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The use of doubled-haploids in cassava breeding

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Abstract. Cassava breeding is difficult and, compared with other crops, inefficient. The problems in cassava breeding relate to the length of the breeding cycle, the large genetic load present in the crop, and the heterozygous nature of the parents and progenies evaluated. The production of doubled haploids, through tissue culture techniques, offers interesting advantages. By definition, any process that involves increased homozygosity will result in a decrease of genetic load. Doubled-haploid lines, therefore, are expected to produce better hybrids. Furthermore, the availability of homozygous lines would allow for a gradual and consolidated breeding to improve parental performance in hybrid combinations. This means that the genetic enhancement will benefit from previous gains, like steps in a staircase. The breeder "*owns*" the genetic superiority of an inbred progenitor but that is not necessarily the case with heterozygous parents, particularly in heterozygous crops like cassava. With the introduction of doubled-haploids, the emphasis of cassava breeding shifts from producing large number of hybrids (hoping to find a superior one) to improving parents for the production of better hybrids that are "*designed*", not just found. In addition to these advantages doubled-haploids will facilitate discovery and exploitation of recessive traits and germplasm conservation. One important additional advantage is that germplasm exchange could be greatly facilitated, thus helping to bridge the relative isolation in which cassava-breeding projects in different countries currently operate.

Introduction

Cassava improvement has not been as consistent and efficient as other crops and has many constraints. A typical scheme implies crossing elite clones to produce segregating families. Each individual produced is highly heterozygous. Once a superior genotype is identified (a process that requires about six years), it is vegetatively multiplied to take advantage of the reproductive habits of this crop (Kawano *et al*., 1998; Jennings and Iglesias, 2002). This system (except for the vegetative multiplication) is similar to the ones used for autogamous crops (beans, wheat, rice, etc.) as well as for the hybrid maize industry. However, there is a major difference because cassava is never pushed to produce inbred (homozygous) lines from the segregating progenies of a given cross. The system also bears some similarities with recurrent selection used in allogamous crops (maize), but there is a significant difference because in cassava there is not a real population whose allelic frequencies are modified through evaluation and selection, as in true recurrent selection schemes.

The differences described imply that cassava breeding is slow and inefficient. Some of the reasons for this inefficiency are listed below:

a. Because no inbreeding is carried out at any stage of cassava breeding, a sizeable genetic load (undesirable or deleterious genes) is expected to prevent the crop fully achieving its actual yield potential.

b. There are no clearly defined populations (as defined by quantitative genetics), allelic frequencies cannot be efficiently modified. Cassava breeding in this regard resembles more the selection of segregating progenies from two parental lines in autogamous crops.

c. Because the highly heterozygous nature of the crop, dominance effects are likely to play a very important role in the performance of materials being selected. The current scheme can exploit dominance effects because, once an elite clone is identified, it can be propagated vegetatively (therefore carrying along the dominance effects). However, selection of progenitors for the production of new segregating material is based on their performance *per se*. In that case, the current procedure has a bias because the breeding values of these clones are unlikely to be well correlated with their performance, precisely because of the distorting effects of dominance.

d. Production of recombinant seed is cumbersome in cassava. Only 0.6 viable seeds per pollination are produced. It takes about 16 to18 months since a given cross is planned until an adequate amount of seed is produced.

e. When a desirable trait is identified, it is very difficult to transfer it from one genotype to another (even if a single gene controlled the trait). The back-cross scheme, one of the most common, successful and powerful breeding schemes for cultivated crops, is not feasible in cassava, because of the constant heterozygous state used throughout the breeding process.

f. Maintenance of genetic stocks is expensive and prone to eventual losses through time. The only proven methods for long term storage of germplasm is through tissue culture procedures (expensive and sometimes require several months to recover plants for planting in the field) or else by maintaining representative plants in the field (expensive when large number of genotypes need to be maintained year after year, and also the stocks are vulnerable to gradual contamination of pathogenic and non-pathogenic organisms).

g. Exchange of germplasm among cassava breeding programs in different countries is very much restricted to a few plants representing few genotypes. Cassava breeding projects, effectively work in very isolated conditions.

h. Lack of inbreeding in cassava breeding implies that there are very little opportunities for identifying useful recessive traits, which could have huge beneficial effects in the crop. For instance, acyanogenesis in the roots has been identified as a very desirable trait to look for, but so far there has been no success in this regard. It has been postulated that this trait may be recessive. Also worth mentioning are the several starch mutations that generally are recessive in most crops.

These may all be a valid explanation for the limited genetic gains for higher productivity or value observed in the crop, compared with that of other crops such as maize or rice. It should be emphasized that because the highly heterozygous condition of cassava in every stage of the breeding process, consolidation of genetic gains is very difficult, due to the inherent genetic instability of heterozygosity.

From the practical point of view, implementing a traditional recurrent selection method in cassava offers some problems. Pollinations are slow and inefficient. It takes about 16 to 18 months since a given cross is planned until the recombinant seed is finally obtained (usually field operations have to adjust to the occurrence of the rainy season, and if that is the case, then planting can only be done 24 months after planning the cross). The third year would be used to grow the plant from the botanical seed. During the fourth year, a clonal evaluation could finally be carried out. Therefore a typical recurrent selection method would require no less than four years. In this case, however, no selfing for reducing genetic load would have been included.

A cassava-breeding scheme based on the production of doubled-haploids. This is an article postulating a new approach for cassava breeding, which is in the process of implementation. It has not been applied to any particular breeding population at CIAT at this point.

The advantages of inbreeding. When an elite clone is self-pollinated two important events occur: a) the unique, specific combination of alleles present in the genotype is broken, therefore loosing the agronomic superiority that the clone might have. b) self-pollination forces that half of the loci on average to become homozygous, thus facilitating the elimination of undesirable, deleterious alleles present in the original clone but hidden because of the predominant heterozygosity. In other words, selfed progenies allow for a reduction of the genetic load originally present in the clone, therefore becoming better progenitors themselves. In a way, selfing allows to "*concentrate*" the desirable genes originally present in the elite clone.

If inbreeding was pursued until near or complete homozygosity, then the transfer of desirable traits through the back-cross scheme becomes feasible. Also homozygosity "*captures*" genetic superiority because of its inherent genetic stability. Therefore each cassava improvement cycle would be a consolidated step that could help further progress in a more consistent and predictable way. On the other hand, each time a hybrid is used as parent, the process goes back to the initial step because of the inherent genetic instability of the heterozygous material. In this case, progress cannot be easily consolidated or sustained through time, but only in a rather inefficient way.

From the quantitative point of view the variability of a given population has traditionally been split into two major components: additive and dominant effects. Additive effects are very important because they define the breeding value of an individual, that is, its relative merit based on the quality of the progeny they produce. Dominance effects are also very important in plant breeding. They are the main contributors to the heterosis or hybrid vigor observed in hybrid cultivars, including cassava. However, contrary to the additive effects, the dominance cannot be transmitted to the progeny. This means that dominance effects cannot be effectively exploited in a breeding program, unless a sophisticated breeding scheme (reciprocal recurrent selection) is employed.

The current breeding scheme is based on the selection of individual genotypes within half- or full-sib families. Table 1 illustrates how the total genetic variance and its components (additive and dominance effects) are partitioned among and within different types of families. It is clear that all genetic effects will influence the selection of plants from half- or full-sib families. That is, 100% of additive variance and 100% of dominance variance emerge during the selection process (this is so because the breeder selects the best families, and then, the best genotype within the best family). This is a convenient situation because, once a given clone is selected, both the additive and dominance effects determining its good performance can be exploited because of the vegetative reproduction of the crop. The specific combination of genes present in this clone can be maintained unaltered generation after generation, as long as there is only vegetative reproduction.

However, only the additive portion of the total genetic variance can effectively be passed on to a next generation, when the same clone is used as parent in a breeding project. It is important to recognize that the dominance strongly influences the selection of the best clones but has no effect on their breeding value. In other words, dominance effects can be beneficial for the *per se* performance of a clone, but it has a confounding effect of its actual value as progenitor.

Inbreeding is advantageous because it erases the dominance effects from the selection process (Table 1). The resulting inbred lines do not possess any dominance effects, and therefore, there will be no heterosis or hybrid vigor expressing in their performance. That is precisely why inbred lines are inferior, agronomically speaking, compared with the non-inbred cassava materials. A striking feature of the data presented in Table 1 is that inbred lines show twice the additive variance originally present. In other words, when selecting among inbred lines the additive variance originally present (say in a F_1-hybrid) has been expanded, thus greatly facilitating the selection process of those effects (additive) that are precisely the only ones that define a superior progenitor.

In summary, inbred lines are better material for selecting progenitors because by definition they carry lower levels of genetic load, no confounding dominance effect influence the selection process and because the additive genetic effects are expanded considerably, making the selection much more efficient. Also if a breeding process is based on the use of inbred lines the transfer of valuable traits is greatly facilitated because the back-cross scheme becomes feasible.

The availability of inbred lines in cassava would also benefit other areas in addition to breeding. Genetic and molecular marker analysis would be greatly facilitated if homozygous lines were produced. The only way to maintain germplasm in cassava is either by growing the plants in the field or by tissue culture. Inbred lines could be maintained and shipped in the form of botanical seed. Phytosanitary problems could be reduced or eliminated if maintenance and/ or multiplication of genetic stocks were partially based on botanical seed. Germplasm exchange among the few cassava breeding projects in the world would be greatly facilitated because exchange would be based on botanical seed rather that *in-vitro* plants. Finally, clones could be reproduced by sexual means. Although time – consuming, the first stage of evaluation could be based on many plants produced by the crossing of selected inbred lines. Currently the evaluation process takes three years to reach a stage for selection based in just 30 plants.

The problems of inbreeding. Cassava, being an outcrossed crop, abhors inbreeding and shows severe depression. As was the case of temperate maize in the early 1900s and tropical maize by the 1970s, cassava will need to be improved for its tolerance to inbreeding depression. A few recurrent selection cycles (self-pollinating each elite clone down to the S_2 level, and recombining the surviving progenies) should prepare elite cassava populations for the trauma of total homozygosity.

A recurrent selection involving the production of inbred lines would be difficult to implement because of the length of each cycle of selection. It is estimated that about nine years will be required from the time a group of elite clones are selected until

Table 1: Distribution of additive and dominance genetic variance among and within full-sib and inbred line families.

Type of family	Among families		Among plants within families	
	Additive variance	Dominance variance	Additive variance	Dominance variance
Half-sib family	1/4	0	3/4	1
Full-sib family	1/2	1/4	1/2	3/4
Inbred lines	2	0	0	0

Source: Hallauer and Miranda Fo, 1988; Vencovsky and Barriga, 1992.

recombinant seed from their inbred lines was obtained. Therefore, if a breeding scheme using inbred lines is to be implemented, a way to reduce the time required for each cycle of selection is urgently needed.

Double haploids have been produced for many crops. Upon producing an F_1 plant, tissue culture techniques are applied to the reproductive tissue (typically anther culture). This process produces a haploid tissue that, quite frequently, doubles spontaneously to produce the doubled-haploid tissue, which by definition, is homozygous. There are other alternatives for producing similar materials (i.e. using inter-specific crosses).

If an efficient protocol for the production of doubled-haploids were available, it could be incorporated into the cassava breeding process with the advantages that inbred lines offer as explained above. From the practical point of view the protocol for the production of doubled-haploids would allow shortening the time required to produce hybrids from inbred lines down to three years.

Advantages of a breeding scheme based on inbred cassava lines. As already explained the current breeding scheme utilized for cassava posses some clear limitations: a) dominance effects confound the selection process for good progenitors; b) the continuous heterozygous state throughout the breeding process make consolidation of genetic gains nearly impossible; c) large genetic load is maintained in cassava germplasm; d) good performing hybrids are not designed, they are just found (eventually) out of several thousand segregating progenies; e) useful recessive traits remain hidden and, therefore, cannot be properly exploited.

The capacity to produce inbred lines in cassava through the use of a dynamic process allows to drastically change the breeding process: a) the emphasis will shift from producing vast number of hybrids hoping that one (or few) will be genetically superior, towards the production of parental lines that will allow *'to design'* outstanding hybrids in a gradual, consistent fashion; b) genetic loads will be quickly reduced in elite cassava populations; c) for several reasons hybrids produced from inbred lines are always better than hybrids produced from non-inbred progenitors (based on widely known experiences in maize); d) germplasm exchange will be greatly facilitated with obvious advantages for the cassava research community; e) gene exchange will also be greatly facilitated. Currently it is very difficult to transfer one valuable gene from its source into an agronomically superior clone. The availability of inbred lines would make the back-cross scheme feasible for cassava; f) inbred materials are genetically stable, they allow the breeder to capture and efficiently exploit the genetic superiority contained in them, therefore, guaranteeing a sustainable and consistent genetic progress that cannot be observed nowadays; g) once a given combination of inbred lines is found (a good performing hybrid) the hybrid could be produced first from botanical seed, and then by vegetative means. This implies not only a faster multiplication rate but also cleaner genetic stocks (from the phytosanitary point of view).

The dominance effects could also be exploited by identifying those inbred lines that produce greater heterotic (hybrid vigor) effects (Hallauer and Miranda, 1988). Although it is not clear from data currently available, a way to identify potential heterotic combinations would be through the use of molecular markers. At least in theory genetic distance has been linked to the occurrence of hybrid vigor, so biotechnology tools would benefit cassava not only by providing the protocol for the production of doubled haploids, but also as a starting point for defining which crosses should have a priority.

Results

CIAT has begun to implement the ideas described in the previous section through a two-way approach, which simultaneously address the major problems related to the introduction of inbreeding in cassava breeding (CIAT, 2003a). Each of these two approaches is described below.

Improvement of tolerance to inbreeding in elite cassava germplasm. As stated above, cassava is likely to show strong inbreeding depression. This is, precisely, because of the large genetic load expected to affect most of cassava germplasm. Maize faced a similar situation when it was started to be self-pollinated. However, after a few cycles of recurrent selection (Hallauer and Miranda, 1988; Pandey and Gardner, 1992) maize developed acceptable levels of tolerance to inbreeding. This is what needs to and will be done with elite cassava germplasm.

The scheme to improve tolerance to inbreeding in cassava has begun with the production of S_1 lines from a set of elite germplasm (around 30 elite clones) as well as other clones selected for particular reasons (i.e. MECU72 because of its resistance to white flies). A relatively large number of S_1 lines will be produced from each elite clone (between 100 and 200 lines from each clone). The best 20 % of the S_1 lines from each clone will be selected and self-pollinated again to produce a group of S_2 segregating lines. The best S_2 lines derived from each elite clone will be recombined to recover full vigor within each clonal lineage. This recombination would complete the first cycle of recurrent selection to improve tolerance to inbreeding in cassava. It would require between 4 and 5 years for completion. The product of this first cycle of selection (full vigor clones derived from the crosses among the best S_2 lines from each elite clone) would then be evaluated for agronomic characteristics and then self-pollinated to start a second cylle of recurrent selection to improve tolerance to inbreeding.

The S_2 lines produced at the end of the first cycle of recurrent selection will also be thoroughly evaluated for different agronomic traits in search of useful recessive traits (for example, absence of amylose in the starch of the roots or waxy cassava, acyanogenesis, etc.). If vigorous S_2 lines are found, they may also be further inbred through additional self-pollinations.

The purpose of this activity is to produce cassava germplasm that will be able to withstand the stress of complete homozygosity on one hand, and search for useful recessive traits on the other.

Development of a tissue culture protocol for the production of doubled-haploid lines. As stated above, the production of completely homozygous cassava lines (> 95% homozygosity) would require between 9 and 10 years. This time requirement is too long if the system is to be adopted in a dynamic and efficient breeding program. The second approach at CIAT is to develop a protocol for the production of doubled-haploid lines through tissue culture techniques. Doubled-haploid lines can be produced within 2 to 3 years, therefore, drastically reducing the time required to reach high levels of homozygosity (which in this particular case would be 100%).

The development of a tissue culture protocol for the production of doubled-haploid lines will take advantage of the experience gained in such endeavors for other crops ranging from maize to tulips (CIAT, 2003b).

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