 (F1378) and BGM1662 (F1662), based on the selection index. S and N: S_1 selected and unselect individuals by the selection index, respectively. PH: plant height (m); DMC: root dry matter content (%); AGY: above ground yield ($t.ha^{-1}$); RY: root yield ($t.ha^{-1}$); SY: starch yield ($t.ha^{-1}$).

dramatically affecting the agronomic performance of the selected individuals (Figure 5). In contrast, by default, cassava and other cross-pollinated crops have a strong correlation between root yield and inbreeding (Ceballos et al. 2015, Freitas et al. 2016, Kaweesi et al. 2016). Therefore, probably the absence of differences in the phenotypic performance of the S_1 individuals with drastic differences in the inbreeding coefficients is due to the number of molecular markers used. However, the results obtained through MAS can greatly contribute in the selection of the most homozygous individuals to compose the S_2 generation and also result in genetic gains when associated with the phenotypic evaluations.

Although the use of inbred lines is an important objective in cassava breeding, its attainment is difficult, since conventional methods of self-pollination are expensive, time-consuming and can still be influenced by environmental effects (Ceballos et al. 2004). As a consequence, the development of cassava inbred lines could take around 14 years. Conversely, the use of MAS can be very efficient in identifying the most homozygous individuals, which can be selected early and then quickly submitted to a new self-pollination cycle to generate Sn populations with fixed loci.

Morris et al. (2003) evaluated the cost-benefit of MAS in comparison to the phenotypic selection for the development of maize inbred lines with

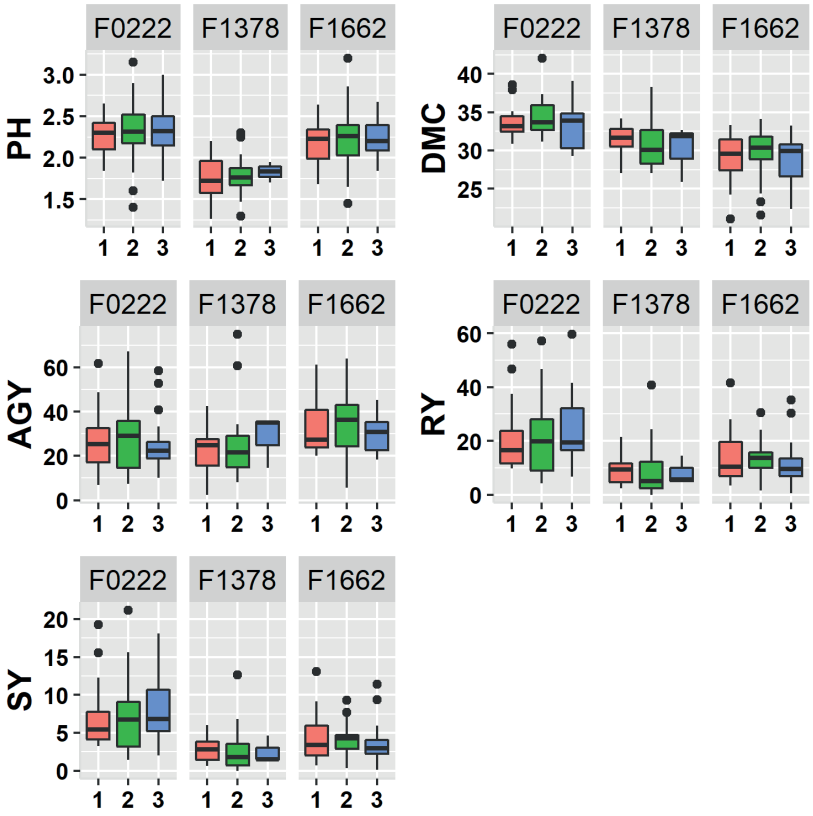

Figure 7 - Boxplot of the agronomic performance of S_1 cassava individuals from the self-pollination of accessions BGM0222 (F0222), BGM1378 (F1378) and BGM1662 (F1662), based on the inbreeding coefficient grouping (*f*). Group 1 ($0.10 < f < 0.39$), Group 2 ($0.40 < f < 0.59$), and Group 3 ($f > 0.60$). PH: plant height (m); DMC: root dry matter content (%); AGY: above ground yield ($t.ha^{-1}$); RY: root yield ($t.ha^{-1}$); SY: starch yield ($t.ha^{-1}$).

introgression of a dominant allele by backcross. The authors stated that 96% of the genome of the recurrent parent was recovered with three cycles of backcross assisted by microsatellites. However, for the phenotypic selection, six backcrosses were required to recover this same proportion of the recurrent parent genome. Therefore, MAS made it possible to reduce three cycles of backcross, which led to saving time and human resources. In another study in rice, MAS allowed the researchers to obtain highly homozygous genotypes with resistance to flooding in three cycles of backcrosses (BC_3F_1), whereas, by phenotypic selection, this would only be possible in the fifth backcross cycle (BC_5F_1) (Iftekharuddaula et al. 2012). Therefore, the inherent advantages of MAS are very clear, and orphan crops such as cassava can greatly benefit from this tool when combined with conventional breeding.

EFFECT OF INCREASING INBREEDING ON AGRONOMIC TRAITS

There was no correlation between the inbreeding coefficient and the agronomic traits in the S_1 cassava individuals. One hypothesis to explain this result may be the lack of linkage between the molecular markers and the expression of these agronomic characteristics. According to Balloux et al. (2004), the number of markers used to accurately determine the correlations between agronomic performance

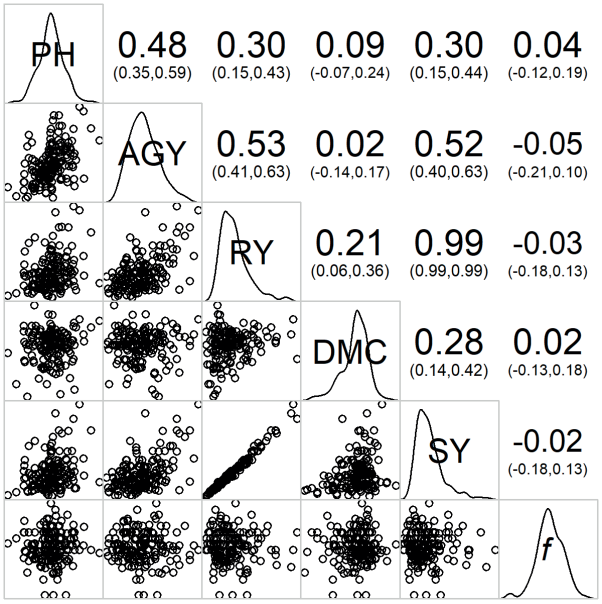

Figure 8 - Pearson correlation estimates for the inbreeding coefficient and agronomic traits in S_1 cassava individuals. PH: plant height (m); DMC: root dry matter content (%); AGY: above ground yield ($t.ha^{-1}$); RY: root yield ($t.ha^{-1}$); SY: starch yield ($t.ha^{-1}$); *f*: inbreeding coefficient.

and inbreeding may be a limiting factor, since a small number of markers may be inadequate to provide sufficient information on heterozygosity in the entire genome. Particularly in the case of cassava, the incorporation of SNPs via genotyping-by-sequencing (GBS) techniques to identify inbreeding in segregating progenies could eliminate the problem of low genomic coverage, since SNPs are widely distributed in the cassava genome (1 SNP for each 121bp – Pootakham et al. 2014).

The absence of a strong association between inbreeding and agronomic attributes identified by molecular markers in cassava indicates that it is possible to obtain genotypes with a higher level of inbreeding without great losses in agronomic performance, although this information should be studied in the S_2 populations. In contrast, some studies regarding inbreeding depression in S_1 cassava individuals have reported that, depending on the progeny evaluated, there are significant losses in some agronomic traits, such as root yield, above ground yield, harvest index and dry matter content due to inbreeding depression (Rojas et al. 2009, Kawuki et al. 2011, Freitas et al. 2016). However, according to these same authors, the self-pollination of cassava accessions also allows the procurement of transgressive individuals with higher phenotypic values in comparison to the parents.

As for the agronomic traits, the results obtained in this study show associations of medium to high magnitude, especially between RY × SY, RY × AGY, AGY × SY and PH × AGY. These results are in agreement with other studies carried out for cassava, which presented correlations between agronomic, morphological and biochemical characteristics with magnitudes and direction similar to those observed in the present study (Ojulong et al. 2008, Ntawuruhunga and Dixon 2010, Gu et al. 2013, Oliveira et al. 2016).

PERSPECTIVES FOR MAS IMPLEMENTATION AND INBREEDING EXPLOITATION IN CASSAVA

Most of the cassava breeding programs use the recurrent phenotypic selection method to drive segregating populations generated from intraspecific crosses between heterozygous individuals (Ceballos et al. 2004), although the use of partial inbred lines has also been an alternative to generate transgressive clones (Freitas et al. 2016). However, recently, the efficiency of cassava breeding programs using conventional breeding strategies has been questioned, since, according to Ceballos et al. (2016), less than 1% of the clones generated reach the final stages of evaluation for the farmer's recommendation. Therefore, the low predictive ability of the parents' breeding values used for crossing is a factor that must be overcome (Ceballos et al. 2016). The use of inbred lines has been pointed out as an alternative to improve the prediction of the individuals' breeding values, besides allowing the use of genomic tools to improve the genetic gain with the selection.

The search for cassava inbred lines is relatively new in breeding programs; however, the potential gains of its use have been debated in the literature (Ceballos et al. 2015, Freitas et al. 2016, Kaweesi et al. 2016). Some theoretical benefits of the use of inbreeding in cassava are: reduction of the genetic load with the elimination of deleterious alleles from the populations; the elucidation of the genetic control of the main agronomic traits; the possibility of using intra- and interpopulation methods in a practical and fast way; the definition of heterotic groups to better explore heterosis; and the use of backcrosses to easily exchange alleles of interest in elite genotypes, especially monogenic or recessive genes (Ceballos et al. 2004).

The development of cassava inbred lines could lead to changes in the way the crop is propagated, since the use of seeds from pure inbred lines would allow the complete reproduction of the genotype of the cultivars/accession (as clonal propagation). In this case, the use of seeds for planting could favor the reduction of pathogens, which are frequently present in the cutting stakes, and allowing the initial evaluation of the clones with replicates in the first stages of selection; whereas this could increase selection efficiency in early cassava breeding stages, since, in general, there is a low correlation of cassava genotype performance in the clonal evaluation and preliminary yield evaluation trials, which directly affects the selection of superior clones and the elimination of individuals with lower genetic potential.

In addition to the aforementioned factors, the attainment of partially inbred cassava lines will depend on the flowering rate of the selected genotypes. It is known that breeding programs' selections tend to advance individuals who are upright when focusing on mechanized planting systems, since both cultural practices and harvest are facilitated, in addition to the fact that the multiplication rate of these genotypes is high. This leads to the selection of plants that do not branch, and since flowering is directly related to the level of branching of the clones (Ceballos et al. 2017), the plants undergo negative selection for flowering. Therefore, advancement of self-pollination generations to obtain cassava inbred lines should be made through the association between molecular (MAS) and phenotypic selection considering the main agronomic traits and, whenever possible, taking into account genotypes with a high flowering rate.

Therefore, the present study demonstrated that the use of MAS was efficient in determining the inbreeding coefficient in S_1 cassava individuals, whose results can be applied to loci fixation in segregating cassava progenies. This enables reduction of the time required to obtain partially inbred lines and to better exploit the inherent advantages of this kind of genetic material.

CONCLUSIONS

Molecular-assisted selection is efficient in identifying individuals with high inbreeding level, providing a selection of approximately 25% of the individuals in cassava self-pollinated progenies. This early identification of the more homozygous individuals hastens the advancement of generations, allowing cost and time reductions compared to conventional selection for the development of inbred lines. There was no correlation between the inbreeding level and agronomic traits in cassava self-pollinated progenies; this is very important, since it is possible to obtain S_1 individuals with high inbreeding and desirable agronomic attributes.

ACKNOWLEDGMENTS

The authors thank the Fundação de Amparo à Pesquisa do Estado da Bahia (FAPESB), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the financial assistance and scholarship support.

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