

Obtaining hybrids of cultivars and wild subspecies of cassava

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Abstract – The objective of this work was to evaluate the crossability between cassava (*Manihot esculenta* subsp. *esculenta*) cultivars and the subspecies *M. esculenta* subsp. *flabellifolia* and *M. esculenta* subsp. *peruviana*. Plants from the BRS Jari, BRS Formosa, BRS Verdinha, and BGM 2050 (Equador 72) cultivars were crossed with the two subspecies. During flowering, at pre-anthesis, pistillate flowers were covered with a nylon bag and staminate flowers on the same inflorescence were emasculated. The staminate flowers were collected and placed in flasks previously labeled and sterilized with alcohol; some fresh flowers were stored at room temperature due to asynchronous flowering. The crosses were made between 10 a.m. and 2 p.m. After pollination, the flowers were covered again. Evaluations were made from the moment of pollination to the dehiscence of the fruit. Of the total flowers pollinated, 38% were fertilized, and 504 developed fruits and 816 produced seeds were observed. As a male parent, *M. esculenta* subsp. *flabellifolia* is more efficient in crosses with *M. esculenta* subsp. *esculenta* and may be recommend for controlled crosses. The crosses between the cassava cultivars and *M. esculenta* subsp. *flabellifolia* and *M. esculenta* subsp. *peruviana* produce viable seeds.

Index terms: *Manihot esculenta*, controlled pollination, hybrid, plant breeding, wild relative species.

Obtenção de híbridos de cultivares e de subespécies silvestres de mandioca

Resumo – O objetivo deste trabalho foi avaliar o potencial de obtenção de híbridos oriundos do cruzamento de cultivares de mandioca (*Manihot esculenta* subsp. *esculenta*) com as subespécies *M. esculenta* subsp. *flabellifolia* e *M. esculenta* subsp. *peruviana*. Os cruzamentos foram realizados entre plantas das cultivares BRS Jari, BRS Formosa, BRS Verdinha e BGM 2050 (Equador 72) e das duas subespécies. Por ocasião do florescimento, no período pré-antese, as flores femininas foram cobertas com saco de pano tipo voal, com posterior emasculação das flores masculinas. As flores masculinas foram coletadas e acondicionadas em frascos previamente identificados e desinfetados com álcool; algumas flores foram armazenadas em temperatura ambiente devido ao florescimento assíncronico. Os cruzamentos foram feitos entre 10 e 14 horas. Após a polinização, as flores foram cobertas novamente. As avaliações foram efetuadas desde a polinização até a deiscência dos frutos. Do total das flores polinizadas, 38% foram fertilizadas, tendo-se observado 504 frutos desenvolvidos e 816 sementes produzidas. *Manihot esculenta* subsp. *flabellifolia* como parental masculino é mais eficiente em cruzamentos com *M. esculenta* subsp. *esculenta* e pode ser indicada para cruzamentos controlados. Os cruzamentos realizados entre as cultivares de mandioca e *M. esculenta* subsp. *flabellifolia* e *M. esculenta* subsp. *peruviana* propiciam a obtenção de sementes viáveis.

Termos para indexação: *Manihot esculenta*, polinização controlada, híbrido, melhoramento de plantas, parentes silvestres.

Introduction

Manihot is native to tropical regions of the New World, located mainly in Brazil and Mexico (Nassar, 2002), and comprises approximately 100 species (Carmo Júnior et al., 2013; Orlandini & Lima, 2014). Cassava (*Manihot esculenta* subsp. *esculenta*) is the

only cultivated species of the genus and is a culture of great importance worldwide, since it is used to feed around 800 million people, especially in countries where food shortages are common (Nhassico et al., 2008). However, despite the great contribution of cassava as a food source to poorer populations, it is necessary to improve the nutritional quality of the root

and to obtain varieties resistant to biotic and abiotic factors (Vieira et al., 2013).

Cassava is an allogamous species; however, with vegetative propagation, self-fertilization can occur when two plants of the same genotype are crossed (Halsey et al., 2008). It is also protogynous, since pistillate flowers open before staminate flowers, and is monocious, with pistillate and staminate flowers on the same inflorescence (Halsey et al., 2008). According to Halsey et al. (2008), pistillate flowers open around ten days before staminate flowers on the same inflorescence; however, pistillate and staminate flowers can open simultaneously on the same plant, allowing self-fertilization and cross-fertilization to occur naturally, which results in strong inbreeding depression (Halsey et al., 2008). The fruit of cassava is an ovoid to ellipsoid, septicidal capsule (Oliveira & Oliveira, 2009), which dehisces two to three months after fertilization (Halsey et al., 2008). The seeds are carunculate, with abundant endosperm and an embryo with thin, flat cotyledons (Orlandini & Lima, 2014).

The genetic pool of cassava is variable for certain economically important characteristics, such as resistance to pests, diseases, drought, and salinity (Nassar et al., 2008; Ojulong et al., 2008; Carabalí et al., 2010; Akinbo et al., 2012b). Nassar et al. (2008) pointed out that wild species of *Manihot* are sources of useful genes for developing better cassava cultivars in order to increase protein contents, seed fertility, resistance to cochineals, drought tolerance, and productivity. Carabalí et al. (2010) and Akinbo et al. (2012b) found that *M. esculenta* subsp. *flabellifolia* and other *Manihot* species show several characteristics that are limited or weaker in *M. esculenta* subsp. *esculenta*, such as high protein contents and resistance to post-harvest physiological deterioration (PPD) (*M. walkerae* Croizat), tolerance to drought [*M. glaziovii* Müll. Arg., *M. catingae* Ule., and *M. carthaginensis* (Jacq.) Müll. Arg.], and resistance to cassava green mite, cochineals, whitefly and hornworm. The lack of resistance leads to large production losses in cassava, which are mainly caused by whitefly (Carabalí et al., 2010). Diseases not present in Brazil also deserve attention. For example, *African cassava mosaic virus* (ACMV) is considered the most economically important threat to cultivated cassava; recently there were reports of serious losses in Africa caused by this disease (Peruch & Andrade, 2015).

These limitations, when cultivating cassava, could be mitigated by obtaining hybrids with wild species. Natural and induced hybridization occur within *Manihot*, indicating that the barriers that isolate species are weak due to the recent evolution of the genus (Duputié et al., 2011).

However, one of the main problems in using wild species of *Manihot* in genetic improvement programs is the asynchrony in flowering periods, which makes it difficult to propagate and maintain these plants in the field (Vieira et al., 2013). Increasingly, there is a need for studies related to the floral biology of cassava and the hybridization process because they facilitate genetic improvement programs that perform cross-pollination.

Allem et al. (2001) proposed that the cultivated species *M. esculenta* Crantz, *M. flabellifolia* Pohl, and *M. peruviana* Müll. Arg. are three subspecies of *M. esculenta*. Genetic and phylogenetic aspects of *M. esculenta* subsp. *esculenta* and *M. esculenta* subsp. *flabellifolia* (Pohl) Ciferri were studied by Carvalho & Schaal (2001), who confirmed the genetic proximity. Olsen & Schall (2001) also corroborated the hypothesis of Allem (1994) that cassava originated from *M. esculenta* subsp. *flabellifolia*. In a study on the genetic similarity between *M. esculenta* subsp. *flabellifolia*, *M. esculenta* subsp. *peruviana*, *M. pilosa* and *M. esculenta* subsp. *esculenta*, based on an isoenzyme analysis, Brondani (1996) concluded that *M. esculenta* subsp. *flabellifolia* and *M. esculenta* subsp. *peruviana* are similar to *M. esculenta* subsp. *esculenta* and that they could be used in crosses to improve cassava.

Crosses between *M. esculenta* subsp. *flabellifolia* and *M. esculenta* subsp. *esculenta* were promising in relation to fertilization (Akinbo et al., 2012a), and hybrids between cassava and *M. glaziovii* were obtained by Nassar et al. (2009). Nassar et al. (2010), when trying to cross four *Manihot* species with *M. esculenta*, observed compatibility only for *M. esculenta* x *M. pilosa* and *M. glaziovii*, but with low levels of interspecific hybridization (1.5 and 2%, respectively). According to Allem et al. (2001), it is possible to cross *M. dichotoma* Ule and *M. glaziovii*, but with difficulties in gene transfer, resulting in a possible sterile generation with weak chromosomal pairing or univalents. Studies related to the compatibility between cassava and wild *Manihot* species were conducted by Nassar (1980) and Nassar et al. (2010), who found a low frequency of compatibility between species.

Normally, crosses made between domesticated cultivars and wild species of *Manihot* enable the transfer of genes that express undesirable characteristics, such as those related to a lower harvest index and, consequently, to a lower productivity of tuberous roots (Ojulong et al., 2008). To eliminate undesirable characters in possible hybrids, gene introgression based on backcrossing is recommended, using a genotype of a cultivated species with high production potential as the recurrent parent (Vieira et al., 2013). However, the fixation of desirable characteristics from the wild parent of cassava is more difficult as the number of genes that control the characteristic in question increases.

Studies related to obtaining cassava hybrids with wild species of *Manihot* are increasingly necessary to support cassava improvement programs so that useful alleles, – that contribute to the reproduction and conservation of species – are not lost.

The objective of this work was to evaluate the crossability between cassava (*Manihot esculenta* subsp. *esculenta*) cultivars and the subspecies *M. esculenta* subsp. *flabellifolia* and *M. esculenta* subsp. *peruviana*.

Materials and Methods

The field work was conducted at the headquarters of the Embrapa Mandioca e Fruticultura in the municipality of Cruz das Almas, in the state of Bahia, Brazil (12°40'19"S, 39°06'22"W, at 220 m altitude). The climate is tropical hot and humid, Aw to AM according to Köppen, with an average annual temperature of 24.5°C and relative humidity of 80% (Agritempo, 2013). The controlled crosses were made from October to December 2012, and 2,114 flowers were pollinated.

For the reciprocal crosses, four cultivars and two subspecies were used. The genotypes of *Manihot esculenta* subsp. *esculenta* selected were: BRS Jari, BRS Formosa, BRS Verdinha, and BGM 2050 (Equador 72), because they perform well agriculturally, have high root productivity, and are considered elite cultivars; they were available at the germplasm bank at Embrapa Mandioca e Fruticultura. The plants of the subspecies *M. esculenta* subsp. *flabellifolia* and *M. esculenta* subsp. *peruviana* belong to the collection of wild *Manihot* species, also at Embrapa Mandioca e Fruticultura, and are from seeds donated by Centro

Internacional de Agricultura Tropical (CIAT). They were selected because they are part of the primary gene pool of *Manihot* (Allem et al., 2001) and presented high seed production in controlled crosses involving wild *Manihot* species in studies carried out from 2007 to 2011 at Embrapa Mandioca e Fruticultura (Ledo et al., 2015).

The hybridization technique involved covering pistillate flowers during pre-anthesis with a nylon bag, when the flowers were nearly mature (visually) and about to open, and emasculating staminate flowers on the same inflorescence (Fukuda, 1980). The staminate flowers were collected and placed in flasks previously labeled and sterilized with alcohol; some fresh flowers were stored at room temperature due to asynchronous flowering. Flowers were pollinated by touching the anthers of a staminate flower to the stigma of a pistillate flower. The crosses were made during the day, from 10 a.m. to 2 p.m., when the sun is the strongest. This period is related to the anthesis of cassava and wild *Manihot* species, which occurs during the day when temperatures are the highest and humidity is the lowest, and is also when pistillate flowers open and the stigmas are the most receptive (Vieira et al., 2013).

After pollination, the flowers were covered again and identified both with the name of their parent plants and the date of the crossing. To detect the occurrence of fertilization, a magnifying glass was used to observe possible changes in the color of the stigma and developing ovary three to five days after pollination. From the moment of pollination to fruit dehiscence, the crossing date, genotypes and plants of *M. esculenta* subsp. *esculenta* used as the female (pistillate flower) and male (staminate flower) parents, number of flowers crossed, and number of developed fruits were recorded. After the fruits matured and dehisced, the seeds were collected and stored – in the seed bank at the cassava genetic program of Embrapa – to be used later.

The absolute and relative frequencies of the number of pollinated flowers, fertilized flowers, developed fruits, produced seeds, and dehisced fruit were analyzed. The relative frequency was calculated based on the number of pollinated flowers. The Kruskal-Wallis non-parametric test was used to compare the averages obtained for the various crosses made, at 5% probability, with the SAS software (SAS Institute Inc., Cary, NC, USA).

Results and Discussion

When analyzing open flowers, it was possible to observe that the wild subspecies and cultivars of cassava are protogynous, because the pistillate flowers opened before the staminate flowers, as reported by Vieira et al. (2013). According to Halsey et al. (2008), on the same inflorescence, the pistillate flowers open around ten days before the staminate flowers.

In all the controlled crosses, the wild subspecies of cassava participated mostly as the male parent (Table 1) because they produced more pollen. These results are similar to those of Vieira et al. (2013), who recorded greater pollen production by wild *Manihot* species compared with cultivars, also finding the same difficulty related to flower synchrony and a large discrepancy in the number of pollinated flowers. In the present study, the only reciprocal crosses performed were: *M. esculenta* subsp. *flabellifolia* x 'BRS Formosa' and *M. esculenta* subsp. *flabellifolia* x 'BRS Jari' (Table 1). No crosses were made between *M. esculenta* subsp. *flabellifolia* (as the female parent) and 'BRS Verdinha' and 'BGM 2050' (Equador 72),

and between *M. esculenta* subsp. *peruviana* and four all cultivars, due to difficulties with flower synchrony, which led to a discrepancy in the number of pollinated flowers. Asynchrony in flowering makes hybridization difficult, limiting reciprocal crosses.

The crosses *M. esculenta* subsp. *flabellifolia* x 'BRS Formosa' and *M. esculenta* subsp. *flabellifolia* x 'BRS Jari' behaved differently (Table 1). When *M. esculenta* subsp. *flabellifolia* was used as the female parent in crosses with 'BRS Formosa', there was no fertilization. As the male parent, however, 40.74% of the flowers were fertilized and 301 seeds were produced. In a previous study, crosses between *M. esculenta* subsp. *flabellifolia* and *M. esculenta* subsp. *esculenta* were promising in relation to fertilization (Akinbo et al., 2012a).

Nassar (1980), in a study on interspecific hybrids of wild *Manihot* species and plants of *M. esculenta* subsp. *esculenta*, observed that the use of wild species as the male parent was more promising. Analogously, Vieira et al. (2012) found that wild *Manihot* species generally produce pollen in greater quantity and with better

Table 1. Absolute and relative frequencies (RF) of pollinated flowers (PF), fertilized flowers, developed fruits, produced seeds, and dehiscence of fruits in days (DF) for controlled crosses between the subspecies *Manihot esculenta* subsp. *flabellifolia* (FLA) and *M. esculenta* subsp. *peruviana* (PER) and four cultivars of *M. esculenta* subsp. *esculenta*.

Crosses		Number (No.) of PF	Fertilized flowers		Developed fruits		Produced seeds			DF ⁽⁶⁾ (day)
Female parent ♀	Male parent ♂		No. ⁽¹⁾	RF (%) ⁽²⁾	No. ⁽¹⁾	RF (%) ⁽³⁾	No. ⁽¹⁾	RF (%) ⁽⁴⁾	RF (%) ⁽⁵⁾	
FLA	BRS Formosa	35	0	0.00	0	0.00	0	0.00	0.00 ⁽⁵⁾	0
BRS Formosa	FLA	459	187	40.74	161	86.1	301	93.5	32.8	67
FLA	BRS Jari	143	31	21.68	9	29.03	11	40.74	2.56	72
BRS Jari	FLA	200	60	30.00	18	30.00	25	46.30	4.17	73
BRS Jari	PER	67	24	35.82	6	25.00	9	50.00	4.48	56
BRS Formosa	PER	262	167	63.74	120	71.86	191	53.06	24.30	74
BGM 2050 ⁽⁷⁾	FLA	66	17	25.76	3	17.65	8	88.89	4.04	76
BGM 2050 ⁽⁷⁾	PER	134	65	48.51	24	36.92	48	66.67	11.94	91
BRS Verdinha	FLA	675	206	30.52	131	63.59	169	43.00	8.35	50
BRS Verdinha	PER	73	47	64.38	32	68.09	54	56.25	24.66	73
Total		2,114	804	38	504	62.69	816	80.95	19.29	-

⁽¹⁾Absolute frequency. ⁽²⁾Relative frequency calculated as a function of the number of pollinated flowers. ⁽³⁾Relative frequency calculated as a function of the number of fertilized flowers. ⁽⁴⁾Relative frequency calculated considering two seeds produced per developed fruit. ⁽⁵⁾Relative frequency calculated considering two seeds formed per pollinated flower. ⁽⁶⁾Average number of days to fruit dehiscence. ⁽⁷⁾BGM 2050 (Equador 72).

viability than plants of *M. esculenta* subsp. *esculenta*. In relation to the reciprocal crosses between the BRS Jari cultivar and *M. esculenta* subsp. *flabellifolia*, fertilized flowers, as well as a good percentage of produced seeds and quantity of developed fruits, were observed (Table 1). For the *M. esculenta* subsp. *flabellifolia* x 'BRS Formosa' and *M. esculenta* subsp. *flabellifolia* x 'BRS Jari' reciprocals, there were better results when *M. esculenta* subsp. *flabellifolia* was used as the male parent. These findings corroborate those of Nassar (1980). Of all the pollinated flowers, 38% were fertilized; the flowers that were not fertilized had undeveloped ovaries, dried up, and fell from the plant. The fertilization rate observed in the present study could have been affected by the low viability of the pollen grains because of the environmental storage conditions that were used for some crosses, such as *M. esculenta* subsp. *flabellifolia* x 'BRS Formosa' and *M. esculenta* subsp. *flabellifolia* x 'BRS Jari', due to the difficulty in synchronizing flowering. Usually, fertilization rates that involve crosses between cassava plants are low and a large quantity of viable pollen is needed, which makes it possible to create various crosses with the same parents to produce more seeds (Nassar et al., 1986). This low fertilization rate could also be related to the "low" pollen viability reported for *Manihot* species, because pollen lasts for only 48 hours after anthesis (Halsey et al., 2008). According to Vieira et al. (2012), the pollen remains viable for six days when conserved in calcium chloride, and loses its viability when stored fresh at room temperature.

When creating hybrids between *M. esculenta* subsp. *flabellifolia* and *M. esculenta* subsp. *esculenta*, Vieira et al. (2013) found high fertilization rates ranging from 17% (FLA005-04 x Col 1725) to 92% (FLA029V x Abóbora), with an average of 47%. In this case, *M. esculenta* subsp. *flabellifolia* was used as the female parent for most of the crosses; however, in three crosses (Paraná x FLA005-03, PER334 x FLA005-02, and TN260 x FLA005-03), it was necessary to use it as a pollen donor (male parent) due to the lack of synchronous flowering of the *M. esculenta* subsp. *esculenta* cultivars. These results were better than those obtained in the present study, for the crosses *M. esculenta* subsp. *flabellifolia* x 'BRS Formosa' and *M. esculenta* subsp. *flabellifolia* x 'BRS Jari', which showed fertilization rates of 0 and 21.6%, respectively (Table 1). The highest fertilization rates were found

when crossing *M. esculenta* subsp. *flabellifolia* and *M. esculenta* subsp. *peruviana* as the male parent, which could be related to the quantity and size of the pollen of these subspecies according to Vieira et al. (2012).

Nassar et al. (1986) created hybrids between cultivated cassava and the wild species *M. pseudoglaziovii* Pax & K. Hoffm., *M. caerulescens* Pohl and *M. pohlii* Wawra, but seeds were only produced when *M. pohlii* was the pollen recipient and the fertilization rate was low (0.09%). In the present study, a higher fertilization rate was observed, i.e., 0.09% vs. 34.72% (Table 1). This was expected because *M. esculenta* subsp. *peruviana* and *M. esculenta* subsp. *flabellifolia* are phylogenetically closer to *M. esculenta* subsp. *esculenta* (Duputié et al., 2011). The fertilization percentage of crosses can reach 100% for species that are part of the primary gene pool of *Manihot* and are between 30 and 90% for species of the secondary gene pool (Ojulong et al., 2008).

However, the total number of fruits that developed (23.84% of the expected) produced 816 seeds, corresponding to 1.62 seed per fruit (Table 1). Vieira et al. (2013) crossed *M. esculenta* subsp. *flabellifolia* and *M. esculenta* subsp. *esculenta*, and the 42 fruits that developed (28% of the expected) produced 94 seeds. For some studies, the rate of fruit production could have been related to the phylogenetic proximity between subspecies (Olsen & Schall, 2001; Duputié et al., 2011).

These results show that the number of seeds a plant produces can be small in relation to what is expected. This could be related to the difficulty of synchronizing flowering because *Manihot* species is protogynous, which limits the number of possible crosses. According to Vieira et al. (2012), conserving pollen could be a strategy to solve this problem. Another alternative is to increase the number of varieties and/or the number of plants of wild *Manihot* species, in order to produce more crosses.

Fruit dehiscence when *M. esculenta* subsp. *flabellifolia* was the male parent occurred in around 66 days; however, for *M. esculenta* subsp. *peruviana*, the average was 73 days (Table 1). When *M. esculenta* subsp. *flabellifolia* was used as the male parent, fruits dehiscenced in around 72 days. In the present study, fruit dehiscence started after 50 days for 'BRS Jari' x *M. esculenta* subsp. *peruviana*. These results agree with those of Halsey et al. (2008), who found that fruits

opened 2 to 3 months after fertilization. For the crosses made with *M. esculenta* subsp. *flabellifolia* and *M. esculenta* subsp. *peruviana*, a higher number of seeds was expected considering there were two seeds per fruit (Table 2). However, the percentage of formed seeds in relation to the quantity of developed fruits was over 50% for both, which is a promising result, due to the observed limitations.

In general, asynchronous flowering was not marked among the species evaluated, which resulted in viable crosses between the cultivars of *M. esculenta* subsp. *esculenta* and the subspecies *M. esculenta* subsp. *flabellifolia* and *M. esculenta* subsp. *peruviana* (Table 1). This also amplified the genetic variability of characters of interest that are of fundamental importance to genetic improvement programs, such as high dry matter and protein contents; resistance to insects, such as the whitefly and hornworm; resistance to diseases, including bacteriose and anthracnose; and resistance to the *African cassava mosaic virus*, which does not occur in Brazil (Carabalí et al., 2010; Akinbo et al., 2012b). It should be noted that there was significant production of hybrid seeds (Tables 1 and 2), which are stored in the seed bank at Embrapa Mandioca e Fruticultura for future use to obtain new hybrids and improve cultivars.

Table 2. Pollinated flowers (PF), fertilized flowers (FF), developed fruits (DF), expected seeds (ES), and produced seeds (PS) for controlled crosses made in the second half of 2012 with the wild subspecies of cassava, *Manihot esculenta* subsp. *flabellifolia* (FLA) and *M. esculenta* subsp. *peruviana* (PER), as parents⁽¹⁾.

Male and female parent	PF	FF ⁽²⁾	DF ⁽³⁾	ES	PS ⁽⁴⁾
FLA	1,578a	501a (27.31)	322a (64.27)	3,156	514a (79.81)(16.28)
PER	536b	303b (56.53)	182b (60.07)	1,072	302b (55.31)(18.78)
Total	2,114	804 (38)	504 (62.69)	4,228	816 (80.95)(19.29)

⁽¹⁾Means followed by equal letters do not differ by the Kruskal-Wallis test, at 5% probability. ⁽²⁾Absolute frequency (AF) and relative frequency (RF, in %) between parentheses calculated as a function of the number of pollinated flowers. ⁽³⁾RF (between parentheses) calculated as a function of the number of fertilized flowers. ⁽⁴⁾AF and RF calculated considering two seeds produced per developed fruit (first parentheses), and RF calculated considering two seeds formed per pollinated flower (second parentheses).

Conclusions

1. The crosses of *Manihot esculenta* subsp. *esculenta* x *M. esculenta* subsp. *flabellifolia* and *M. esculenta* subsp. *esculenta* x *M. esculenta* subsp. *peruviana* result in fertile hybrids.

2. The use of *M. esculenta* subsp. *flabellifolia* as the male parent is more efficient for crosses with *M. esculenta* subsp. *esculenta* and is best for controlled crosses.

3. Crosses made between cassava cultivars and *M. esculenta* subsp. *flabellifolia* and *M. esculenta* subsp. *peruviana* produce fertile seeds.

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