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FRUIT SET AND PLANT REGENERATION IN CASSAVA FOLLOWING INTERSPECIFIC POLLINATION WITH CASTOR BEAN

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ABSTRACT

The increasing demand for cassava (*Manihot esculenta* Crantz) for food and non-food uses in the tropics necessitates that its breeding for increased root productivity be made faster. The characteristic long breeding cycle and heterozygous nature of this crop, pose a major obstacle to its rapid genetic improvement. This study aimed at inter-pollinating cassava with castor bean (*Ricinus communis*), with a purpose of inducing and regenerating cassava doubled haploids (DHs). A total of 3,349 flowers from twelve elite cassava varieties were inter-pollinated with caster bean. A total of 803 fruits were harvested for early embryo rescue and/or ovule culture. Of these, three were dissected to obtain seven unique embryos, while 800 were dissected to obtain 1312 young ovules, all of which were cultured *in vitro*. Overall, 82 (6.25%) of the cultured ovules formed callus that originated from the embryosac region, which is haploid. Four out of seven rescued embryos (57.1%) regenerated into plantlets. Ploidy analyses of 24 samples using flow cytometry revealed that 23 of the analysed samples were diploid. However, one callus sample was anueploid. Only one sample had an exceptionally high level of homozygosity (84.2%). These findings lay a foundation for future research aimed at induction of haploids in cassava.

Key Words: Doubled haploid, embryo rescue, ovule culture, ploidy

RÉSUMÉ

La demande croissante de manioc (*Manihot esculenta* Crantz) à usage alimentaire et non alimentaire dans les tropiques nécessite que sa reproduction pour une productivité accrue des racines soit faite plus rapidement. Le long cycle de reproduction et le caractère hétérozygote de cette plante constituent un obstacle majeur dans la rapidité de son amélioration génétique. Cette étude visait à inter-polliniser

le manioc avec le haricot (*Ricinus communis*), dans le but d'induire et de régénérer le manioc d'haploïdes doublé (HD). Un total de 3 349 fleurs de douze élites variétés de manioc ont été inter-pollinisées avec le haricot. Un total de 803 fruits ont été récoltés pour les embryons prématurés qui etaient sauves et / ou la culture d'ovules . Parmi ceux-ci, trois ont été disséqués pour obtenir sept embryons uniques , tandis que 800 ont été disséqués pour obtenir 1312 jeunes ovules, qui ont tous été cultivés *in vitro* . Un total de 82 (6,25%) des ovules en culture ont formé des cals provenant de la région embryonnaire , qui est haploïde. Quatre parmi sept embryons sauvés (57,1%) se sont régénérés en plantules. Les analyses de ploïdie de 24 échantillons par cytométrie en flux ont révélé que 23 des échantillons analysés étaient diploïdes.Cependant, un échantillon de cals était anueploïde. Un seul échantillon présentait un niveau d'homozygotie exceptionnellement élevé (84,2 %). Ces résultats sont les bases des recherches dans le futur sur la cause des haploïdes dans le manioc.

Mots Clés: haploïde doublé, embryon sauvé, culture d'ovules, ploïdie

INTRODUCTION

Cassava (*Manihot esculenta* Crantz), a member of the family *Euphorbiaceae* (Allem, 2002), is a major source of calories and is rapidly becoming an important crop in economic development in many tropical areas (Prakash, 2013). It grows well under marginal conditions such as in drought-prone and low-fertility soils where few other crops can survive (FAO, 2001); because of its ability to adapt to almost all climatic conditions. Therefore, it is imperative that research efforts should be tailored towards its genetic enhancement so as to improve its productivity in terms of root quantity and quality.

The major drawback of cassava breeding is the length of its breeding cycle (~ 8 years) and its high heterozygosity perpetuated by vegetative reproduction by which it is normally seeded (Wang *et al*., 2014; Ceballos *et al*., 2015). Consequently, production challenges such as diseases and pests, which would be ably addressed *via* breeding still cause enormous yield losses. It is difficult to transfer traits of interest (including those under monogenic inheritance) from one genotype to another because of the high heterozygosity of the crop. Nonetheless, some research progress has been achieved in the control of cassava mosaic disease (CMD) (Hahn *et al*., 1980), CBSD (Kanju *et al*., 2007), enhanced nutritional levels (Stupak, 2008; Nassar and Ortiz, 2010); yield increase (Ortiz, 1992; Nassar and Ortiz, 2010) and drought resistance (Okogbenin *et al*., 2013). However, it is worth noting that this has been attained with enormous difficulty, owing to the high heterozygosity and long breeding cycles of the crop.

Interspecific hybridizations to induce haploids, first exploited in barley (*H. vulgare* × *H. bulbosum)* (Kasha and Kao, 1970), has continued to gain prominence (Ren et al 2017). Thus, herein, we explored its suitability to induce doubled haploid (DH) in cassava, as an approach to overcome its breeding challenges.It can be estimated that, with the use of DHs the breeding cycle can be shortened to at least 3 years (Ceballos *et al*., 2007).

A doubled haploid plant is a haploid plant that has undergone spontaneous or induced chromosome duplication (Germanà, 2011). The value of DH lines in breeding programmes and their role in fixing traits without the routine requirement of multiple generations of selfing cannot be underrated (Begheyn *et al*., 2016). Due to their genetic purity and homozygosity, their use in breeding programmes has been reported to be precise, in that specific hybrids can be attained by design, thus making attainment of genetic gains and enhancement feasible (Chen *et al.,* 2011). Further still, they are significant in basic genetic research, molecular studies, and practical applications

in plant breeding. DHs can improve the efficiency and speed of the usually cumbersome, time-consuming, laborious and sometimes rather inefficient conventional breeding methods. They expedite the breeding process thus leading to an increase in crop yield (Piosik *et al.,* 2016).

Doubled haploids and/or haploids have been known to originate spontaneously in nature or as a result of various induction techniques (Murovec and Bohanec, 2012). Several techniques have been reported to induce haploids and DHs in plants, e.g., wide crossing (Wêdzony *et al*., 2009; Mishra and Goswami, 2014); use of pollen from a haploid inducer line (Murovec and Bohanec, 2012); use of irradiated pollen (Godbole *et al*., 2012); androgenesis (Forster *et al*., 2007) and culture of unfertilised ovules (Mishra and Goswami, 2014). Unfortunately, no DH induction and/or generation has been reported in cassava (Perera *et al*., 2014; Buttibwa *et al*., 2015), a crop that continues to experience old and emerging threats such as pests and diseases (Vanessa *et al*., 2011).

Use of interspecific pollinations and/or wide crosses has been reported as one of the most successful methods for haploid induction in many plant species (Mishra and Goswami, 2014). The fertilisation of polar nuclei and production of functional endosperms can induce the parthenogenic development of haploid embryos (Murovec and Bohanec, 2012), which mature normally and then propagated through seeds, e.g., potato (Rokka, 2009). In other cases (e.g., barley, *Hordeum vulgare*), fertilisation of ovules with distant pollen is followed by paternal chromosome elimination in hybrid embryos (bulbosum method) (Acquaah, 2007). In this case, the endosperms are either absent or poorly developed, leading to early fruit abortions. This therefore, necessitates early embryo rescue and further *in vitro* culture of embryos for onward growth and development (Kasha and Kao, 1970). Successful induction of haploids through wide crosses (induced parthenogenesis) has been reported in some crops; for example, in barley, *Hordeum vulgare* (Berzonsky *et al.,* 2003); lettuce, *Lactuca sativa* L. (Piosik *et al*., 2016); carrot, *Daucus carota* L. (Kielkowska *et al*., 2014); cucumber, *Cucumis sativus* L. (Galazka *et al*., 2013); and wheat, *Triticum aestivum* (Inagaki and Hash, 1998).

No DH cassava has been reported. Therefore, the aim of this study was to inter-pollinate cassava with castor bean (*Ricinus communis*), with a purpose of inducing and regenerating cassava DH lines to be used for breeding.

MATERIALS AND METHODS

Donor plants and castor bean pollen source. Twelve diverse elite cassava varieties were selected as mother plants for this study, due to their good flowering behaviour. Variety-dependent responses have been observed in interspecific pollination studies (Mishra and Goswami, 2014), and thus this panel of diverse lines was included to increase chances of fruit set, DH induction and plant regeneration. Each of the donor plants was established in a crossing nursery at the National Crops Resources Research Institute (NaCRRI), Namulonge in central Uganda in the first growing season. Each variety was represented by 100 plants. Meanwhile, castor bean bulk pollen was sourced from an established field, and occasionally from neighbouring castor fields at Namulonge, Uganda. Castor bean was selected in this study because it is a distant relative of cassava in the family *Euphorbiaceae*. Just like cassava, it is an outcrossing plant with separate sex flowers on the same plant. The male flowers shed copious amounts of pollen and this provides an opportunity for undertaking controlled pollinations (Salihu *et al.,* 2014).

Pollination using castor bean pollen. For each cassava variety, mature female flowers were bagged in muslin bags for 1 to 3 days

prior to anthesis to avoid contamination by pollen from unknown sources. Bagging was done on any inflorescence that had a reasonable number (> 5) of mature female flowers. Freshly opened mature male castor bean flower buds were collected by hand-picking in the morning on the day designated for pollination. Pollination was done only on cassava female flowers that had fully opened flowers (at anthesis). Pollen was applied by gently brushing the castor anthers on the stigmas, using hands. All the pollinated flowers were re-bagged for at least three days to avoid contamination with unwanted pollen.

For comparison purposes, one additional treatment was imposed as control. In this case, for each of the test varieties, mature cassava pollen from freshly opened flowers of the same variety was applied on a few bagged flowers (self-pollinated). A total of twelve rounds of pollination were undertaken with a total of 4,267 flowers pollinated. For each round, field observations and records were made on the total number of pollinations made per variety, the numbers of surviving fruits per inflorescence after 3-42 days after pollination, number of fruits, ovules, seeds and embryos rescued. Records were also taken on any plantlets and callus generated.

***In vitro* and *in vivo* germination of castor bean pollen.** To determine the germination ability and behavior of castor bean pollen, *in vitro* and *in vivo* germination tests were done. *In vitro* germination was investigated using modified Brewbaker and Kwack (1963) medium, with pH adjusted to 5.9 and occasionally solidified with 0.2% agar. Approximately 2-3 ml of the medium was dispensed in small petri dishes (45 x 10 mm). Castor bean pollen grains were distributed in the medium with the aid of a brush. After inoculation, the pollen grains were incubated in darkness at a temperature of 28 and 40 ºC for 24 hours, before counting the germinated pollen grains under a microscope. A pollen grain was scored as germinated if a pollen tube was present. Pollen germination frequencies were established by counting germinated and non-germinated pollen grains in three different views per sample. Out of the 12 rounds of pollination undertaken, germination tests were only conducted for five rounds, since the results were consistently similar. For each test, three petri dishes (three replications) were considered.

A few flower samples of selected cassava varieties were used for *in vivo* pollen germination tests. This was done using aniline blue method modified from Kho and Baer (1968) and Adamus (2010). In this case, two to three cassava flowers, pollinated with castor bean pollen were detached from the plants at one, two and three DAP. Bracts were removed and pistils placed in a fixative containing glacial acetic acid and 96% ethanol (in a ratio of 1:3). Thereafter, these were kept in darkness at 4^0C for at least 3 hr. Fixative was decanted off and 8M NaOH added to macerate the pistils for 4 hr in darkness and at room temperature. The NaOH was decanted off and pistils were washed three times with basic 0.5N buffered potassium phosphate (pH 10-12) for 5 minutes each to neutralise effects of NaOH.

Pistils were then washed thrice with 0.1% aniline blue in buffered potassium phosphate and then stained with basic 0.1% aniline blue solution in buffered potassium phosphate solution (pH 10-12) for 1 hour at 4^0C in darkness. The pistils were removed from the stain, placed in a drop of glycerol on a glass slide, stigmas separated off by cutting and then ovaries dissected to extract ovules. Ovary wall tissues were discarded, a drop of basic 0.1% aniline blue solution added on the ovules and stigmas, covered with a cover slip and then gently squashed. Observations of pollen germination and pollen tube growth were made with a fluorescence microscope and images taken using a camera head (Nikon DS-L3). Pistils pollinated with cassava pollen were used as control.

***In vitro* embryo rescue and ovule culturing.** Surviving fruits after pollination were harvested for *in vitro* embryo rescue. Initially, embryo rescue was undertaken at 42 DAP for the fruits in the first seven rounds of pollination, and then adjusted to 7-14 DAP in the subsequent five rounds. The change was made because embryo rescue at 42 DAP was associated with higher abortion rates (>85% fruit abortion), and, hence the need to reduce it by undertaking the earlier rescue strategy.

For comparison purposes, a few fruits from self-pollinated flowers were harvested. All fruits were taken through surface sterilisation by being washed in soapy tap water (2-3 times), rinsed and immersed in 70% alcohol for 1 minute. Thereafter, the fruits were soaked (while being shaken) twice in 2% sodium hypochlorite (NaOCl) containing 2-3 drops of Tween 20 added as a surfactant for 20 minutes (10 minutes each soaking). The fruits were then rinsed three times with sterile distilled water in a laminar flow hood.

The fruits harvested at 42 DAP were dissected and embryos excised. The embryos were cultured *in vitro* on modified Murashige and Skoog (MS) (1962) (M6 or $^1/_2$MSREm) basal medium in glass jars with radicles pushed down into the medium. The M6 medium contained half MS basal salts, supplemented with 1.0 mg L^{-1} gibberellic acid (GA3), 2% sucrose and 0.2% gelrite or agar as a gelling agent, as described by Huabing *et al*. (2014). Meanwhile, for the fruits at 7-14DAP, ovules were excised and cultured *in vitro* on modified MS (MS2 or MS3) induction medium supplemented with 2 mg L^{-1} 2,4D, 0.5 mg L^{-1} kinetine, 1 mg L^{-1}GA3, 0.2 mg L^{-1} benzylaminopurine (BAP), MS vitamins, 8% sucrose and 0.3% gelrite or 0.6% agar in petri dishes (90 x 14.2 mm). In all cases, the pH of media was adjusted to 5.8 before autoclaving at 121 ºC for 15 minutes.

The immature embryos at 42 DAP were incubated at 28 ± 1ºC under a 12/12 hr (day/night) photoperiod with light supplied by white fluorescent tubes (25 ìmol m-2s-1) in a growth room for four weeks. Plantlets that resulted from these embryos were sub-cultured using basic MS medium containing 4.3g L^{-1} of solid medium of MS salts supplemented with 2% sucrose, 1.0 ml L^{-1} MS vitamins and 0.3% phytagel with pH adjusted to 5.8 in glass jars. Meanwhile, the dishes containing ovules were cultured in darkness (improvised by enclosing the dishes in aluminium foils) at 28 ºC for one month and then sub-cultured until regeneration of callus from embryosac region. In all cases, the regenerated plantlets and some calli were subjected to ploidy and homozygosity analyses.

For curiosity purposes, *in situ* histological analyses were also done on a few ovules in selected cassava varieties to track embryo developmental changes following pollination with castor bean pollen. The ovules were excised from fruits at 7, 14, 21 and 28 days after pollination and then fixed in glacial acetic acid and 96% ethanol (in ratio of 1:3) in falcon tubes, and kept in darkness at 4 ºC for at least 3 hours. The ovules were processed using a tissue processor (Leica TP 1020), embedded in Paraffin wax (Histowax), and then sectioned using a rotary microtome (Leica RM 2235; section thickness: 5 µm). They were then stained with Schiff's reagent and counterstained with Naphthol Blueblack, NBB (5% w/v). Stained sections were mounted using Depex to make permanent slides. Examination of slides was performed under an inverted light microscope and images taken using a camera head (Nikon DS-L3).

Ploidy level and homozygosity analyses. Flow cytometry was performed using Partec GmbH ploidy analyser (Otto-Hahn-Str. 32, D-48161 Münster) to determine the ploidy level of plantlets generated from rescued and cultured embryos, and some calli from cultured ovules following the method described by Dole•el (1995). In brief, approximately 25 mg of sample plus control were chopped with a sharp razor blade in a petri dish containing 0.5 ml of cold OTTO1 buffer (O.1M citric

acid monohydrate and 0.5% v/v of Tween-20). The homogenate was filtered through a 50 µm nylon filter into a cuvette. In each case, the diploid parental cassava lines were used as internal controls. The samples were incubated for about 5 minutes before 1 ml of OTTO II buffer (0.4 M anhydrous Na_2HPO_4, 4 µg ml^{-1} of DAPI (4, 6´-diamidino-2-phenylindole), and 1µl ml^{-1} â-metcaptoethanol) were added. The flow cytometer was adjusted so that the peak representing 2n or 2C DNA in a diploid at first growth (G1) phase of the control was localised at channel 100. The ploidy level of the sample was determined by comparing the relative position of the sample's G1 peak and that of the control. A total of 24 samples together with seven controls were analysed.

For the homozygosity analysis, 31 genomic DNA samples were extracted and assayed. These comprised of: (a) plantlets generated from rescued and cultured embryos (4 samples); (b) calli derived from cultured ovules (20 samples); and (c) mother plants that were used as controls (7 samples). DNA was extracted using the QIAGEN (DNeeasy) plant kit following manufacturers instructions. DNA concentration and quality was determined using a NANODROP 2000 (Thermo SCIENTIFIC, USA) and then analysed on 0.8% agarose gel stained with ethidium bromide. The DNA samples were shipped to the Laboratory of the Government Chemist (LGC) Genomics Ltd, UK for SNP genotyping to ascertain homozygosity. A panel of 34 heterozygous and polymorphic SNPs developed and validated for cassava (Ferguson *et al*., 2011) was used to assay the DNA samples.

Data analysis. Data sets generated in the field and laboratories included number of flowers pollinated, *in vitro* pollen germination rate, fruit set and survival up to harvest, number of seeds or ovules and embryos excised, number of plantlets and calli regenerated, ploidy and homozygosity levels of generated calli and plantlets. From these sets, mean number of flowers, fruits, ovules, seeds, embryos, for each pollination event were computed for 12 rounds of pollination and 12 varieties using Excel 2013 software. For *in vitro* pollen germination, mean numbers and percentages of germinated pollen grains were computed for five rounds of pollen germination tests using Excel 2013. Ploidy comparisons between progeny samples and controls (mother plants) were done by using channel mean and mean ratio of sample to diploid parental lines. The channel and/or peak means of diploid cassava were used to compute ratios that were used to discriminate the ploidy levels of the samples. In this analysis, channel mean for a diploid was set at 100, ploidy level was computed by multiplying the mean ratio of target sample to diploid mother by diploid number of mother used as a control; 2x=2n=36.When channel mean is 50 (or mean ratio is 0.5) it is expected that the target sample is a haploid (Dole•el, 1995; Ochatt, 2006). Percentage homozygosity was simply computed by summing up the number of homozygous loci in each progeny sample and then dividing it by the sum of heterozygous loci in the corresponding mother sample.

RESULTS

Castor bean pollen germination tests. Over 80% pollen germination *in vitro* was observed across the two temperature regimes (Table 1). It was further noticed that castor bean pollen germinates and forms pollen tubes which penetrate through the cassava pistil, up to the nucellar beak, in some cases. Indeed, castor pollen tube penetration into the embryo sac region was observed only in ovules of NASE 18 and NASE 19, at two days after pollination (Fig. 1). In other varieties, pollen tubes penetrated only up to the style and/or nucellar beaks of ovules.

Fruit and seed set. A total of 4,267 flowers were pollinated; of these 3,349 flowers were

TABLE 1. *In vitro* germination of castor bean pollen at different temperatures

Round of germination test	Temperature of culture (^{0}C)	Number of pollen grains in a view		Germination (%)
		Total	Germinated	
1	40	98	65	66.3
2	28	82	80	97.6
2	40	91	87	95.6
3	28	70	58	82.9
4	28	90	83	92.2
4	40	104	93	89.4
5	28	131	124	94.7
5	40	146	129	88.4

Each value is an average obtained from 2-3 plates after taking three views per plate

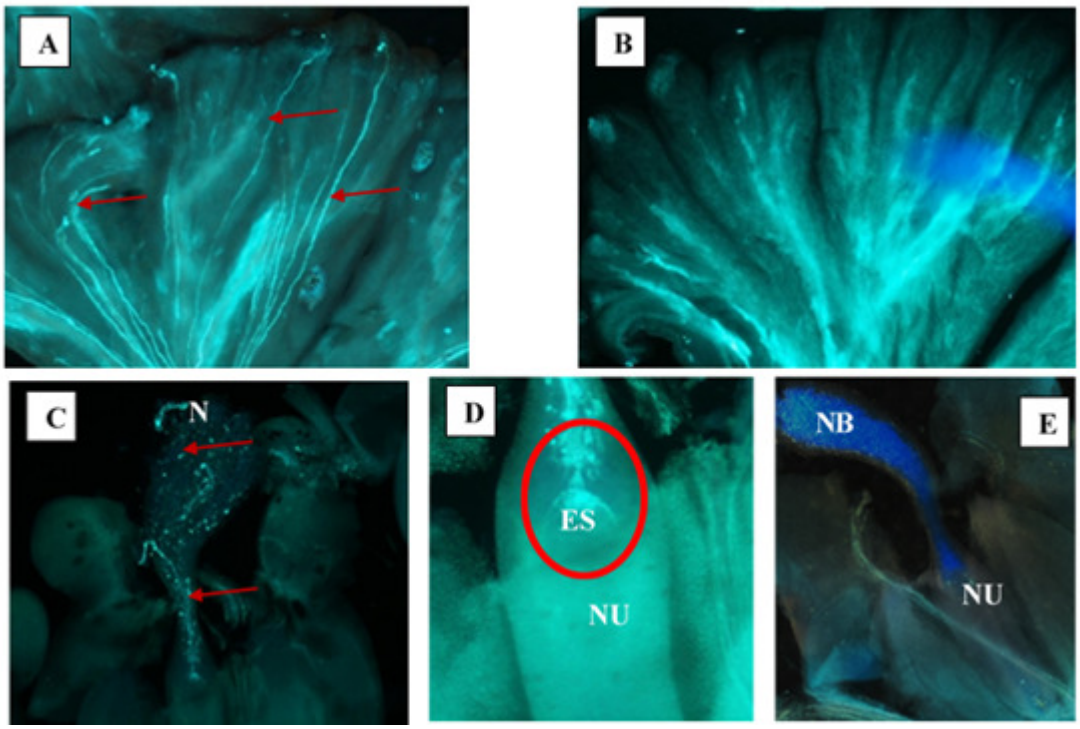

Figure 1. *In vivo* castor pollen germination on cassava pistil at two days after pollination: Red arrows point at pollen tubes; A)-pollen tubes in stigma and style tissue (x40); B)-tissue of unpollinated stigma; C)-pollen tubes in nucellar beak (NB) (x40); D)-pollen penetration into embryo sac (ES) and fertilisation (x100); E)-nucellus (NU) of an unfertilised ovule (x40). Castor pollen tube penetration into embryo sac was observed once in NASE 18 and NASE 19.

inter-pollinated using castor bean pollen, while 918 were self-pollinated (Table 2). The fruits harvested were few in all cases, with 19.81 and 33.23%, respectively, from interspecific and self-pollinations. Following pollination with castor bean, low fruit survival was observed. For example, at 42 DAP only three fruits (0.21%) were harvested compared to 158 fruits harvested from self-pollinated cassava flowers. For pollination rounds 8-12 during which fruits were harvested at 7-12 DAP, 800 (41.07%) fruits were harvested from flowers of interspecific pollination (Table 2).

The number of flowers pollinated per fruits, seeds, ovules embryos rescued per variety, and plantlets generated are presented in Table 3. The number of fruits that survived by harvest time varied between varieties. For example, at 42 DAP only 3 fruits were harvested, one from each of the three varieties (52TME14, NASE 15 and NASE 18). This was because many flowers aborted following pollination with castor bean. These were generated after undertaking 1401 interspecific pollinations using castor bean pollen, from which seven unique embryos were rescued (rounds 1-7). Out of these, only four subsequently developed into unique plants. Cassava varieties NASE 19, NASE 16, NASE 13 and NASE 12 had all their fruits aborted by 42DAP. However, at 7-14 DAP 800 young fruits were harvested and 1312 ovules excised for early ovule culture, from which 82 developed calli from the embryo sac region (Fig. 2).

Time in days after pollination (DAP) influenced fruit survival following pollination of cassava flowers with castor bean pollen. The number of surviving fruits decreased drastically, and by 17 DAP over 90% fruits had aborted. NASE 14 had the highest number of pollinated flowers (801), and Nase12 had the least (44), but in either case, no fruit survived up to 42DAP, not until the harvest period for rescue was revised to 7-14 DAP.

TABLE 2. Fruit set following interspecific pollination of cassava with castor bean

Round	Castor pollen	Cassava pollen	Total flowers	Fruit set (xCP)	Fruit set (xCassava)	% fruit set (xCP)	% fruit set (xCassava)
1	272	66	338	0	28	0.0	42.4
2	269	33	302	0	15	0.0	45.5
3	359	40	399	3	18	0.8	45.0
4	161	169	330	0	52	0.0	59.8
5	164	98	262	0	32	0.0	32.7
6	150	64	214	0	12	0.0	18.8
7	26	17	43	0	01	0.0	5.9
8	460	131	591	230	69	50.0	52.7
9	534	120	654	99	10	18.5	8.3
10	153	19	172	106	5	69.3	26.3
11	583	49	632	238	16	40.8	32.7
12	218	112	330	127	32	58.3	28.6
Total	3349	918	4267	803	290		
Mean	279.08	76.50	355.58	66.92	24.17	19.81	33.23

In rounds 1-7 fruits were harvested at 42 DAP for embryo rescue, but due to high fruit abortion rates, harvest period was revised to 7-14 DAP for ovule culture in round 8-12.xCP = pollination with castor bean pollen, xCassava = pollination with cassava pollen (self-pollination), % = percentage. Percentage fruit set was computed as (number of fruits at harvest/number of flowers pollinated) x 100

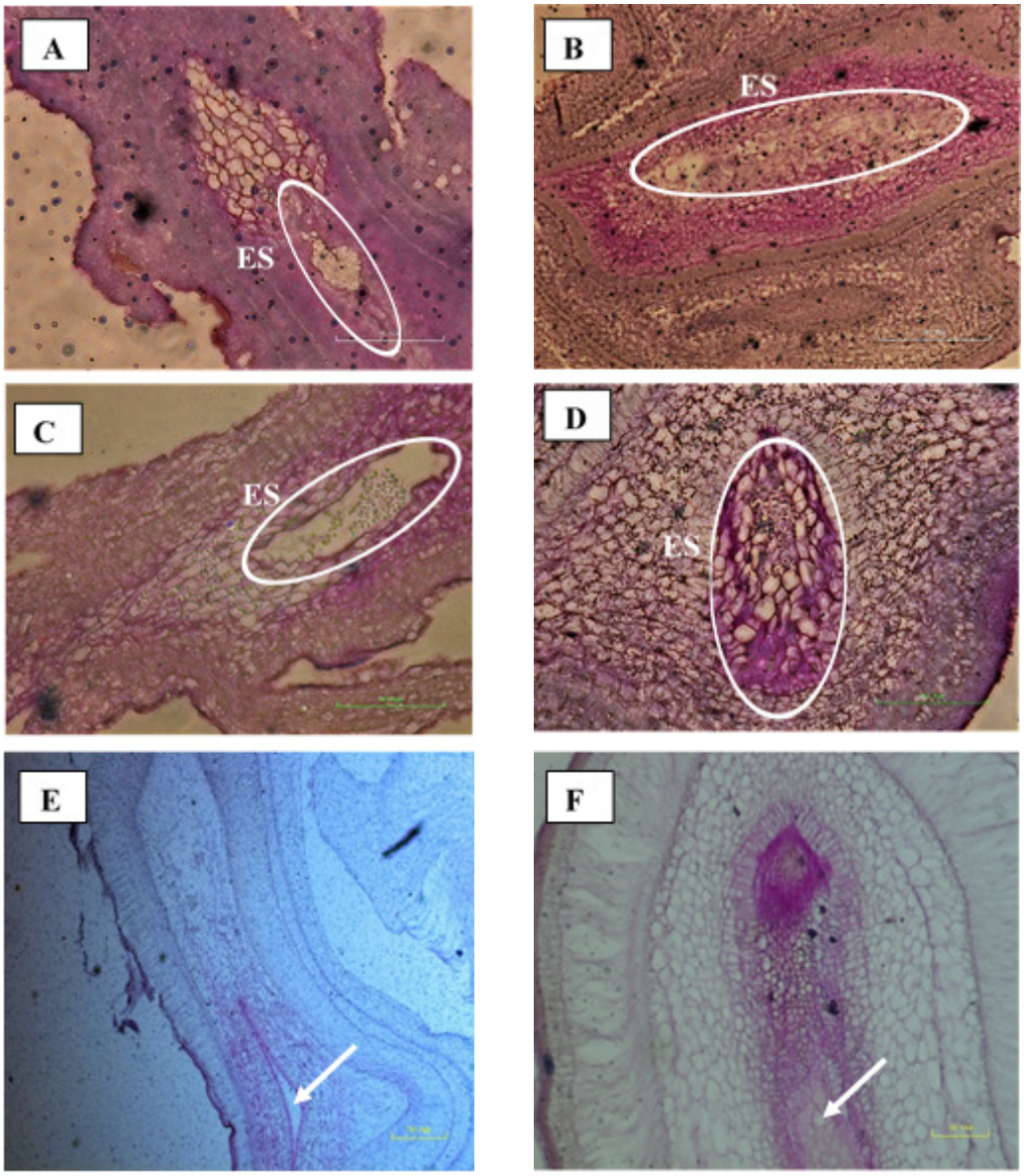

Figure 2. Comparison of developmental stages in inter-pollinated and self-pollinated cassava flowers: (A) ovule at 7 DAP in a flower pollinated with castor bean pollen showing a degenerating egg apparatus in embryo sac; (B) ovule at 7 DAP in a self-pollinated flower shows cell proliferation in embryo sac; (C) ovule at 14 DAP in a flower pollinated with castor bean pollen showing embryo sac filled with starch grains; (D) ovule at 14 DAP in a self-pollinated flower showing continued cell proliferation; (E) ovule at 28 DAP in a flower pollinated with castor bean pollen shows degenerated embryo sac and surrounding tissues (white arrow); (F) ovule at 28 DAP in a self-pollinated flower shows embryo and surrounding tissues developing (white arrow). ES = embryo sac.

A few varieties (NASE 14, NASE 16, NASE 18, NASE 19 and NAROCASS 1) in which self-pollination was done were selected for comparison purposes (Table 3). It was also noted that fruit abortion occurred as days after pollination progressed, but the extent of fruit survival was higher by 42DAP (Table 3). For example, fruit survival for self- and castor-pollinated flowers was 26% and 9% in NASE 18 and 83% and 23% in NAROCASS 1 respectively. Overall, self-pollinated flowers resulted in better fruit survival than those pollinated with castor bean pollen.

Histological examinations done on selected ovules revealed accelerated degeneration of embryo sac after 14DAP in ovules of flowers pollinated with castor bean pollen (Fig. 2). This is manifested in the presence of many empty ovules with disorganised tissues. However, in the self-pollinated flowers there was evidence of normal embryo development (Fig. 2).

Ploidy and homozygosity analysis. Ploidy analyses by flow cytometry revealed that 23 of the 24 samples analysed were diploids just as the controls (Table 4). They produced one peak in G1 phase, except callus derived from an inter-pollination between NASE 3 and castor bean (sample C22), which produced a peak at channel 74 relative to the control peak at channel 99 (Fig. 3). It is very likely that this sample was not haploid, but rather an aneuploid.

For homozygosity analysis, we limited our comparisons by focusing on heterozygous alleles in the mother and progeny samples. Among plantlets the lowest was 10% in NASE 18 (sample C25) and the highest being 84.2% in the plantlet of 52TME14 (sample C27). Meanwhile, in most calli no increase in homozygosity was noted (0%), i.e., the level of heterozygosity was similar to that of the mother samples, the highest was 43.5% in TME 204 (sample C2).

DISCUSSION

Castor bean pollen germination. This study reports successful *in vitro* and *in vivo* germination tests on castor bean pollen, with the latter being reported for the first time in cassava. Efficient *in vitro* pollen germination was confirmed at both 28 and 40°C (Table 1). Copstein *et al*. (2015) obtained over 80% pollen germination rate between 20 and 25°C. Similarly, Diamantino *et al*., (2016) obtained germination rates of up to 90.37% in a medium containing 150 g L^{-1} of sucrose across 15 varieties of castor bean. These findings are consistent with the findings of this study which revealed castor pollen germination rates of up to 97.6% at 28°C (Table 1). On the other hand, our results are not congruent with the findings of Vargas (2006) who obtained low percentages of *in vitro* pollen germination ranging between 0.4 to 0.82%. This notwithstanding, *in vitro* pollen germination in other species of the same family such as cultivated cassava (*Manihot esculenta* Crantz) has been reported to be low and sometimes zero (Vieira *et al*., 2012a and b).

Pollen grains of all the castor bean species used in this study also germinated *in vivo* on stigmas of cassava within one day after pollination. Long and well-developed pollen tubes penetrated the cassava styles (Fig. 1) in all the varieties used. These results confirm that the castor bean pollen used in this study was viable. Relatedly, in lettuce (*Lactuca sativa* L.) *in vivo* pollen germination was also observed following distant pollination with *Helianthus annuus* L. or *H. tuberosus* L. (£ukasz, 2012; Piosik *et al.*, 2016). In a study on intergeneric cross-ability, pollen of poplar, *Populus* species, was seen to germinate on stigmas of willow, *Salix viminalis* and some pollen tubes were observed growing into the ovary (Zenkteler *et al*., 2005). It is apparent from literature and this study that castor bean

TABLE 3. Cassava fruits, ovules, embryos rescued and calli generated from different varieties after interspecific and self-pollination in cassava

Variety	No. of flowers pollinated		Number of fruits at harvest		% fruit survival		Ovules (xCP)	Embryos (xCP)	Callus (xCP)	Plantlets (xCP)
	xCP	xCassava	xCP	xCassava	xCP	xCassava				
52TME14[1]	87	-	01	-	1.15	-	03	03	-	01
NASE 15[1]	215	-	01	-	0.47	-	03	02	-	01
NASE 18[1]	342	174	31	46	9.06	26.44	06	02	02	02
NASE 19	144	51	13	22	9.03	43.14	27	-	-	-
NASE 16	504	167	03	52	0.60	31.14	09	-	-	-
NASE 3	474	154	302	61	63.71	39.61	553	-	34	-
NASE 14	801	211	222	40	27.71	18.96	394	-	35	-
NASE 13	47	19	10	02	21.28	10.53	13	-	-	-
NASE 12	44	30	28	23	63.63	76.67	34	-	-	-
NASE 4	405	57	115	05	28.40	8.77	126	-	01	-
TME 204	163	37	39	13	23.93	35.14	73	-	05	-
NAROCASS 1	163	18	38	15	23.31	83.33	74	-	05	-
Total	3349	918	803	279			1315	07	82	04
Mean	282.4	91.8	66.9	27.9	22.7	37.4	109.6	2.3	13.7	1.3

Inter-pollinations were made on test cassava varieties across the twelve rounds of pollination. For comparison purposes a few self-pollinations were also made among the test cassava varieties; only fruit counts were recorded at the time of harvest and a few fruits were harvested. [1]Embryos from these varieties were obtained from fruits harvested at 42DAP. The calli developed from ovules excised from fruits harvested at 7-14DAP and only those that developed from the embryo sac regions were recorded. In this table: xCP = pollination with castor bean pollen, xCassava = pollination with cassava pollen (self-pollination), %= percentage. Percentage fruit survival was computed as (number of fruits at harvest/number of flowers pollinated) x 100.

TABLE 4. Ploidy and homozygosity levels in callus and plantlets obtained from interspecific pollination of cassava with castor bean

Sample ID of DNA	Variety mean	Source	Channel sample to	%CV	Ratio of level diploid mother	Ploidy	No. of heterozygous loci		No. of homozygous loci in progeny	Percentage homozygosity in progeny
							Mother sample	Progeny sample		
C1	TME 204	Callus	98.19	5.86	0.999	2x	23	20	3	13.0
C2	TME 204	Callus	97.79	8.95	0.995	2x	23	13	10	43.5
C7	NASE 3	Callus	98.02	4.85	0.981	2x	21	20	1	4.8
C8	NASE 3	Callus	100.01	5.75	1.001	2x	21	21	0	0.0
C9	NASE 3	Callus	98.88	4.80	0.990	2x	21	21	0	0.0
C10	NASE 3	Callus	99.62	5.77	0.997	2x	20	19	1	5.0
C11	NASE 3	Callus	99.07	3.79	0.992	2x	21	20	1	4.8
C12	NASE 3	Callus	99.43	5.28	0.995	2x	20	18	2	10.0
C13	NASE 14	Callus	99.72	6.27	1.021	2x	10	10	0	0.0
C14	NASE 3	Callus	98.00	4.85	0.981	2x	20	20	0	0.0
C15	NASE 3	Callus	98.56	3.80	0.986	2x	17	17	0	0.0
C16	NASE 19	Callus	100.30	5.73	1.023	2x	11	9	2	18.2
C17	NASE 14	Callus	98.89	6.42	0.992	2x	10	9	1	10.0
C18	NASE 7	Callus	96.10	9.62	1.00	2x	16	16	0	0.0
C19	NASE 3	Callus	98.96	4.80	0.990	2x	21	21	0	0.0
C20	NASE 3	Callus	99.39	4.78	0.995	2x	21	21	0	0.0
C21	NASE 3	Callus	99.99	4.75	1.001	2x	21	21	0	0.0
C22	NASE 3	Callus	74.78	5.01	0.748	1.5x	21	21	0	0.0
C23	NASE 3	Callus	98.01	4.84	0.981	2x	18	18	0	0.0
C24	NASE 3	Callus	98.50	6.34	0.986	2x	8	8	0	0.0
C25	NASE 18	Leaf lobes	99.58	5.27	0.998	2x	8	7	1	12.5
C26	NASE 18	Leaf lobes	99.83	4.76	1.000	2x	9	7	2	22.2
C27	52TME14	Leaf lobes	103.19	5.09	1.032	2x	19	3	16	84.2
C28	NASE 15	Leaf lobes	99.75	5.26	1.016	2x	11	8	3	27.3
Mean							17.13	15.3	1.7	10.6

Channel mean for a diploid was set at 100; ploidy level was computed by multiplying the mean ratio of target sample to diploid mother by diploid number of mother used as a control; 2x=2n=36. Sample C22 with ploidy level 1.5x is suspected to be an aneuploid. Percentage homozygosity was computed as (number of homozygous loci in progeny/number of corresponding heterozygous loci in mother sample) x 100. Genotyping was done using 34 SNPs. CV=coefficient of variation, % = percentage.

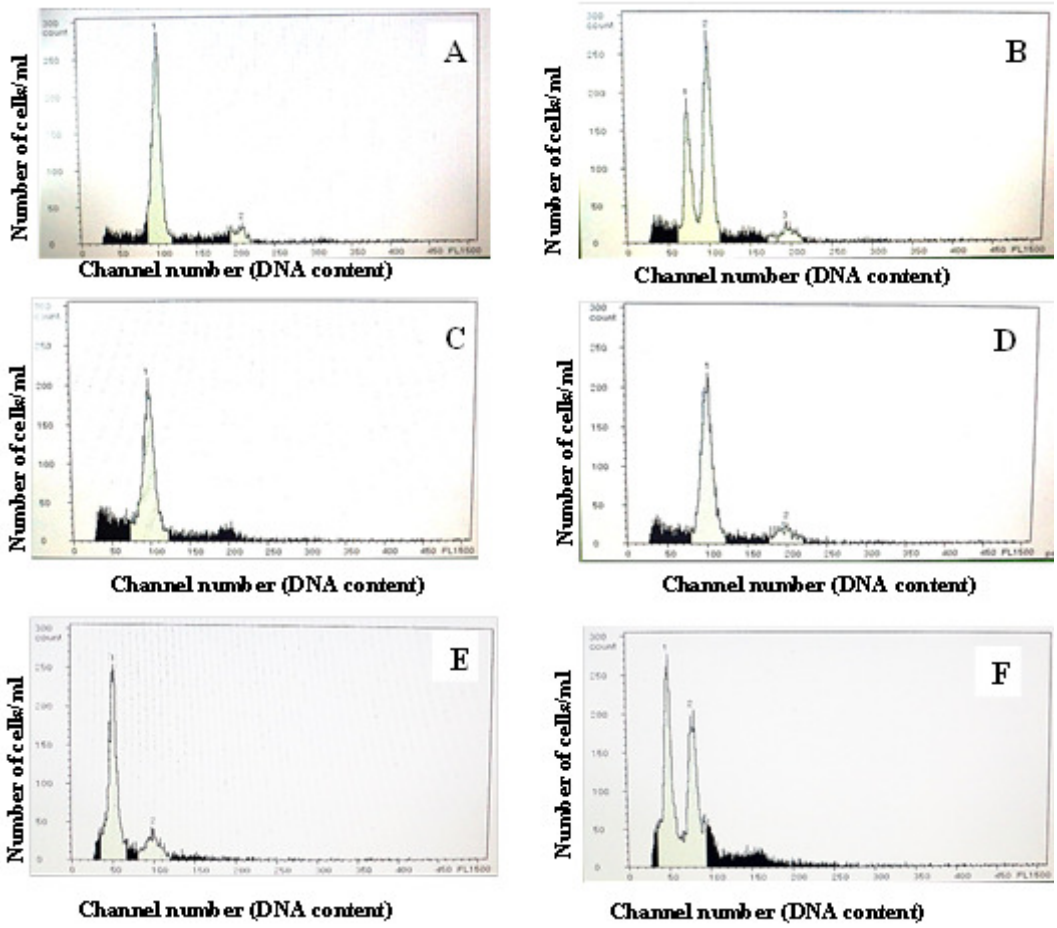

Figure 3. The channel number (distribution of DNA content) of nuclei isolated from selected cassava regenerants following pollination with castor bean pollen: (A) NASE 3 diploid mother sample (*2n=36*) used as a standard, peak 1 represents *G1* nuclei with *2C* DNA and peak 2 represents *G2* nuclei with *4C* DNA. (B) sample C22 from callus mixed with NASE 3 as a standard, peak 1is at channel number 74.78 with ratio of 0.748 corresponding to DNA aneuploidy (*1.5C* DNA content); peak 2 (of NASE 3 diploid mother) represents *G1* DNA, while peak 3 represents *G2* DNA; (C)-NASE 14 diploid mother (leaf lobes); (D) NASE 14xCP (callus) with mother sample; (E)- castor bean mother plant, its *2n*DNA content in a diploid at *G1* phase is at channel 50; (F) castor bean mother sample (peak 1) mixed with NASE 18 mother sample (peak 2). A peak at channel 50 would represent *1n* or *1C* DNA content in a haploid.

pollen germinates on a number of species, but to varying levels owing to intrinsic differences such as physical and chemical nature of exine wall, morphology and viability of the pollen among species.

A key step in undertaking interspecific pollinations is the ability of the alien pollen to germinate, and where possible penetrate the embryosac. In the present study, castor pollen was able to germinate on the cassava stigma. The observed penetration of castor bean pollen tube in to the cassava embryo sac (Fig. 1) (only in two sampled flowers out of 3,349 inter-pollinated flowers) indicates probable fertilisation and/or hybridisation of cassava. Several factors such as meiotic pairing behaviour of cassava and castor bean are likely to explain pollen tube penetration but this but these merit further studies.

Fruit and seed set. The observed reduction in fruit and seed set, following pollination with castor bean pollen (Table 2) is mainly attributed to the incompatibility of gametes, resulting in failure of fertilisation to produce viable zygotes and/or failure of normal endosperm development. Since the *in vivo* pollen germination results obtained in this study confirmed castor pollen germination on cassava stigma, the high abortion rates could further be attributed to post-pollination and/or post-fertilisation barriers probably associated with abnormal development of endosperm or endosperm incompatibility and early inhibition of embryo development. The revelations from histological examinations done in this study (Fig. 2), confirm this attribution. It is also possible that the aborted cassava fruits had fragile haploid embryos, but this attribute was not measured under this study.

These results corroborate the findings of many of the studies in which interspecific pollinations or wide crossing have been done. Classic examples include: crosses between cultivated barley (*Hordeum vulgare* L.) and uncultivated type, *H. bulbosum* (Kasha and Kao, 1970). In that study seeds set from the pollinations aborted in about ten days after induction and only five out forty (12.5%) developed to form plantlets through embryo rescue and culture methods. So, owing to the distant relationship between cassava (*n =18*) and castor (*n =10*), it can be hypothesized that castor does not readily cross with cassava, rather its pollen germinates on the cassava stigma and induces parthenogenic embryo development. So, it is likely that the seven unique embryos obtained in this study from the three fruits at 42 DAP (Table 2) resulted from parthenogenic induction following germination of castor bean pollen on cassava stigmas. This is probably true, since the generated plantlets were morphologically similar to cassava plants. Although not confirmed at DNA level, this rules out the possibility of hybridisation. According to the "wound-hormone or necrohormone theory", an egg cell can be stimulated to develop by necrohormones emitted from dying cells or tissues in the vicinity (Asker and Jerling, 1992). In line with this theory, it can be assumed that the rescued embryos in this study developed as a result of a similar stimulation since no endosperm tissue is believed to have been formed, the consequence of which was death or degeneration of some embryo sac cells.

Results of this study corroborate with those of Gedil *et al*., (2009), who also obtained intergeneric hybrids between cassava and castor bean, however, analysis using molecular markers revealed that the hybrids contained cassava DNA, but not castor bean DNA. In other crops, crosses of carrot (*Daucus carota* L.) x parsley (*Petroselinum crispum*) haploids and embryo-derived homozygotic diploids were obtained (Kielkowska *et al*., 2014). Similarly, haploids were obtained in Lettuce (*Lactuca sativa* L.) x *Helianthus annuus* L. or *H. tuberosus* L. (Piosik *et al*., 2016). On the other hand, cross-incompatibility in interspecific and intergeneric hybridisations has hampered the development of hybrids in Brassicaceae crops (Kaneko and Bang, 2014).

The improvement in the percentage fruit survival in round 8 to 12 observed in this study (Table 2) can be attributed to reduced fruit abscission since fruit harvest (for early ovule culture) was done not later than 14 DAP. Owing to the fact that this was one of the few pioneer studies to inter-pollinate cassava with castor bean, there are no obvious comparisons to make since many experiments are still on-going such as at National Crops Resources Research Institute, Namulonge (NaCRRI) in Uganda and the International Centre for Tropical Agriculture (CIAT) in Colombia. In comparison with related studies on other plants such as bread wheat (*T. aestivum* L.) hybridised with pearl millet (*Pennisetum glaucum* L.), seed development percentage was 79.8, (Inagaki and Hash, 1998). In interspecific crosses between auto-tetraploid cultivated barley (*Hordeum vulgare* L.) and tetraploid *H. bulbosum,* the percentage haploid seed set obtained was 51.5% (Kasha and Kao, 1970) and embryo development was 24.29% when carrot (*Daucus carota* L.) was pollinated with parsley pollen (Kielkowska *et al*., 2014). Clearly, this shows that fruit set and/or haploid induction varies considerably between species, cassava inclusive as observed in the current study.

The calli regenerated from ovules are still under observation at NaCRRI for the possibility of embryogenesis and/or plant regeneration. At the time of writing this report, no embryo and/or plantlet had been regenerated. However, one callus line had developed root-like structures (Fig. 4). Failure of embryo and plant regeneration from the calli could be attributed to the general slow response of cassava or the effect of several media components on the regeneration (Szabados *et*

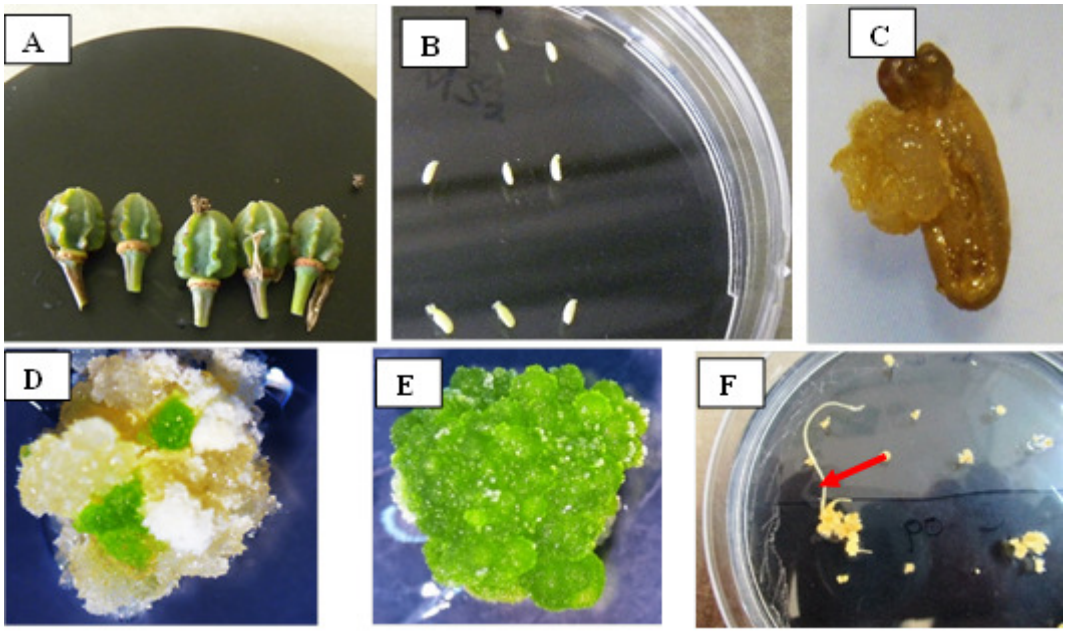

Figure 4. Callus regeneration and its progress following early culture of ovules from fruits of cassava inter-pollinated with castor bean at 12DAP: (A)-young fruits at 12DAP; (B)-excised ovules on modified MS; (C)-protruding callus from embryo sac region at after five months of culture on MS3 medium; (D)-greening callus at about 6 months of culture on MS3 medium; (E)- callus at 63 days on modified MS for regeneration; (F)-callus developing root-like structures (red arrow) at about three months on regeneration medium. No shoots had formed at the time of compiling this report.

al., 1987) or probably the calli initially isolated were not friable and embryogenic.

Ploidy and homozygosity. Ploidy analysis of the 4 plantlets rescued from embryos at 42 DAP indicated that none was haploid (Table 4) suggesting the development of normal diploid zygotes following pollination with castor bean pollen. However, analysis of 20 callus lines from ovules at 7-14 DAP revealed an aneuploid; while the majority were diploids (Fig. 3). These results suggest that parthenogenesis, was induced, but the resulting diploid embryos could have been due to spontaneous doubling of chromosomes in the induced embryo from the egg cell, or from fusion of two haploid cells in the embryo sac (either the synergids, or the antipodals, or the polar nuclei). Relatedly, in lettuce, *Lactuca sativa,* distant pollination technique was successfully used to induce haploid embryos, using pollen of *Helianthus* species and the ploidy level of the regenerated plants was easily detected by flow cytometry (Piosik *et al.,* 2016). Intergeneric hybrids were also obtained in a cross of *Salix aegyptica* and *Populus caspica (*Ahmadi *et al*., 2010).

The aneuploid sample observed in one of the callus sample C22, points to an assumption of hybridisation between cassava and castor bean, followed by elimination of some chromosomes or the unbalanced pairing of chromosomes at fertilisation given the fact that in cassava *n=18* and in castor bean *n=10.* Gedil *et al.* (2009) reported that the relative amount of nuclear DNA in castor bean is considerably less than that of cassava; a trend which was confirmed by the results of their experiment in which repeated measures showed the mean channel number for castor to be slightly less than half that of cassava (23 vs 50). Aneuploids were also obtained in an intergeneric cross involving pollination of F_1 interspecific cotton hybrids (*Gossypium barbadense* × *Gossypium. hirsutum*) with pollen from *Abelmoschus esculentus* (Kantartzi and Roupakias, 2008).

The diploid calli could have originated from the somatic cells of the ovule integuments and/ or nucellus tissue, despite the fact that care was taken to isolate callus that emerged only from the embryo sac regions of the ovules. The calli with *4C* DNA content must have contained cells at the second growth *(G2)* phase of the cell cycle. On the other hand, the observed deviations in peak positions (Fig. 3) could be attributed to instrument instability as well as due to variation in sample preparation and the intrinsic differences in DNA content. This is a finding that is consistent with previous studies (Dolezel *et al.,* 1995; ; Bohanec 2003). Through *in vitro* cultures, haploid calli and consequently haploid plants of *L. sativa* were regenerated following distant crossing with pollen of *Helianthus* species (Piosik *et al*., 2016). In this study however, all calli generated were diploid, except for one aneuploidy. Further, regeneration of plants from these calli was not successful.

The diploid nature and increased homozygosity revealed by SNP genotyping in some samples (Table 4), confirms doubled chromosome numbers, since all the loci had paired alleles. Since no fertilisation by castor pollen can be assumed to have occurred, it is likely that automictic parthenogenesis (automixis) occurred. Mogie (1986) defines automixis as a process in which a new individual is formed from a product or products of a single meiotically dividing cell. In this case, the diploid chromosome number may have been spontaneously restored by a mutation process which involved fusion of two haploid nuclei, or formation of a restitution nucleus or endomitosis as described in Asker and Jerling (1992).

Relatedly, in maize, a certain mutation is known to increase homozygosity through causing additional replication of chromosomes in the interphase between the first and second meiotic division and formation of a restitution nucleus after the first or second meiotic division (Asker and Jerling, 1992). Buttibwa, *et al*. (2015) also observed increased

homozygosity of up to 68% in cassava progeny obtained from flowers pollinated with irradiated pollen.

CONCLUSION

Results of this study indicate successful fruit and seed set, induced parthenogenesis, spontaneous diploidisation and increased homozygosity in cassava embryos, following interspecific pollination of cassava with castor bean. Additionally, the successful regeneration of four cassava plantlets *via* embryo rescue and an aneuploid callus line *via* ovule culture demonstrate a major step towards the development of cassava inbred lines. We also learned that the rescue of embryos at an advanced period after pollination, rather than ovule culture at an earlier period after pollination is a better strategy, since efforts of plant regeneration from callus were futile. This study opens up more opportunities to explore interspecific pollination and/or other techniques of DH breeding and genetic enhancement in cassava.

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