academicJournals

Vol. 14(27), pp. 2191-2201, 8 July, 2015
DOI:10.5897/AJB2015.14577
Article Number: C758D4354080
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

In vitro embryo rescue and plant regeneration following self-pollination with irradiated pollen in cassava (*Manihot esculenta* Crantz)

Mary Buttibwa[1,2], Robert S. Kawuki[1], Arthur K. Tugume[2]*, Jacinta Akol[1], Stephen Magambo[1], Hellen Apio[1], Erwin Heberle-Bors[3], Maria Wedzony[4], Hernán Ceballos[5], Clair Hershey[5] and Yona Baguma[1]

[1]National Crops Resources Research Institute (NaCRRI), Namulonge, P. O. Box 7084, Kampala, Uganda.
[2]Department of Biological Sciences, College of Natural Sciences, Makerere University, P. O. Box 7062, Kampala, Uganda.
[3]Max-F. Perutz Laboratories, University of Vienna, Dr. Bohrgasse 9, 1030 Vienna, Austria.
[4]Institute of Biology, Pedagogical University of Kraków, Podchorążych, 30-084, Kraków, Poland.
[5]International Center for Tropical Agriculture (CIAT), Apartado, Aéreo, 6713, Cali, Colombia.

Received 19 March, 2015; Accepted 17 June, 2015

Cassava is a highly heterozygous species; hence, current methods used in classical cassava breeding cannot match the urgent need to high yielding varieties. Recently, progress was made through androgenesis and gynogenesis as pathways for raising doubled cassava haploid lines to overcome problems associated with cassava's inherent reproductive biology, but these efforts were limited (no candidate cassava plantlets were regenerated). For the first time, this study shows that pollen irradiation coupled with self-pollination and embryo rescue regenerated 62 candidate cassava plantlets. Plants of an elite cassava variety, Nase14, served as a mother plant and as the pollen donor for the irradiation. Irradiation dosages of 50 to 250 Gray studied across five pollination events and 300 or 500 Gray in one pollination event caused a reduction in pollen germination up to 67.0%. By 15 days after pollination (DAP) with irradiated pollen, up to 89.7% of the pollinated flowers had aborted. By embryo rescue time (42 DAP), significant differences were observed in number of fruits, seeds and embryos generated, with the non-irradiated pollen treatments having significantly higher numbers. Sixteen (16) heterozygous SSR markers in the parent and ploidy analysis showed that none of the regenerated plants was haploid or homozygous. However, the plantlets resulting from pollination with non-irradiated pollen had 56.2% homozygous loci, while progeny derived from irradiated treatments had frequencies of homozygous loci between 28.1 and 55.0%. This is the first time to use irradiated pollen in cassava as a pathway to generate candidate plantlets as an initial step in double haploid production.

Key words: Cassava, doubled haploids, embryo rescue, plant regeneration, pollen germination, pollen irradiation.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is the world's second most important root crop that is grown in over 100 countries in Africa, Asia, the Pacific, and South America (FAOSTAT, 2014). Cassava is becoming popular for

three main reasons: (i) Its yields are the highest with respect to the amount of starch per unit area when compared to other staples; (ii) cassava starch is amenable to diverse food and non-food uses; and (iii) it has a flexible harvesting schedule that is suitable for most rural and subsistence farmers. Also, the high resilience of cassava and its adaptation to climatic changes when other major food staples fail is an attribute for sustainable food security (Burns et al., 2010; Jarvis et al., 2012).

Globally cassava yields have increased by ca. 1.8% per annum over the last decade to 12.8 tons per hectare, resulting into a global cassava harvest of more than 280 million tons in 2012, reflecting a 60% increase since 2000 (FAO, 2013). This output is set to accelerate further due to enhanced recognition of cassava's potential. Therefore, the current and anticipated demands for cassava starch necessitate concerted and systematic efforts tailored to the development of higher yielding cassava varieties (Aerni, 2006; Bull et al., 2011).

Although conventional breeding may still deliver high yielding varieties (Ceballos et al., 2004; 2012), systematic progress in the genetic improvement of cassava is challenged by the heterozygous nature of the crop (Kawano et al., 1978; Meireles da Silva et al., 2003; El-Shakaway 2004; Bull et al., 2011). Similar limitations in maize (*Zea mays*) were overcome by introducing inbreeding and use of inbreds over a century ago (East, 1908; Shull, 1908; 1909; Crow, 1998). Inbred lines (or pure, homozygous lines) have two copies of the same genome, such that after self-pollination (same genotype is both male and female); all the offsprings are identical to the parent. Similarly, double haploids (DHs) (genotypes formed when haploid cells undergo chromosome doubling), eliminate heterozygosity and achieve approximate complete homozygosity in one generation, compared to many generations in conventional inbreeding (Jain et al., 1996; Maluszynski et al., 2003a, b). The success attained in maize hybrid breeding (Crow, 1998) spurred the use of inbred lines or doubled haploids for several other economically important crop species such as cucumber, flax, oil palm, barley, solanaceous crops (Magoon and Khanna, 1963; Maluszynski et al., 2003a, b; Nasertorabi et al., 2012; Nasution et al., 2013; reviewed in Andersen, 2005; Dunwell, 2010; Germanà, 2011).

Based on the fact that cassava is outcrossing and monoecious, utilization of heterozygous parents to generate F_1's for onward evaluation and selection is the norm since the inception of cassava breeding (reviewed in Ceballos et al., 2004; 2012). However, several advantages in using inbred progenitors in cassava have been reported (Ceballos et al., 2012). In maize, genetic homogeneity and inbred lines can be obtained in one generation through use of doubled haploid inducers, which are attained by crossing target lines with a DH inducer line and thereafter selection among progeny individuals that are doubled haploids (Kebede et al., 2011).

The use of DH inducer technology boosted maize breeding as inbred lines are readily tested and used within a shorter time. In contrast, cassava has a long breeding cycle (that is, generation of sexual seeds) of more than a year, followed by five to six years of evaluation and selection to identify best performing genotypes (Kawuki et al., 2011). Therefore, conventional breeding approach has limited genetic precision particularly when responding to emerging threats such as cassava brown streak and cassava mosaic diseases in Africa and thus remains limited to match the emerging demands for cassava resources (Aerni, 2006; Bull et al., 2011; Ceballos et al., 2012).

Doubled haploids (DHs) are a preferred method for developing homozygous lines in a timely fashion (Jain et al., 1996; Maluszynski et al., 2003a, b; Wedzony et al., 2009). DHs can be produced by tissue culture methods that use immature pollen (microspores) or anthers (androgenesis), ovules (gynogenesis), immature embryos from distant crosses and after pollination with irradiated mature pollen to produce a fully homozygous plant in a short time, enabling the production of pure lines from a donor plant within one generation (reviewed in Forster et al., 2007; Wedzony et al., 2009). The outstanding contributions of DH technology have been reported in several crops, especially in *Brassica* spp., maize, barley and wheat (Alison et al., 2011). The possibility to raise DH lines in cassava has just been investigated by Perera et al (2014a, b) focusing on androgenesis, gynogenesis and crosses with castor bean (*Ricinus communis* L.). While all the three methods gave promising results in terms of ability to generate calli, neither of them has so far made a breakthrough to generate cassava plantlets (Perera et al., 2014a, b). Moreover, there is no optimized method to regenerate microspore or anther-derived embryos and plants.

The use of irradiated pollen in combination with embryo culture is an efficient method for induction of haploids in several plants notably plum (*Prunus domestica* L; Peixe et al., 2000), oil pumpkin (*Curcurbita pepo*; Košmrlj et al., 2013) and melon (*Cucumis melo*; Sari et al., 1994; Nasertorabi et al., 2012). In cassava, embryo rescue methods are available (Fregene et al., 1999; Akinbo et al., 2010; Yan et al., 2014), which further motivates the use of the pollen irradiation technology. Recently, considerable progress was made for early embryo rescue (two to three weeks after pollination) in cassava by

*Corresponding author. E-mail: aktugume@cns.mak.ac.ug. Tel: +256772514841, +256704514841 Fax. +256414531061.

cassava crosses (Restrepo, 2014). Taken together, the prevailing challenges in cassava breeding arising from cassava reproductive biology can be overcome using DH technology to facilitate attainment of homozygosity in a short time.

Regardless of the method chosen for doubled haploid production, fast and inexpensive identification of candidate plants and the development of accurate methods or tools chromosomal duplication are critical. Flow cytometry is widely used to evaluate DNA content and determination of the efficiency of haploid-induction (Nasution et al., 2013).

Other tools used in the identification of haploids include molecular markers, especially microsatellites (simple sequence repeats, SSR), due to their stability and co-dominance, allowing the separation of homozygotes from heterozygotes (F_1) (Drumeva et al., 2005; Belicuas et al., 2007).

The aim of this study was to regenerate cassava plantlets from embryos after self-pollination with irradiated cassava pollen of cassava genotype Nase 14. To our knowledge, this is the first attempt of using irradiated pollen to generate plantlets for on-ward development of doubled haploid cassava lines.

MATERIALS AND METHODS

Plant material

A cassava variety, Nase 14, was used in this study. Isolated plots of ca. 1.5 acres (0.60 ha) were established at National Crops Resources Research Institute (NaCRRI), Namulonge, Uganda (~ 200 m away from the nearest cassava plants of another genotype. The variety Nase 14 was selected because it flowers profusely and is highly responsive to tissue culture (*unpublished personal observation*); it is thus likely that its explants or progeny will respond well in tissue culture during downstream applications. Mature male flowers (unopened buds) were harvested from cultivar Nase 14 and subjected to gamma irradiation.

Pollen irradiation

Harvested mature male flowers were irradiated using a Co^{60} gamma ray source (GWGP-80, Cobalt-60, Nuclear Power Unit Institute, China, Installed at Mulago Hospital, Kampala, Uganda). This irradiation was performed in small cardboard boxes (5 × 7 × 2 cm). The irradiation source had a half-life T½ of 5.26 years and the mean energy of 1.2 MV with the maximum source to surface distance of 80 cm. The field size was set using a hand control panel. The treatment couch table was adjusted by moving it vertically, laterally and horizontally to position the samples in the center of the radiation beam.

Based on the fact that this was a pioneering pollen irradiation experiment in cassava, there was no prior knowledge of the most appropriate dosage to be used. Therefore, the most frequently used ranges in other crops were used (Peixe et al., 2000; Nasertorabi et al., 2012), taking keen interest in dosage rates that have been reported to induce haploids in those crops.

Accordingly, mature male flowers were subjected to the irradiation dosages of: 50, 100, 150, 200 and 250 Gy; 1 Gray (Gy) = 100 Rad). This translated into approximately 74, 148, 221, 295 and 369 min of exposure, respectively. At one point, dosages of 300 Gy (443 min) and 500 Gy (738 min) were also used. Non-irradiated male flowers/buds (0 Gy treatments) were included in the experiment as controls.

For each irradiation treatment, ~150 male buds were detached from the plants in the morning (0630 h local time) including buds for the control treatment and placed in (90 × 30) mm Petri dishes. Each irradiation treatment was represented by five Petri dishes, with each Petri dish having ~40 male buds. Only mature male buds (just before anthesis) were irradiated (treatments running from 0800 to 1500 h) and their extracted pollen used for pollination in the evening (1700 to 1830 h). Thus, pollen detachment from mother plants, irradiation and pollination were all done on the same day (within 13 h). Pollination with non-irradiated pollen was also done in the evening, as stated above.

Pollen germination

Pollen was cultured on modified Brewbaker and Kwack (1963) medium containing 100 mg/l boric acid; 300 mg/l calcium nitrate; 200 mg/l magnesium sulfate; 100 mg/l potassium nitrate; and 5% sucrose adjusted to pH 5.8. Petal-like bracts were removed from the male flowers and pollen was brushed into the medium. Thereafter, the pollen was incubated in an oven maintained at 40°C and 100% relative humidity. Aniline blue was used for staining, prepared by dissolving 1 g of aniline blue in 100 ml of 85% ethyl alcohol. Aniline blue stains callose that is located in the inner layer of the pollen tube, and although it is not commonly used to stain pollen from the genus *Manihot;* evidence showed that it is an effective method for observing and studying pollen tube development, pollen morphology, longevity, viability and cross-ability in species of *Manihot* and other genera (Fang, et al., 2015; Vieira et al., 2015). Pollen germination and viability were assessed after 24 h using a sample of 300 pollen grains per treatment. Three replicates were performed per treatment. Pollen was considered to have germinated when pollen tube length was at least equal to or greater than the grain diameter. Pollen viability and germination ability were observed by direct microscopy through an inverted microscope (Nikon Alphaphot-2 YS2, Japan) equipped with a mercury lamp of 100 W and an excitation UV light.

Pollination with irradiated pollen

Female flowers were bagged for one day (with white interwoven a nylon bag that is routinely used in cassava pollinations) to avoid undesirable pollinations. Only bagged and opened (receptive) female flowers were pollinated. Pollination was done by hand by brushing the stigmas with irradiated pollen, and thereafter the pollinated flowers were re-bagged for three to seven days to eliminate contamination by foreign pollen. This is the same protocol used in controlled pollinations in cassava (Kawano, 1980). Control pollinations with non-irradiated pollen were performed at the same time. All pollinations experiments were conducted in the field at NaCRRI as described above. The cassava field was laid out in plots such that each irradiation dosage rate was assigned a separate set of plants/plot onto which the irradiation treatment was only applied. Field records were taken on the total number of pollinations made per dosage rate. Beginning with three days after pollination (DAP), the numbers of surviving fruits (pollinated female flowers) per treatment were recorded up to 42 days, when embryo rescue was done. Records were also taken on seed-set (seeded or seedless fruits), and number of embryos recovered. Five rounds of pollinations with irradiated pollen were undertaken.

In vitro embryo rescue

Fruits were harvested 42 DAP, washed under running tap water, and surface-sterilized for 20 min with 15% sodium hypochlorite (NaOCl) containing two to three drops of Tween 20. Thereafter, the

fruits were rinsed three times with sterile distilled water in a laminar flow hood as previously described (Fregene et al., 1999). Some fruits did not have seeds. Setted seeds were excised from the fruits under sterile conditions in a laminar flow hood and the immature embryos were isolated under a stereomicroscope using sterile forceps.

The immature embryos were each separately cultured in culture tubes containing 15 ml of solid medium (M6) containing modified Murashige and Skoog (MS) medium; 2% sucrose, 1 mg/l GA3, 0.7% agar, adjusted to pH 5.8. This methods was adopted from Catano et al. (1993). Immature embryos were cultured for approximately one month in the growth room with a temperature of 28±2°C for 16 h in the light and 8 h of darkness. Embryos that had experienced reasonable growth (with 1-2-cm long shoots) were transferred to baby jars containing 40 ml of solid medium of MS supplemented with 2% sucrose, vitamins and 0.3% phytagel adjusted to pH 5.8. Subsequently, each plantlet was sub-cultured by putting the single stem node cutting on new medium containing solid MS-medium with 2% sucrose. The generated plantlets were subjected to microsatellite genotyping and ploidy analysis.

Microsatellite genotyping

To ascertain zygosity status of the 62 plantlets generated from the rescued embryos, DNA was extracted using the Qiagen kit (Qiagen, Venlo, The Netherlands), following manufacturer's instructions. The DNA was assayed using 16 genomic simple sequence repeat markers (SSRs; EME425, NS158, NS169, SSRY100, SSRY103, SSRY106, SSRY135, SSRY148, SSRY179, SSRY181, SSRY19, SSRY20, SSRY215, SSRY240, SSRY28 and SSRY59) (Mba et al., 2001). All these markers/loci were heterozygous in the mother genotype (Nase 14), and thus deviations from the original heterozygous state to homozygous state (for the generated plantlets) would indicate attainment of homozygosity. The increased homozygosity attained through inbreeding could be inferred from the plantlets derived from the non-irradiated treatments. Amplifications with SSR primers were performed in 10 µl reactions containing 50 ng of DNA, 1 pmole of each primer, 1x *Taq* DNA Polymerase buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs and 0.375 U *Taq* DNA Polymerase (New England Biolabs Inc.). The PCR cycling parameters were 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, 55-57°C for 1 min, and 72°C for 1 min and a final extension at 72°C for 30 min. Differently-sized amplification products at the different loci and variable fluorescent labels [(NED, 6-FAM, PET and VIC (by manufacturers, MWG-Biotech)] on the forward primer allowed multiplexing of amplicons from the same individual.

For each co-loading set, 1 to 2 µl, depending on amplification efficiency, of the different amplicons were mixed and briefly vortexed. Aliquots of 1 µl of the mixture were added to 9 µl of a master-mix containing Hi-Di formamide and GeneScan 500 LIZ size standard (1 ml of Hi-Di and 12 µl of 500-LIZ). Amplicons were denatured at 95°C for 3 min, and subjected to capillary electrophoresis using the ABI 3730 DNA sequencer (Applied Biosystems) and allele calls made using GENEMAPPER® software version 3.7 (Applied Biosystems). All genotyping was done at the Biosciences Eastern and Central Africa hub in Nairobi, Kenya. The DNA of the heterozygous Nase 14 was used as a control. In addition, genetic relationships among the generated plantlets were calculated using the simple matching Euclidean distance (D_E) followed by cluster analysis using the weighted neighbour-joining algorithm. Relationships were visualised as a dendrogram. This analysis was done using PowerMarker version 3.25 (Liu and Muse, 2005).

Ploidy analysis

Eight-week old plantlets (since initiation onto embryo rescue medium) were obtained from rescued embryos and subjected to ploidy analysis using flow cytometry technique according to Otto (1990). For each plantlet, the fresh innermost part of approximately 25 mg of a young leaf tissue was cut with a sharp razor blade in a glass Petri dish containing 0.5 ml of cold OTTO1 buffer (0.1 M citric acid monohydrate and 0.5% Tween-20). The contents were then passed through a nylon filter of 50 µm pore size. The filtrate was centrifuged at 1000 rpm for 5 min, and the pellet was re-suspended in OTTO I buffer. Thereafter, 400 µl of OTTO II buffer [containing 0.4 M anhydrous Na_2HPO_4, 4 µg/ml of DAPI (4,6-diamidino-2-phenylindole), and 1µl/ml mercaptoethanol] was added and the samples run for analysis. Diploid mother plants of cassava (cv. Nase 14) were used as internal controls for ploidy determination. The channels or peak mean of diploid cassava were used to compute a ratio that could be used to discriminate the ploidy levels of the samples.

RESULTS

Effects of radiation on pollen, fruits, and embryos

Pollen germination done 13 h after irradiation treatments showed that irradiation doses used did not prevent pollen germination. Microscopic observations also indicated that pollen grains germinated across all irradiation doses (Figure 1). However, the frequency of pollen germination was reduced for irradiated pollen as compared to fresh or non-irradiated pollen (Table 1). Indeed, compared to fresh pollen, the 13 h old non-irradiated pollen (0 Gy) had the lowest reduction in pollen germination (24%) while the irradiated pollen registered reductions of up to 67% with 200 Gy irradiation dose (Table 1).

Five sets of pollinations with irradiated pollen were undertaken with the number of pollinated female flowers ranging from 494 (for irradiation dosage of 250 Gy) to 595 pollinations (for dosage of 50 Gy) (Table 1). A total of 660 pollinations were made with non-irradiated pollen (Table 1). Thus, across the five irradiation sets, mean numbers of pollinations made with irradiated pollen were 98.8 (for 250 Gy), 108.2 (for 200 Gy), 112.4 (for 150 Gy), 117.2 (for 100 Gy), 119 (for 50 Gy), and 132 pollinations (for non-irradiated pollen) (Table 1). By 15 DAP, most of the pollinated flowers (fruits) had aborted (Table 1). The highest abortion (89.7%) was observed in flowers pollinated with pollen irradiated with 250 Gy, while the least abortion rate (72.6%) was in flowers pollinated with non-irradiated pollen (Table 1).

At the time of embryo rescue (42 DAP) (Figure 2A-E), less than 10 fruits per irradiation treatment were still surviving following pollination with irradiated pollen (Table 1). Fruit length and width did not differ significantly ($P > 0.05$) across the irradiation doses. However, average fruit length (1.4 cm) and fruit width (1.2 cm) were consistently lower than those recovered from non-irradiated pollen treatments, which were 1.6 and 1.5 cm, respectively (Table 1). Significant differences ($P < 0.05$) were observed in number of fruits, seeds and embryos generated with the non-irradiated pollen treatments having significantly higher numbers (Table 1).

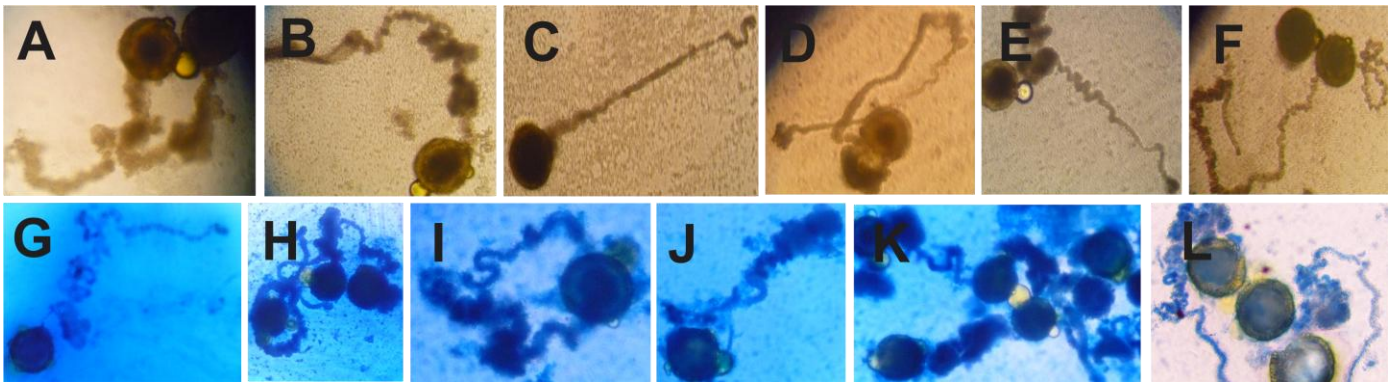

Figure 1. *In vitro* germination of pollen grains of cassava (*M. esculenta* Crantz) cv. Nase 14 after irradiation with various dosages. Pollen tubes (thick thread-like structures) emerging out of the germinating pollen grains (darkened circular structures) are shown. Upper panel (A-F) shows germination without aniline blue staining, while the lower panel (G-L) shows germination with aniline blue staining. The dosage used was 0 Gy (no irradiation, A and G), 50 Gy (B and H), 100 Gy (C and I), 150 Gy (D and J), 200 Gy (E and K), and 250 Gy (F and L). Images for dosage of 300 and 500 Gy are not shown.

Under natural conditions, the cassava pistil is trilocular with a single ovule in each locule, producing a maximum of three seeds from each fruit at maturity (Ogburia et al., 2000). However, for all pollinations undertaken with irradiated pollen, some fruits did not contain seeds while some recovered seeds did not contain embryos. For example, at 42 DAP in 100 Gy treatment, an average of 9.8 (ca. 10) fruits were rescued containing 21.2 (ca. 21) seeds (instead of the expected maximum 30 seeds in a trilocular pistil) from which only 5.4 (ca. 5) embryos were recovered (Table 1). On the other hand, the non-irradiated pollen treatment had on average 26.2 (ca. 26) fruits at 42 DAP from which 64.8 (ca. 65) seeds were recovered and 45.6 (ca. 47) embryos were rescued (Table 1, Figure 2D-F). On average, fruits resulting from pollinations with irradiated pollen had 1.9 seeds per fruit, while those from non-irradiated pollen had 2.5 seeds per fruit (Table 1). For the seeds that had an embryo, one embryo was found for each seed. Two weeks after embryo rescue treatments, the first leaf appeared (Figure 2F) and by eight weeks, a total of 62 cassava plantlets were recovered from embryo rescue procedures (Figure 2H-J).

Ploidy and zygosity analysis

The regenerated 62 plantlets (Figure 2I-J) were subjected to zygosity and ploidy analysis. Each plantlet was considered a different genotype and thus analyzed separately. On calibration using diploid Nase 14 as the internal control, the channel or peak mean was 78.8 (Table 2). Ideally, a haploid plant should have half of this mean (that is, channel mean of 39.4). The ratio of channel means of mother plant, Nase 14, were all above 39.4 (Table 2), indicating that none of the plantlets was haploid.

When analyzed with 16 SSR markers that are heterozygous in the mother plant (Nase 14), none of the 62 plantlets was completely homozygous (Supplementary Table S1). However, varying levels of homozygosity were observed. For instance, the four plantlets resulting from 150 Gy had homozygous loci in a frequency varying between 6.0% (one locus out of 16 loci) and 50.0% (eight loci out of 16 loci), while the four plantlets from 250 Gy had homozygous loci varying between 12.5 and 68.0% (Table 2, Supplementary Table S1). The five plantlets from 0 Gy had percentages of homozygous loci ranging from 43.0 to 68.0% (Table 2). On average the highest homozygosity was observed from plantlets from 0 Gy and the lowest was from plantlets from 200 Gy (Table 2). These results suggest that the plantlets obtained may have originated in self-pollinations rather than through parthenogenesis as it was hoped.

DISCUSSION

This study reports for the first time in the history of cassava breeding the use of irradiated pollen and *in vitro* embryo rescue procedures to regenerate cassava plantlets as candidate materials for the development of doubled haploids. Irradiation dosages 50 to 250 Gy were studied across five pollination events, while dosages 300 and 500 Gy were used in one pollination event. Although irradiation reduced pollen germination (between 30-67%), none of the irradiation dosages was able to completely inhibit pollen germination completely. Non-irradiated 13 hour-old pollen showed a 24% reduction in pollen germination, a finding that has strong implications towards ongoing efforts to address flowering and fruit-set bottlenecks in cassava crossing nurseries. Pollination with irradiated pollen and subsequent embryo rescue resulted in the recovery of 62 diploid cassava plantlets

Table 1. Influence of irradiation dosage on cassava pollen germination, mean number of fruits, seeds, and embryos generated following pollination with irradiated pollen.

Sample Identity	Total no. of pollen grains	No. of pollen grains with pollen tube[a]	Percent reduction in pollen germination	Total pollinations	Mean pollinations	Percent abortion, 15 DAP	Average no. of fruits at 42 DAP	Fruit length (cm)	Fruit width (cm)	No. of seeds	Average no. of seeds per fruit	No. of embryos
Fresh pollen	898	242.3 (80.8)										
Dose 0 Gy	890	184 (61.3)	24.1	660	132.0	72.6	26.2	1.7	1.6	64.8	2.5	45.6
Dose 50 Gy	880	167 (55.7)	31.1	595	119.0	85.0	8.2	1.3	1.1	16.2	2.0	5.6
Dose 100 Gy	815	90.6 (30.2)	62.6	586	117.2	81.1	9.8	1.7	1.3	21.2	2.2	5.4
Dose 150 Gy	520	140.6 (46.9)	42.0	562	112.4	85.4	5.6	1.2	1.0	9.2	1.6	1.8
Dose 200 Gy	161	80.0 (26.7)	67.0	541	108.2	81.8	6.2	1.3	1.2	8.6	1.4	4.0
Dose 250 Gy	852	170.6 (56.9)	29.0	494	98.8	89.7	7.2	1.7	1.5	15.0	2.1	3.4
LSD					134.2	11.8	8.7	0.7	0.6	19.2		11.9
CVs					89.7	38.7	63.8	34.8	35.0	65.5		83.4

[a]Numbers in parentheses indicate percent germination based on counts of 300 pollen grains across replicates. Assessments of number of fruits, fruit length, fruit width, number of seeds and embryos rescued, all done at 42 DAP. Data for dosages 300 and 500 Gy are not presented.

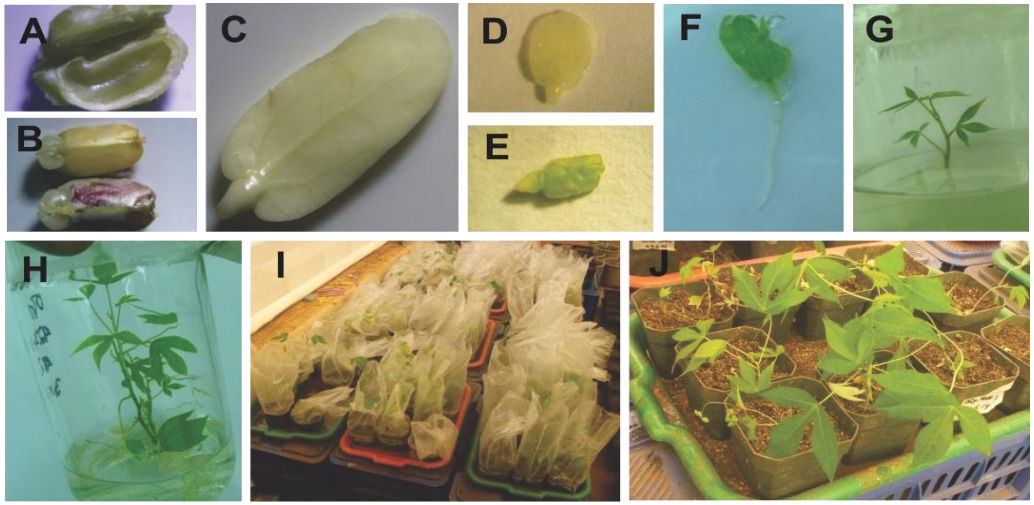

Figure 2. Embryo rescue at 42 days after pollination (DAP) and regeneration of cassava plantlets. A and B: Opened and unopened cassava locules containing one embryo each for extraction; C: Freshly extracted embryo; D, E and F: Embryo germination after three days, 1 week, and 2 weeks of culture, respectively; G and H: cassava plantlets after 4 and 16 weeks of culture, respectively, from the time embryo rescue was done; I and J: cassava plantlets from embryos rescued from irradiated pollinations for three and eight weeks, respectively, of being hardened in the screen house. A total of 62 cassava plantlets were successfully regenerated from the rescue embryos.

Table 2. Zygosity and ploidy analysis of 62 cassava plantlets recovered from embryos that were generated from crosses with irradiated pollen.

Dosage	No. of plants	No. of homozygous loci	Mean (%) homozygous loci	Channel mean	Mean ratio of sample to diploid mother plant
Dose 0 Gy	5	7 to 11	56.2	76.6	0.972
Dose 50 Gy	9	7 to 11	55.0	77.2	0.980
Dose 100 Gy	6	2 to 6	28.1	77.9	0.989
Dose 150 Gy	4	1 to 8	32.8	77.5	0.984
Dose 200 Gy	2	2 to 5	28.1	77.5	0.984
Dose 250 Gy	4	2 to 11	48.4	75.7	0.961
Dose 300 Gy	25	2 to 12	38.0	77.9	0.989
Dose 500 Gy	7	6 to 8	42.8	76.9	0.976
Nase 14[a]	1	16	100.0	78.8	1.000

[a]Cassava mother genotype that was considered under this study. Number of homozygous loci out of the 16 loci that were assayed in each of the samples.

that are currently undergoing *in vitro* growth to allow future field evaluations and characterization.

Successful *in vitro* pollen germination in the family Euphorbiacea is rare with limited success (for example, Orrego and Hershey, 1984; Mbahe et al., 1994; Chavarriaga-Aguirre and Halsey, 2005; Viera et al., 2012); however, efficient pollen germination was achieved in our study using modified Brewbaker and Kwack (1963) medium at pH 5.8. These results annul an earlier hypothesis by Viera et al. (2012) claiming the presence of a specific substance found in the stigma but not in the medium that induces pollen germination, which opens up opportunities to study the impact of irradiation in cassava. The effects of ionizing radiation on pollen germination have been studied in a number of plants (Visser and Oost, 1981; Pooler and Scorza, 1997; Musial and Pryzwara, 1998; Yue and Zou, 2012; Košmrlj et al., 2013). These studies generally concluded that irradiation results in a reduction of pollen germination. Similarly, our study show that pollen germination in cassava is possible up to 500 Gy, although there was a general reduction in the frequency of pollen germination with irradiated pollen. Studies in peach (*Prunus persica*) have shown that pollen germination is still possible at 1500 Gy (Pooler and Scorza 1997). Furthermore, huge differences in pollen germination frequency between non-irradiated pollen (that is, freshly collected pollen and 13 h old pollen) indicate that viability of cassava pollen after shedding drops rapidly, something that is indirectly implied by poor fruit set observed in cassava crossing nurseries (Nassar and Ortiz, 2006). Future experiments will reduce the time between pollen collection and pollination; develop protocols for preserving pollen viability, and to determine a clear dose response. Nonetheless, irradiation may have had an effect on the pollen as revealed by the zygosity patterns of the generated plantlets. In addition, source activity (for example, Gy/min) along with total radiation is an important factor to take into account. Ideally, future studies should use high-activity sources that will require a short time of exposure to achieve the desired and probably higher levels of radiation.

Pollinations with irradiated pollen ranged between 494 (for 250 Gy) to 595 (for 50 Gy), potentially translating to 494 and 595 fruits, respectively. However, by 15 DAP over 80.0% of the fruits had aborted (highest abortion in the 250 Gy treatments). By embryo rescue time at 42 DAP, fewer fruits and seeds were obtained from irradiated treatments as compared to the non-irradiated treatments ($P < 0.05$). Due to the fact that this was the first time to use irradiated pollen in cassava, no direct comparisons can be made. Studies on other plants, notably peach (*Prunus persica*) have reported fruit set of less than 15 % for pollinations with pollen irradiated with 530, 820 and 5000 Gy (Pooler and Scorza, 1997). This suggests that more than 85% fruits or seed loss is observed following pollination with irradiated pollen. In kiwifruit (*Actinidia deliciosa*), the number of abnormal seeds drastically increased in fruits when pollinations with irradiated pollen were performed, that is by 68% for 700 Gy and 74% for 900 Gy (Musial and Pryzwara, 1998). Related studies show that the number of seeds generated by pollination with irradiated pollen is a function of genotype (Nasertorabi et al., 2012), while the frequency of haploids generated using irradiated pollen is a function of the recipient female parent and the pollen donor (Košmrlj et al., 2013). Also, haploid production from the same genotype is not consistent across different pollination events with irradiated pollen (Košmrlj et al., 2013). Our study consisted of only one cassava genotype, Nase 14. This decision was made in order to get an initial insight based on one genotype to guide follow-up studies on the use of irradiated pollen on many other cassava genotypes.

High fruit abortion was observed in all cases resulting from pollination with irradiated pollen, irrespective of low (50 Gy) or five-fold higher doses (250 Gy). Each irradiation

dose was associated with a significantly lower number of seeds that had no embryos. In kiwifruit, seeds resulting from pollination with irradiated pollen at 700 and 900 Gy had 90% of the seeds containing only endosperm and 9.6% seeds containing both endosperm and embryos (Musial and Pryzwara, 1998). On the other hand, in plum (*Prunus domestica*), there was a continuous fruit drop in number from 30 to 70 DAP due to absence or occurrence of fertilization, followed by a rapid rejection of the male genome (Peixe et al., 2000).

Fruit abortion can be a result of rapid collapse of the zygote due to problems related to mitosis in irradiated pollen (Peixe et al., 2000), a phenomenon that could also explain the high abortion rates observed in our study. It is also possible that the aborted cassava fruits had fragile haploid embryos, but this was not determined under this study. However, without systematic efforts to rescue and analyze ploidy and zygosity levels of the embryos, it is rather difficult to make accurate judgments on what is happening *in vivo*. In cassava elite lines HMC-1, SM1219-9 and MCOL1505, natural open pollination by insects showed that the ideal stage for embryo rescue was 32 to 36 DAP (Yan et al., 2014) compared to 42 DAP in our study. Thus, our current efforts are tailored towards optimizing embryo rescue protocols for cassava embryos that are 10-14 DAP, a period associated with high fruit abortion as observed in our study. This similar strategy could be helpful in rescuing cassava embryos from wild crosses, which are also associated with significantly high abortion rates (Restrepo, 2014). These efforts are critical as the lack of normal fertilization process (due to the irradiation of pollen) would prevent the normal development of endosperm which would result in the abortion of the haploid embryos that would otherwise develop.

Ploidy and zygosity analysis of the 62 plantlets rescued from embryos at 42 DAP indicated that none was haploid, suggesting that the irradiated pollen from each of the dosages was able to germinate on stigma, grow within the style, reach the embryo sac, and thereafter fertilize the egg cell. Alternatively, the development of normal diploid zygotes may have originated in pollinations happening after the bags covering the flowers have been removed. It is because of the interest to develop doubled haploids through different approaches that recent studies have demonstrated that stigmas remain viable for up to three days after anthesis (*unpublished data*). This information was not available at the time this current study was conducted. Thus, no parthogenesis was initiated in the recovered plantlets. It is also likely that the irradiation doses partially affected the pollen sperm nuclei but did not affect its capacity to fertilize the egg cell. In kiwifruit, the frequency of abnormal seeds was shown to increase following pollination with irradiated pollen (Musial and Pryzwara, 1998). On the other hand, it is also possible that parthenogenesis may have been induced but the resulting embryos would have failed to develop fully as reflected by the high fruit abortion. In kiwifruit, parthenocarpic fruits were obtained at varied doses, that is, at 200 Gy (hexaploids), 500 to 900 Gy (hexaploid and triploids), and at 1500 Gy (only triploids) with embryo rescue done between 50 and 72 DAP (Musial and Pryzwara, 1998). In cassava, the deployed irradiation doses (50 to 500 Gy) were probably not sufficient. Thus, follow-up studies could consider exploring higher irradiation doses that should be accompanied by optimal protocols for embryo rescue within the first 14 days after pollination. Equally important is the need to have short time requirements for attaining irradiation doses and undertaking subsequent pollinations.

The possibility that some mutations or aberrations occurred in the pollen grains and were transferred to the progeny is implied by the genetic diversity observed among the S_1-inbreds as revealed by heterozygosity data. For example, the S_1-inbreds resulting from pollination with non-irradiated pollen had 56.2% homozygous loci (implying that some 43.8% could potentially be heterozygous). On the other hand, S_1-progeny derived from pollinations with irradiated pollen had an average frequency of homozygous loci between 28.1% (100 and 200 Gy) to 55.0% (50 Gy). This translates to about 71.9 to 45.0% loci being in a heterozygous state. One generation of inbreeding of a completely heterozygous plant is expected to generate 50.0% loci in a homozygous state and the other 50.0% loci in heterozygous state. Indeed, the results from the non-irradiated control approximate this expectation with 56.2% loci being in homozygous state, but not the results of the irradiation treatments (Table 2, Supplementary Table S1). This suggests that new genetic variability may have resulted from radiation-induced mutations; however, we plan to fully characterize these plantlets and to establish whether or not novel traits have been added to the already existing native traits in the mother parent.

Taken together, this study provides evidence that pollen germination in cassava though reduced is not completely inhibited by irradiation dosages of up to 500 Gy, which opens up opportunities to explore the effects of higher irradiation dosages. Despite the high incidences of fruit abortions and seeds without embryos after self-pollination with irradiated pollen, the successful regeneration of 62 diploid cassava plantlets via embryo rescue demonstrates a major advancement towards the development of doubled haploid plants via irradiated pollen for pure line breeding schemes in cassava. As such, the placement of cassava in the current and future agriculture merits adoption of this technology to generate genotypes that match emerging needs. Current follow-up studies are aimed at weaning and hardening of the plantlets to allow field evaluations, self-pollination experiments, implementing these procedures to other genotypes, exploring higher irradiation doses by use of high activity sources, and rescuing embryos at earlier stages of development.

Conflict of interests

The authors did not declare any conflict of interest

ACKNOWLEDGEMENTS

The funds for undertaking this work were provided by the International Centre for Tropical Agriculture (CIAT). We thank personnel of Mulago Hospital, Kampala, Uganda, who assisted with the gamma irradiation. We thank Mr. Moses Mugisha of the International Institute of Tropical Agriculture (IITA-Uganda) for technical assistance in ploidy analysis. We also thank each of the six anonymous reviewers for their helpful comments that improved this manuscript and Professor Silvester Nyakaana (Makerere University) for his critical comments and assistance in the English editing of the manuscript.

REFERENCES

Aerni P (2006). Mobilizing science and technology for development: the case of cassava biotechnology network (CBN). AgBioForum. 9(1): 1-14.

Akinbo O, Labuschagne M, Fregene M (2010). Embryo rescue as a method to develop and multiply a backcross population of cassava (*Manihot esculenta* Crantz) from an interspecific cross of *Manihot esculenta* ssp. Flabellifolia. Afr. J. Biotechnol. 9(42):7058-7062.

Alison M, Ferric R, Möllers C (2011). Haploids and doubled haploids in *Brassica spp* for genetic and genome research. Plant Cell Tiss. Organ Cult. 104(3): 375-386.

Andersen SB (2005). Haploids in the improvement of woody species. In: Haploids in Crop Improvement II, Vol. 56 (Palmer CE, Keller WA, Kasha KJ, eds), pp. 243-257. Heidelberg, Germany: Springer.

Belicuas PR, Guimarães CT, Paiva LV, Duarte JM, Maluf WR, Paiva E (2007). Androgenetic haploids and SSR markers as tools for the development of tropical maize hybrids. Euphytica 156(1-2):95-102.

Brewbaker JL, Kwack BH (1963). The essential role of calcium ion in pollen germination and pollen tube growth. Am. J. Bot. 50(9):747-758.

Bull SE, Ndunguru J, Gruissem W, Vanderschuren H (2011). Cassava: constraints to production and transfer of biotechnology to African laboratories. Plant Cell Rep. 30(5): 779-787.

Burns A, Gleadow R, Cliff J, Zacarias A, Cavagnaro T (2010). Cassava: the drought, war, and farmine crop in a changing world. Sustainability 2(11):3572-35607.

Catano K, Plazas J, Roca WM (1993). Development of methodologies for the isolation and culture of cassava immature pollen and zygotic embryos. In: Roca WM, Thro AM (eds) Proc Ist lilt Sci Meet of the Cassava Biotechnology Network. CIAT, Cartagena de Indias, Columbia, pp. 185-190.

Ceballos H, Hershey C, Becerra-López-Lavalle LA (2012) New approaches to cassava breeding. Plant Breeding Rev. 36:427-504.

Ceballos H, Iglesias CA, Perez JC, Dixon AGO (2004). Cassava breeding: opportunities and challenges. Plant Mol. Biol. 56(4):503-516.

Chavarriaga-Aguirre P, Halsey M (2005). Cassava (*Manihot esculenta* Crantz): Reproductive biology and practices for confinement of experimental field trials. Report prepared for the Program for Biosafety Systems. Washington, DC: Program for Biosafety Systems.

Crow JF (1998). 90 years ago: The beginning of hybrid maize. Genetics 148(3):923-928.

Drumeva M, Berville A, Ivanov P, Nenova N, Encheva J (2005) Molecular investigations on the double haploid origin of sunflower lines (*Helianthus annuus* L.) developed through gamma-induced parthenogenesis. Biotech. Biotechnol. Equip. 19(3):46-50.

Dunwell JM (2010) Haploids in flowering plants: origins and exploitation. Plant Biotechnol. J. 8(4):377-424.

East EM (1908). Inbreeding in corn. Rep. Conn. Agric. Exp. Stn. pp. 419-428

El-Shakawy MA (2004). Cassava biology and physiology. Plant Mol. Biol. 56(4): 481-501.

Fang X, Turner NC, Yan G, Li F, Siddique KHM (2015). Flower numbers, pod production, pollen viability, and pistil function are reduced and flower and pod abortion increased in chickpea (*Cicer arietinum* L.) under terminal drought. J. Exp. Bot. 61(2): 335-345.

FAO (2013). Cassava's huge potential as a 21st century crop. http://www.fao.org/news/story/en/item/176780/icode/ (Accessed on 8th October 2014).

FAOSTAT (2014). Food and Agriculture Organization of the United Nations, Statistics Division. http://faostat3.fao.org/faostat-gateway/go/to/compare/Q/QC/E (Accessed on 8th October 2014).

Forster BP, Heberle-Bors E, Kasha KJ, Touraev A (2007). The resurgence of haploids in higher plants. Trends Plant Sci. 12(8): 368-375.

Fregene M, Ospina JA, Roca W (1999). Recovery of cassava (*Manihot esculenta* Crantz) plants from culture of immature zygotic embryos. Plant Cell Tiss. Organ Cult. 55(1): 39-43.

Germana MA (2011). Gametic embryogenesis and haploid technology as valuable support to plant breeding. Plant Cell Rep. 30(5): 839-857.

Jain SM, Sopary SK, Veilleux RE (1996). *In vitro* haploid production in higher plants, volumes 1-5. Kluwer Academic Publishers, Dordrecht Boston London.

Jarvis A, Ramirez-Villegas J, Campo BVH, Navarro-Racines C (2012). Is cassava the answer to African climate change adaptation? Trop. Plant Biol. 5(1): 9-29.

Kawano K (1980). Cassava. In: Fehr WR, Hadley HH (eds.), Hybridization of Crop Plants. ASA, CSSA. Madison, Wisconsin. pp. 225-233.

Kawano K, Amaya A, Daza P, Rios M (1978). Factors affecting efficiency of hybridization and selection in cassava. Crop Sci. 18(3):373-376.

Kawuki RS, Pariyo A, Amuge T, Nuwamanya E, Ssemakula G, Tumwesigye S, Bua A, Baguma Y, Omongo C, Alicai T, Orone J (2011). A breeding scheme for local adoption of Cassava (*Manihot esculenta* Crantz). J. Plant Breed. Crop Sci. 3(7): 120-130.

Kebede AZ, Dhillon BS, Schipprack W, Araus JL, Banziger M, Semagn K, Alvarado G, Melchinger AE (2011). Effect of source germplasm and season on the *in vivo* haploid induction rate in tropical maize. Euphytica 180(2): 219-226.

Košmrlj K, Murovec J, Bohanec B (2013). Haploid induction in hull-less seed pumpkin through parthenogenesis induced by X-ray irradiated pollen. J. Am. Soc. Hort. Sci. 138(4): 310-316.

Liu K, Muse SV (2005). Power Marker: Integrated analysis environment for genetic marker data. Bioinformatics 21(9): 2128-2129.

Magoon ML, Khanna KR (1963). Haploids. Caryologia 16: 191-234.

Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) (2003a). Doubled Haploid Production in Crop Plants: a Manual. pp. 480. Dordrecht, Netherlands: Kluwer Academic Publishers.

Maluszynski M, Kasha KJ, Szarejko I (2003b). Published double haploid protocols in plant species. In: *Haploid Production in Crop Plants: a Manual* (Maluszynski, M, Kasha KJ, Forster BP, Szarejko I, eds), pp. 309-335. Dordrecht, Netherlands: Kluwer Academic Publishers.

Mba REC, Stephenson P, Edwards K, Melzer S, Nkumbira J, Gullberg U, Apel K, Gale M, Tohme J, Fregene M (2001). Simple sequence repeat (SSR) markers survey of the cassava (*Manihot esculenta* Crantz) genome: towards an SSR-based molecular genetic map of cassava. Theor. Appl. Genet. 102(1): 21-31.

Mbahe RE, Aken'ova ME, Hahn SK (1994). Germination of Cassava (*Manihot esculenta* Crantz) pollen. ISHS Acta Hort.380: 172-177.

Meireles da Silva R, Bandel G, Martins PS (2003). Mating system in an experimental garden composed of cassava (*Manihot esculenta* Crantz) ethnovarieties. Euphytica 134(2): 127-135.

Musial K, Pryzwara L (1998). Influence of irradiated pollen on embryo and endosperm development in kiwifruit. Ann. Bot. 82(2): 747-756.

Nasertorabi M, Madadkhah E, Moghbeli E, Grouh MSH, Soleimani A (2012). Production of haploid lines from parthenogenetic Iranian melon plants obtained of irradiated pollen (*Cucumis melo* L). Int. Res.

J. Appl. Bas. Sci. 3(8): 1585-1589.
Nassar NMA, Ortiz R (2006). Cassava Improvement: Challenges and Impacts. J. Agric. Sci. 145(2):163-171.
Nasution O, Sitorus AC, Nelson SPC, Forster BP, Caligari PDS (2013). A high-throughput flow cytometry method for ploidy determination in oil palm. J. Oil Palm Res. 25(2):265-271.
Ogburia MN, Adachi T, Yabuya T (2000). Ovule dichotomy per locule of the trilocular pistil in Cassava, *Manihot esculenta*: a useful abnormality for increased seed production. Plant Breed. 119(2):191-192.
Orrego JI, Hershey CH (1984) Almacenamiento del polen de yuca (*Manihot esculenta* Crantz) por medio de liofilizacion y varios regimenes de humedad y temperatura. Acta Agronomica 34(1):21-24.
Otto F (1990). DAPI staining of fixed cells for high-resolution flow cytometry of nuclear DNA. Meth. Cell Biol. 33:105-110.
Peixe A, Campus MD, Cavaleiro C, Barroso J, Pias MS (2000). Gamma-irradiated pollen induces the formation of 2n-endosperm and abnormal embryo development in European plum (*Prunus domestica* L, cv "Rainha Claudia Verde"). Scientia Hort. 86(4):267-278.
Perera PIP, Ordoñez OA, Becerra Lopez-Lavalle LA, Dedicova B (2014a). A milestone in the doubled haploid pathway of cassava (*Manihot esculenta* Crantz): cellular and molecular assessment of anther-derived structures. Protoplasma 251(1):233-246.
Perera PIP, Ordoñez OA, Dedicova B, Ortega PEM (2014b). Reprogramming of cassava (*Manihot esculenta*) microspore towards sporophytic development. AoB Plants, 6.
Pooler MR, Scorza R (1997). Irradiation and heat affect peach pollen germination and fertility. HortiScience 32(2):290-291.
Restrepo G (2014). Desarrollo del saco embrionario asociado a la formación del embrión de yuca *in vitro.* Tesis de Grado, Universidad ICESI, Cali. Colombia.
Sari N, Abak K, Pitrat M, Rode JC, Dumas de Vaulx R (1994). Induction of parthenogenetic haploid embryos after pollination by irradiated pollen in watermelon. HortiScience 29(10):1189-1190.
Shull GH (1908). The composition of a field of maize. Am. Breed. Assoc. Rep. 4:296-301.
Shull GH (1909). A pure line methods of corn breeding. Am. Breed. Assoc. Rep. 5: 51-59.
Vieira LJ, Soares TL, Rossi ML, Alves AAC, Santos FAR, Souza FVD (2012). Viability, production and morphology of pollen grains for different species in the genus *Manihot* (Euphorbiaceae). Acta Bot. Brasilica, 26 (2): 350-356.
Vieira LJ, de Santana JRF, Alves AAC, da Silva Ledo CA, Souza VD (2015) Use of aniline blue stain to observing pollen tubes development in different *Manihot* Mill. species. Afr. J. Agric. Sci. 10(15): 185-1809.
Visser T, Oost EH (1981). Pollen and pollination experiments III. The viability of apple and pear as affected by irradiation and storage. Euphytica, 30(1): 65-70.
Wędzony M, Forster BP, Żur I, Golemiec E, Szechyńska-Hebda M, Dubas E, Gotębiowska G (2009). Progress in doubled haploid technology in higher plants. In: Advances in Haploid Production in Higher Plants (Touraev A, Forster BP, Jain SM, eds), pp. 1-34. Heidelberg, Berlin: Springer-Verlag.
Yan H, Lu L, Alzate A, Ceballos H, Hershey C, Chen S, Li K (2014). Fruit, seed and embryo development of different cassava (*Manihot esculenta* Crantz) genotypes and embryo rescue. Afr. J. Biotech. 13(14): 1524-1528.
Yue J, Zou J (2012). Study of radiation effects on upland cotton (*Gossypium hirstum* L.) pollen grain irradiated by ^{60}Co-γ ray. J. Agric. Sci. 4(7):85-94.

Supplementary Table S1: Genotypes of 62 cassava plantlets assayed with 16 heterozygous SSR loci.

Sample Identity	Number of Homozygous Loci	% homozygosity loci
0 GY plant 1	10	62.50
0 GY plant 2	7	43.75
0 GY plant 4	9	56.25
0 GY plant 5	8	50.00
0 GY plant 6	11	68.75
100 Gy plant 1	6	37.50
100 Gy plant 2	2	12.50
100 Gy plant 3	4	25.00
100 Gy plant 4	5	31.25
100 Gy plant 5	4	25.00
100 Gy plant 6	6	37.50
150 Gy plant 1	6	37.50
150 Gy plant 2	6	37.50
150 Gy plant 3	1	6.25
150 Gy plant 4	8	50.00
200 Gy plant 2	2	12.50
200 Gy plant 3	5	31.25
250 Gy plant 1	11	68.75
250 Gy plant 2	2	12.50
250 Gy plant 4	7	43.75
250 Gy plant 5	11	68.75
300 GY plant 1	6	37.50
300 GY plant 10	6	37.50
300 GY plant 11	4	25.00
300 GY plant 12	10	62.50
300 GY plant 13	10	62.50
300 GY plant 14	4	25.00
300 GY plant 15	9	56.25
300 GY plant 16	8	50.00
300 GY plant 17	2	12.50
300 GY plant 18	10	62.50
300 GY plant 19	4	25.00
300 GY plant 2	5	31.25
300 GY plant 20	4	25.00
300 GY plant 21	4	25.00
300 GY plant 22	5	31.25
300 GY plant 23	2	12.50
300 GY plant 24	6	37.50
300 GY plant 25	5	31.25
300 GY plant 3	4	25.00
300 GY plant 4	9	56.25
300 GY plant 5	6	37.50
300 GY plant 6	9	56.25
300 GY plant 7	12	75.00
300 GY plant 8	3	18.75
300 GY plant 9	5	31.25
350 Gy plant 1	3	18.75
50 Gy plant 1	10	62.50
50 Gy plant 2	9	56.25

Supplementary Table S1 Cont.

50 Gy plant 3	9	56.25
50 Gy plant 4	8	50.00
50 Gy plant 5	7	43.75
50 Gy plant 6	8	50.00
50 Gy plant 7	10	62.50
50 Gy plant 8	10	62.50
50 Gy plant 9	9	56.25
500 Gy plant 1	7	43.75
500 Gy plant 2	8	50.00
500 Gy plant 3	6	37.50
500 Gy plant 4	6	37.50
500 Gy plant 5	6	37.50
500 Gy plant 6	8	50.00
500 Gy plant 7	7	43.75