

Full Length Research Paper

Fruit, seed and embryo development of different cassava (*Manihot esculenta* Crantz) genotypes and embryo rescue

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Fruit, seed and embryo developments of different cassava (*Manihot esculenta* Crantz) genotypes, as well as embryo rescue, were investigated. The fruits of three genotypes after uncontrolled open pollination presented the same progressive development with similar sizes at different stages. There are large differences in the fruit set as well as the embryo development between different genotypes. Days after pollination (DAP) was found not to be an adequate predictor of embryo size as their size ranged from almost invisible to 8.7 mm in length at 32 DAP even within the different locules of the same fruit. The ideal stage for embryo rescue in cassava was from 32 to 36 DAP, because at that stage most embryos are visible (> 0.7 mm); and their excision without injury is feasible. Also, *in vitro* germination of the cotyledonary embryos at that stage had a high success rate. A half Murashige and Skoog (MS) medium supplemented with 1.0 mg/l GA₃, 2% sucrose and 0.2% gel rite proved to be adequate for embryo rescue.

Key words: *Manihot esculenta* Crantz, day after pollination (DAP), fruit set, seed size, embryo size, embryo rescue.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz), a member of the Euphorbiaceae family, is a perennial vegetative propagated shrub widely planted in tropical and subtropical regions of the world. Cassava, along with maize, sugarcane and rice, constitutes the most important sources of energy in the diet of most tropical countries of the world (Ceballos et al., 2004). It has played an

important role in food security as a famine reserve crop historically, and also become potentially highly resilient to future climate changes (Jarvis et al., 2012). With the worldwide increasing importance of cassava for human consumption, animal feed and industrial applications, there will be an increasing need to develop cultivars with specific characteristics and for adaptation to different

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ecologies. Conventional breeding efforts have attempted to address many of the constraints facing cassava productivity, with outstanding success. Average productivity of cassava in countries like Thailand and Vietnam has doubled in the last two decades. Biotechnology provides new tools for overcoming some of the problems that currently hinder cassava improvement. Major efforts are currently undergoing to develop protocols for the production of doubled haploids with the ultimate objective of introducing inbreeding in the genetic enhancement of cassava. Embryo rescue is to nurture the immature or weak embryo, thus allowing it the chance to survive, and plays an important role in modern plant breeding (Sharma et al., 1996). Embryo rescue also demonstrates potential roles in cassava breeding: (1) rescue plantlets from the younger fruits when confronted with adverse climate (Uma et al., 2011); (2) rescue *in vitro* plantlets following interspecific hybridization (Clarke et al., 2011; Cisneros et al., 2013); (3) shorten the breeding cycle by recovering the plantlets from younger seeds, replace germinating the seeds harvested after breaking the dormancy (Kagithoju et al., 2013; Gbadamosi, 2013). But initial work required considerable amount of basic research because of the scarcity of information related to the biology or structural development of flower buds in cassava (Perera et al., 2013). So, this research was conducted to explore fruit, seed and embryo development of different cassava genotypes, and then recover the plantlets from younger seeds. This information is crucial for ongoing research to obtain doubled haploids through wide crosses with *Ricinus communis*.

MATERIALS AND METHODS

This study was carried out in the field and at the tissue culture laboratory of cassava genetics in the International Center for Tropical Agriculture (CIAT), Cali-Palmira, Colombia, from February to September, 2012. Three elite lines (HMC-1, SM1219-9 and MCOL1505) being used by the cassava program of CIAT were selected because of their profuse flowering and planted in the field. All three lines were grown in the field in clay loam soil, pH 7.2, at CIAT headquarters in Cali, Colombia (3°30'N, 76°19'W; 965 m above sea level). During 1993 to 2010, mean daily temperatures ranged from a maximum of 29.7^L to a minimum of 19.2^L, with a mean monthly temperature of 23.8^L. The relative humidity averaged 78% and annual rainfall, 936 ± 34 mm. Each of the two experiment, at anthesis day, 100 female flowers on 15 to 20 inflorescences (in members of the Euphorbia family whose correct name is cyathium) from 10 healthy plants of three lines were marked with plastic labels (identifying them and stating date of anthesis) and left for natural, open pollinations carried out by insects. In the first experiment, the width of fruits at different development stages (7, 14, 21 and 28 days after pollination (DAP) was measured by using the electronic digital caliper (01407A, Neiko). Fruit set at different stages was calculated as the ratio of fruits that remained attached to the mother plant to the number of female flowers initially marked. When determining the width of fruits, those fruits with atypically smaller size were not considered, except at 7 DAP.

At 32 DAP, parts of the remaining fruits were harvested, and number of fruits scored. Fruits were then dissected and number of seeds and cotyledonary embryos counted. The width of fruits, the

length of seeds, and cotyledonary embryos were then measured. The width of fruits was measured by using the electronic digital caliper. The length of both seeds and cotyledonary embryos were measured with a Wild M7A stereomicroscope connected to a digital micrometric ocular Wild MMS 235 (Heerbrugg, Switzerland). The fruit width, seed length and embryo length were presented as mean ± standard error. In a second experiment, fruits were harvested at different number of days after anthesis (26, 28, 30 and 32 DAP), and counted. Fruits were dissected and number of seeds and cotyledonary embryos was counted to determine the percentage of seeds with visible cotyledonary embryos that could be observed under Wild M7A stereomicroscope (10 × ocular with 6 × magnification). Parts of harvested cotyledonary embryos were used to recover plantlets via embryo rescue culture.

Embryo rescue was done at the tissue culture laboratory of cassava genetics in CIAT. The immature seeds dissected out of fruits were surfaced-sterilized by immersion in 70% alcohol for 1 min, followed by immersion in 0.5% sodium hypochlorite for 8 min, and then washed three times with sterile water. The seeds were split along the longitudinal axis utilizing sterile forceps and scalpel, using aseptic conditions, under the stereomicroscope. For each line, 50 excised cotyledonary embryos were used for embryo rescue. Excised cotyledonary embryos were placed radicle down on the preferred medium (M6 medium), that is, half Murashige and Skoog (1962) (MS) basal medium, supplemented with 1.0 mg/L GA₃, 2% sucrose and 0.2% gelrite (Sigma). The pH of media above was adjusted to 5.8 ± 0.1 before autoclaving at 121°C for 20 min. The embryo cultures were incubated at 28 ± 1°C under a 12/12 h (day/night) photoperiod with light supplied by white fluorescent tubes (25 μmol m⁻²s⁻¹). After *in vitro* culture for two weeks, the germination of the cotyledonary embryos was scored.

RESULTS AND DISCUSSION

Fruit set and development data are summarized in Table 1. The fruits of three genotypes (HMC-1, SM1219-9 and MCOL1505) after uncontrolled open pollination presented the same progressive development with similar sizes at different stages. In contrast, there were marked differences for fruit set among the three cultivars employed (Table 1). At the initial stage (7 DAP), there were no apparent differences on fruit set. But at 14 DAP, fruit set of HMC-1 and MCOL1505 sharply declined to 62.1 and 39.7%, respectively, contrasting with that of SM1219-9 (95.7%). And at 28 DAP, the fruit set of SM1219-9 remained at 91.9%, much higher than that of the other two genotypes (48.5 and 38.2% respectively). The difference in fruit set is mainly attributed to the abscission of fruits during the second weeks probably due to the occurrence of abnormal gametes and/or (most likely) problems in the germination and growth of pollen tubes or in the fertilization to produce viable zygotes.

The genotype differences on fruit set had also been reported in other crops (Esen et al., 1978). There are also other factors affecting the fruit set, such as insects and diseases (Figure 1C). In one particular batch, the marked female flowers encountered moderate rain during the afternoon of anthesis day, and fruit set of all three genotypes dropped down at 14 DAP (data not shown). As illustrated in Table 2 and Figure 1A, there was considerable variation in seed and embryo developments among the

Table 1. Fruit development of different cassava genotypes at different days after pollination (DAP).

Genotype	7 DAP		14 DAP		21 DAP		28 DAP	
	Fruit width (mm)	Fruit set (%)	Fruit width (mm)	Fruit set (%)	Fruit width (mm)	Fruit set (%)	Fruit width (mm)	Fruit set (%)
HMC-1	5.4 ± 0.9	98.5	15.1 ± 3.1	62.1	19.9 ± 1.8	53.0	22.6 ± 1.3	48.5
SM1219-9	6.8 ± 1.0	100	13.9 ± 1.0	95.7	19.1 ± 0.7	95.7	20.5 ± 0.7	91.9
MCOL1505	5.3 ± 0.7	100	14.4 ± 0.8	39.7	17.9 ± 2.1	38.2	19.3 ± 1.9	38.2

**Figure 1.** A) Seeds and embryos of cassava (HMC-1) at 30 to 38 days after pollination (DAP). B) Seedlings germinated from young cotyledonary embryos. C) Seeds affected by insects and diseases in the field.**Table 2** Fruit, seed and embryo development of different cassava genotypes at 32 days after pollination (DAP).

Genotype	Number of fruits collected	Number of seeds	Number of embryos	Fruit width (mm)	Seed length (mm)	Embryo length (mm)		
						Average	Maximum	Minimum
HMC-1	35	71	51	22.8 ± 1.8	10.9 ± 0.6	5.6 ± 2.2	8.7	0.7
SM1219-9	31	79	52	20.7 ± 0.5	9.2 ± 0.3	4.2 ± 1.2	6.6	1.8
MCOL1505	31	23	18	19.3 ± 1.3	9.9 ± 0.4	3.6 ± 1.6	7.3	1.5

different genotypes considered. Cassava ovaries have three locules, each with one ovule (Alves, 2002). At 32 DAP, the average seed number of each fruit of HMC-1, SM1219-9 and MCOL1505 were 2.1, 2.6 and 0.7, respectively. Mostly, only two seeds were harvested in fruits from HMC-1, with the third locule containing a brown tiny ovule. Fruits from SM1219-9, often produced three seeds, as apposed to an average of less than one seed per fruit of MCOL1505.

Fruit and seed sizes of different genotypes were similar

at the same number of days after anthesis; but the embryo size showed marked differences. On the other hand, there was wide variation of embryo length when harvested at 32 DAP. For example, in the case of HMC-1 embryo length ranged from 0.7 to 8.7 mm (Table 2). Moreover, large variation in embryo sizes was observed among the different locules of the same fruit, which would suggest that the embryo development of cassava is highly un-synchronized. It is worthy to emphasize the relative uniformity in embryo size as for SM1219-9, compared with

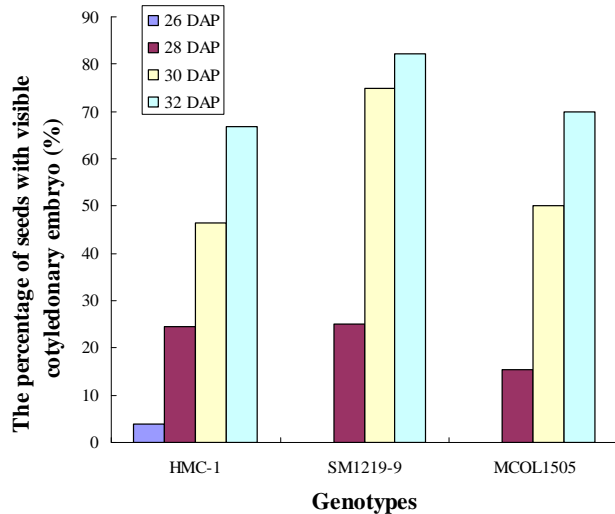


Figure 2. Embryo development of three cassava genotypes at different days after pollination (DAP).

that of HMC-1 and MCOL1505. This observation would further point SM1219-9 as an ideal elite parental material for the ongoing work to develop a protocol for the production of doubled haploids in cassava. In addition to genetic variation, other factors, such as temperature (Lang and Sutka, 1994), might affect the development of seed and embryo. However these factors would affect equally the three genotypes involved in this study (unless genotype x environment interactions are markedly significant in the reproductive biology of cassava) and therefore, would not properly explain the differences observed between the three genotypes.

An important and critical preliminary result of this work was the observation that the embryo and endosperm development remains very slow till the fourth week after pollination; and only at 22 DAP, it was possible to observe embryos at early developmental stage (globular phase) (L.N. Ramos, personal communication). Our results are showed in Figure 2, it was very difficult to visualize cotyledonary embryos inside the seed at 26 DAP, even under stereomicroscope. This was the case for the three genotypes used. Even at 28 DAP; the percentage of seeds with visible cotyledonary embryo was less than 30%. At 30 DAP, this figure rose up to over 70% in SM1219-9, and around 50% for both HMC-1 and MCOL1505. It is interesting to note that abortive embryos were found in the relatively smaller size of seeds of all three genotypes. During fruit development of each genotype, the color of seeds changed progressively from cream white to brown from 30 to 38 DAP; and also various sizes of embryo were observed (Figure 1A). At 38 DAP, most of the seeds are too hard to dissect out the embryo without injury. Seeds from fruits harvested at an earlier age are, in this regard, more suitable for embryo rescue purposes.

The cotyledonary embryos excised from the immature fruits were cultured on M6 medium, which was screened out by comparative experiments. Every cultured cotyledonary embryo could germinate and develop roots, internodes and shoots, independent of the genotype (Figure 1B). Our protocol improved the recovery efficiency of the seeds in cassava embryo rescue culture, compared to the 66% results reported in the literature (Fregene et al., 1999; Akinbo et al., 2010). The presence of GA₃ in the M6 medium probably promoted the development of immature embryos in agreement with the results reported by Anuradha and Rout (2005). It also found that the embryo without visible cotyledon was too small to germinate, and demonstrated that embryo size was positively correlated with success in embryo rescue and recovery of plantlets (Kapila and Sethi, 1993). Rescuing and culturing younger embryos when they are not readily visible may require the inclusion of other tissue such as embryo sac and even part of the ovary. Likewise, parental genetic, phenological and physiological differences can also contribute to variations in the *in vitro* responses of rescued embryos (Clarke et al., 2006).

Conclusion

In conclusion, this is an important contribution to our understanding of cassava fruit, seed and embryo development of different genotypes and an improvement on previous protocols for embryo rescue in cassava (Fregene et al., 1999). Large differences were found in fruit set and developmental rate as well as the embryo development between different genotypes. The ideal stage for embryo rescue in cassava was found to be from 32 to 36 DAP. A half MS medium supplemented with 1.0 mg/l GA₃, 2% sucrose and 0.2% gelrite proved to be adequate for embryo rescue. Embryo rescue is likely to play an important role in the production of doubled haploid plants from wide crosses or pollinations with irradiated pollen, and also shorten the breeding cycle.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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