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Exploring the induction of doubled haploids in cassava through gynogenesis

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Production and use of double haploids is important for attainment of systematic genetic gains, and indeed several plant breeding programmes have benefited from it. Gynogenesis, the *in vitro* culture of unfertilized ovules and/or embryos has specifically been exploited in several economically important crops but not cassava. In this study, we examined possibilities of generating doubled haploids (DH) in cassava through gynogenesis, by bagging female flowers of selected varieties to prevent pollination. A total of 2,466 flowers across 32 elite cassava varieties were bagged for a period of one-to-three days. Early embryo rescue and ovule culture were done at 7 to 42 days after anthesis. Consequently, 517 fruits (21%) were harvested and dissected to obtain 97 seeds from which 47 unique embryos and 18 callus lines were obtained *in vitro*. Only six of the rescued embryos (12.8%) regenerated into plantlets. Upon undertaking ploidy analysis and single nucleotide polymorphism (SNP) genotyping, it was established that all samples analyzed (regenerated plants and calli) were diploid. SNP genotyping revealed increased homozygosity (up to 85.7%), but no doubled haploids were noticed. The knowledge generated is a significant contribution towards understanding cassava flowering biology and thus a foundation to on-going efforts towards developing protocols for generation of cassava DH.

Key words: Anthesis, embryo rescue, gynogenesis, homozygosity, ploidy.

INTRODUCTION

Parthenogenesis (a form of “apomixis”) refers to the development of a haploid embryo from a reduced egg nucleus (*n*) in an asexual embryo sac without fertilization by the sperm nucleus (Palmer and Keller, 2005; Acquaah, 2007). This has been investigated in various crops since the 1940s (Freitas and Nassar, 2013). It occurs naturally or spontaneously and is widely distributed in plants including a few species of agricultural importance. In addition to parthenogenesis, other methods of producing embryos without fertilization have been observed, notably: apospory, diplospory, adventitious embryony, androgenesis and semigamy (Acquaah, 2007).

The regeneration of haploid embryos and plants through unpollinated female gametophytes has been described as gynogenesis (Chen et al., 2011). *In vitro*

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culture of unfertilized ovules has long been recognized as an important tool to produce haploid and homozygous double haploid (DH) plants for genetic studies and plant breeding programmes (Godbole and Murthy, 2012). In this case megaspores or female gametophytes of plants have been successfully triggered to undergo sporophytic development leading to plant regeneration. Production of haploid plants through gynogenesis by culturing unfertilized ovaries was first described in barley (Chen et al., 2011). Similarly, successful gynogenesis has been reported in many plant species, e.g., Egyptian henbane (*Hyoscyamus muticus*) (Chand and Basu, 1998), onion (*Allium cepa)* (Alan et al., 2007; Fayos et al., 2015), sweet potato (*Ipomoea batatas*), tulip (*Tulipa generiana*), maize (*Zea mays*) (Leblanc et al., 2009), sugar beet (*Beta vulgaris*), cucumber (*Cucumis sativus*) (Tantasawat et al., 2015), and wheat *(Triticum durum)* (Berzonsky et al., 2003).

Indeed, haploids of 21 angiosperm species have been obtained from *in vitro* unfertilized ovules or ovary culture since 1976; in most cases using explants at uninucleate to mature embryo sac stages (Wu et al., 2004). However, the success of the method and its efficiency is greatly influenced by several biotic and abiotic factors. The variety of donor plants, combined with growth conditions, is among the crucial factors (Murovec and Bohanec, 2012). In recent advances involving tissue culture and embryology, there has been successful induction of haploid plants by the culture of unpollinated ovaries or ovules (Yang and Zhou, 1982; Chen et al., 2011). It is on this premise that this study was initiated to explore the effect of no pollination on fruit and seed set and its potential in inducing cassava haploids (Hs) and DHs.

Reports from studies so far carried out on cassava indicate there is evidence that apomixis occurs in different varieties, though, at low rates (Nassar et al., 1998). Some other studies have suggested that the apomixis genes have also been successfully transferred to cassava through hybridizing with the wild species, *Manihot glaziovii* (Nassar and Collevati, 2013). Use of molecular markers on a few varieties has revealed that apomixis is controlled by genes which occur naturally in the cassava gene pool, though at a low frequency (Freitas and Nassar, 2013).

Therefore, the purpose of this study was to explore the effect of no pollination on fruit and seed set and the possibility of inducing haploid and/or doubled haploid cassava plants through gynogenesis.

MATERIALS AND METHODS

Parental varieties and nurseries

A total of 32 elite cassava varieties were selected for this study based on their profuse flowering behaviour. These included 52TME14, NAROCASS1, NASE12, NASE13, NASE14, NASE15, NASE16, NASE18, NASE19, NASE3, NASE4, TME204, UG120004, UG120014, UG120035, UG120078, UG120106, UG120109, UG120114, UG120125, UG120149, UG120156, UG120174, UG120192, UG120211, UG120219, UG120223, UG120243, UG120261, UG130005, UG130048 and UG130107. Some of these cassava varieties have been officially released by National Agriculture Research Organisation (NARO) while others are still undergoing evaluation. Pedigree information of these varieties can be accessed at www.cassavabase.org. Due to the differences in variety responses observed in several DH induction and apomixes studies, a diverse selection of varieties was preferred to increase chances of haploidy induction and recovery. Each of the donor plants was established in a crossing nursery at National Crops Resources Research Institute (NaCRRI), Namulonge and Rwebitaba Zonal Agricultural Research and Development (ZARD) in the first growing season of 2014. Each variety was represented by about 100 plants.

Experimental lay out and pollination control

In each test, variety plants were purposely selected, and on each plant some inflorescences were selected for the test treatment and others for the control. The inflorescences for the test treatment were bagged to prevent pollination while the ones for control were left for natural open pollination. On each selected plant some inflorescences were bagged at one to three days before anthesis while others were left un-bagged for open pollination to occur. The bags were left on the inflorescences for three to seven days after anthesis (DAA) to ensure that no pollination occurred. At intervals of three to seven days from the day of bagging the numbers of surviving fruits per inflorescence, plant and variety were recorded up to harvest time between 7 and 42 DAA, after which fruit harvest and embryo rescue were done. This was done at two to seven levels of flowering in the selected varieties. In a revised design, the test and control inflorescences were selected and fruits harvested at 7 to 14 DAA, ovules excised and cultured. Nine rounds of bagging and not bagging were carried out, and this involved up to 2,644 bagged and 1,990 un-bagged flowers. In all the rounds, only plants with good and mature inflorescences (before anthesis) were purposely selected.

In vitro embryo rescue and culturing

Surviving fruits of bagged inflorescences were harvested for *in vitro* embryo rescue or ovule culture. Initially embryo rescue was undertaken at 42 DAA for the fruits in the first six rounds and then adjusted to 7 to 14 DAA. For comparison purposes, a few fruits from open-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 min. Thereafter, the fruits were soaked (while being shaken) twice in 2% sodium hypochlorite (NaOCl) containing 2 to 3 drops of Tween 20 added as a surfactant for 20 min (10 min each soaking). The fruits were then rinsed three times with sterile distilled water in a laminar flow hood.

The fruits at 42 DAA were dissected and embryos excised. The embryos were cultured *in vitro* on modified Murashige and Skoog (MS) basal medium (1962) (M6 or $^{1}/_{2}$ MSREm) in glass jars with radicles pushed down into the medium. The M6 medium contained half MS basal salts, supplemented with 1.0 mg/L 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 to 14 DAA, ovules were excised and cultured *in vitro* on modified MS (MS2 or MS3) induction medium supplemented with 2 mg/L 2,4 D, 0.5 mg/L kinetine, 1 mg/L GA3, 0.2 mg/L benzylaminopurine (BAP), MS vitamins, 8% sucrose and 0.3% gelrite or agar in petri dishes (90 × 14.2 mm). In all cases the pH of media was adjusted to 5.8 before autoclaving at 121°C for 15 min.

The embryos at 42 DAA were incubated at 28 ± 1°C under a 12/12 h (day/night) photoperiod with light supplied by white

fluorescent tubes (25 µmol $m^{-2}s^{-1}$). The immature embryos were left in the growth room for four weeks for regeneration into plantlets. The resulting plantlets were sub-cultured and transferred to basic MS medium containing 4.3 g/L of solid medium of MS salts supplemented with 2% sucrose, 1.0 ml/L MS vitamins and 0.3% phytagel with pH adjusted to 5.8 by adding a base in glass jars. Meanwhile, the dishes containing ovules were cultured in darkness (improvised by enclosing the dishes in aluminium foils) at 28°C for one month and then taken through callus formation and plantlet regeneration process.

Histological analyses

Ovules excised from fruits at 7, 14, 21 and 28 DAA were fixed in glacial acetic acid and 96% ethanol (in ratio of 1:3) in falcon tubes and kept in darkness at 4°C for at least 3 h. 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 Blue black, NBB (5% w/v). Stained sections were mounted using Depex to make permanent slides. Examination of slides for embryological analyses was performed under an inverted light microscope (Nikon, Eclipse TS100-F) and images taken using a camera head (Nikon DS-L3).

Ploidy level and homozygosity analyses

Flow cytometry was performed using Partec GmbH ploidy analyzer (Otto-Hahn-Str. 32, D-48161 Münster) to determine the ploidy level of plantlets generated from rescued and cultured embryos, and some calluses from cultured ovules following the method described by Dolezel et al. (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 (0.1M 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 min before 1 ml of OTTO II buffer (0.4 M anhydrous Na_2HPO_4, 4 µg/ml of 4, 6´-diamidino-2-phenylindole (DAPI), and 1 µl/ml β-metcaptoethanol) was added. The flow cytometer was adjusted so that the peak representing 2n or 2C DNA in a diploid at G1 phase of the control was localized 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 22 samples together with six controls were analysed.

For the homozygosity analysis, twenty eight samples of genomic DNA were extracted from young fresh leaves of regenerated plantlets (07 samples) generated from rescued and cultured embryos, selected calluses (15 samples) derived from cultured ovules and the mother (parental) lines as controls (06 samples) using the QIAGEN (DNeasy) plant kit, following the manufacturer's instructions. DNA concentration was determined using a NANODROP 2000 (Thermo SCIENTIFIC, USA) and then analyzed 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., 2012) was used to assay the 28 genomic DNA samples.

Data analysis

Different kinds of data sets were generated both in the field and laboratories. These included number of flowers bagged, fruit set and survival at different levels of flowering, number of seeds or ovules and embryos excised, number of plantlets and calli regenerated, ploidy and homozygosity levels of generated calli and plantlets. From this, mean number of flowers, fruits, ovules, seeds, and embryos, for each round of experiment were computed. Further still, the data were subjected to analysis of variance (ANOVA) at the significant level of 5% ($P \leq 0.05$) using Genstat statistical software, edition 12 and R-Studio Statistical Programming software (R Core Team, 2017). Additionally, ploidy level comparisons between progeny samples and controls (mother plants) was 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 (Dolezel et al., 1995; Ochatt, 2006). Computation of percentage homozygosity was done by summing up the number of homozygous loci in each progeny sample followed by division with the sum of heterozygous loci in the corresponding mother sample.

RESULTS

Fruit and seed set from non-pollinated and open-pollinated flowers

A total of 2,466 flowers were bagged from which only 517 fruits were harvested representing a survival rate of 21% (Table 1). On the other hand, 1,990 flowers were not bagged (open-pollinated) out of which 895 fruits were surviving at the time of harvest, that is, 7 to 42 DAA representing a survival rate of 45%. In rounds 1 to 6 fruits were harvested at 42 DAA for embryo rescue. Due to high fruit abortion rates of the non-pollinated flowers, harvest period was reduced to 7 to 14 DAA for ovule culture in round 7 to 9. Only fruits from non-pollinated (NP) flowers were harvested for embryo rescue or ovule culture.

By 42 DAA, only 100 fruits out 1,891 bagged flowers (5%) had survived compared to 895 out of 1,798 (42.7%) fruits from open-pollinated (OP) flowers in rounds 1 to 6. Highest fruit survival rates from bagged flowers were registered in rounds 7 to 9 in which fruit harvest was done at 7 to 14 days after anthesis. On the whole, there were significant differences in the mean number of fruit-set between non-pollinated and open-pollinated flowers ($P<0.001$) (Table 1)

Although the results in Table 2 show more flower production between second and fourth branching levels, there is no consistent trend in fruit set in the non-pollinated flowers. The percentage fruit set varied across levels of branching, with the lowest and highest percentages noted at second and seventh levels, respectively. Meanwhile in the open-pollinated flowers, there was a notable declining trend with increase in the branching level, except at seventh level. Overall percentage fruit and seed set was lower in the non-

Table 1. Fruit set following bagging to prevent pollination of cassava flowers.

Round	Number of flowers		Number of fruits set		Percentage fruit set	
	NP	OP	NP	OP	NP	OP
1	201	61	43	22	21.4	36.1
2	93	68	4	31	4.3	45.6
3	1419	1490	464	797	32.7	53.5
4	100	98	0	32	0.0	32.7
5	61	64	6	12	9.8	18.8
6	17	17	0	1	0.0	5.9
7[1]	173	86	0	0	0.0	0.0
8[1]	72	-	0	-	0.0	-
9[1]	330	106	0	0	0.0	0.0
Total	2,466	1,990	517	895	-	-
Mean	274.0	248.8	57.4	112.3	7.6	24.1

[1]Rounds in which fruits were harvested for ovule culture at 7-14 DAA. Most fruits harvested at 42 DAA had no developed seeds.

Table 2. Comparison of fruit set in non-pollinated and open-pollinated flowers at different branching levels.

Branching level	Number of flowers		Number of fruits set		Percentage fruit set	
	NP	OP	NP	OP	NP	OP
2	708	597	258	326	36.4	54.6
3	854	771	124	342	14.5	44.4
4	441	383	76	170	17.2	44.4
5	226	184	36	50	15.9	27.2
6	69	49	6	7	8.7	14.3
7	45	6	0	0	0	0
Unknown	123	-	17	-	13.8	-
Total	2,466	1,990	517	895		
Mean	352.30	331.70	73.9	179.2	15.2	30.8

Data sets generated from 32 cassava varieties.

pollinated (bagged) flowers than in the open-pollinated (un-bagged) ones. However, in both cases, the mean number of fruit survival reduced with progress of the experiment (Figure 1). There was significant difference in both length and width of fruits from non-pollinated (NP) flowers and open-pollinated (OP) flowers ($P<0.001$).

The number of fruits that survived by harvest time varied significantly ($P < 0.001$) among the varieties (Table 3). At 42 DAA, a total of 47 unique embryos were rescued, out of which only seven unique plantlets were regenerated. NASE 19 had the highest number of rescued embryos and regenerated plantlets. From the ovules excised for early ovule culture to induce gynogenesis, only 18 of them developed calluses from the embryo sac region.

Histological examinations done on a few selected ovules revealed accelerated degeneration of embryo sac after 14 DAA in ovules of unpollinated flowers. This is noticed by the presence of many empty ovules with disorganizing tissues. However, in the open-pollinated flowers there was evidence of normal embryo development (Figure 2).

Ploidy and homozygosity analysis results

Ploidy analysis by flow cytometry revealed that all of the 22 samples (obtained from six out of 32 varieties) were diploids just as the controls (Table 4). They all produced one peak in G1 phase (Figure 3). For homozygosity analysis, comparisons were limited to heterozygous alleles in the mother and progeny samples. It was observed increased by homozygosity at varying degrees. Among plantlets the lowest was 45.5% in NASE19 (sample P25) and the highest being 85.7% in the plantlet of UG141658 (sample P22). This variety was not in the original selection of this study. Meanwhile, in most calli no increase in homozygosity was noted (0%), that is, the

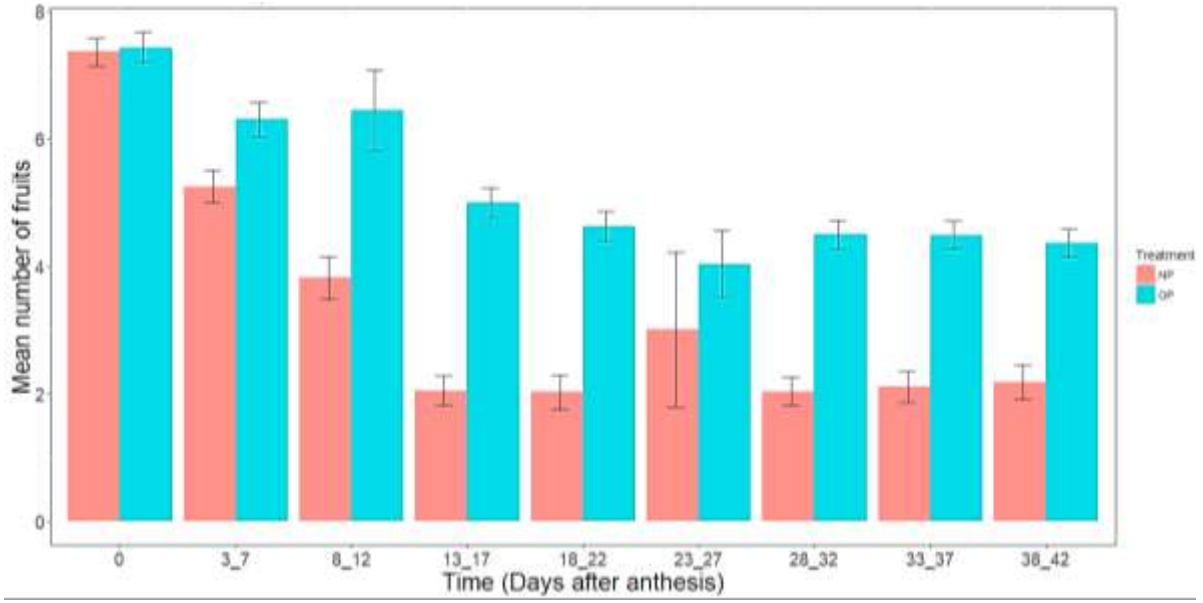

Figure 1. Mean number of surviving fruits during the experiment. Red bars = fruits harvested from non-pollinated flowers (NP); Blue bars = fruits of open-pollinated flowers (OP). The vertical bars represent the error deviation.

Table 3. Number of cassava fruits, seeds, ovules, embryos rescued, calli and plantlets generated from different cassava varieties following non-pollination and open pollination.

Variety	Number of flowers		Number of fruits set		Number of seeds	Number of embryos	Number of calli	Number of plantlets
	NP	OP	NP	OP	NP	NP	NP	NP
52TME14	49	-	7	-	0	0	-	0
NAROCASS 1	31	11	0	0	-	-	-	-
NASE 12	45	6	0	0	-	-	-	-
NASE 13	23	19	0	0	-	-	-	-
NASE 14	323	172	2	31	6	0	6	0
NASE 15	74	-	10	-	0	-	-	-
NASE 16	81	80	0	25	-	-	-	-
NASE 18	60	35	9	12	0	-	-	-
NASE 19	57	37	21	16	20	11	-	3
NASE 3	207	106	4	11	12	0	12	0
NASE 4	23	-	0	-	-	-	-	-

Table 3. Contd.

TME 204	74	34	0	3	-	-	-	-
UG120004	76	82	44	55	13	13	-	0
UG120014	72	66	47	43	6	0	0	-
UG120035	86	69	15	47	0	-	-	-
UG120078	48	58	27	48	2	0	-	-
UG120106	70	88	28	46	5	3	-	1
UG120109	112	120	28	11	2	0	-	-
UG120114	110	101	26	59	13	6	0	2
UG120125	121	138	63	86	13	10	0	0
UG120149	81	60	54	3	0	-	-	-
UG120156	51	48	13	24	0	-	-	-
UG120174	43	62	9	32	0	-	-	-
UG120192	131	104	32	69	0	-	-	-
UG120211	75	90	7	65	3	3	-	0
UG120219	76	95	3	44	0	-	-	-
UG120223	41	38	2	16	0	-	-	-
UG120243	72	87	27	47	2	1	0	0
UG120261	61	87	9	66	0	-	-	-
UG130005	36	40	2	11	0	-	-	-
UG130048	16	16	10	15	0	-	-	-
UG130107	41	41	18	10	0	-	-	-
Total	2466	1990	517	895	97	47	18	6
Mean	77.1	68.6	16.2	30.9	3.7	3.6	3.0	0.6

Only varieties that had surviving fruits at harvest time are presented. For comparison purposes some inflorescences were left for natural open-pollination and only fruit counts were recorded at the time of harvest. The embryos and plantlets were obtained from fruits harvested at 42 DAA. Ovules were excised from fruits harvested at 7-14 DAA and these have so far developed calluses. Only calluses that developed from the embryo sac regions were recorded.

level of heterozygosity was same as that of the mother samples, the highest was 10.5% in NASE3 (sample P15).

DISCUSSION

Fruit and seed set

Nine rounds of bagging to prevent pollination were carried out across 32 test varieties in order to induce parthenogenic fruit and seed set. Since cassava has unisexual and protogynous flowers, detection of seed production in absence of pollination is easy as only the precaution required is to avoid pollen contamination (Leblanc and Mazzucato, 1980). It is therefore rational to conclude that fruits and seeds obtained from un-pollinated flowers in this study are apomictic. The noted drastic reduction in fruit retention and seed set following non-pollination is primarily attributed to the abscission of fruits and failure of endosperm development that is required to support embryo growth. The reductions observed in open-pollinated fruits, point to inherent obstacles to pollination. The improvement in the percentage fruit survival observed in rounds 7 to 9 is due to reduced fruit abscission since fruit harvest (for early ovule culture) was done between 7 and 14 DAA.

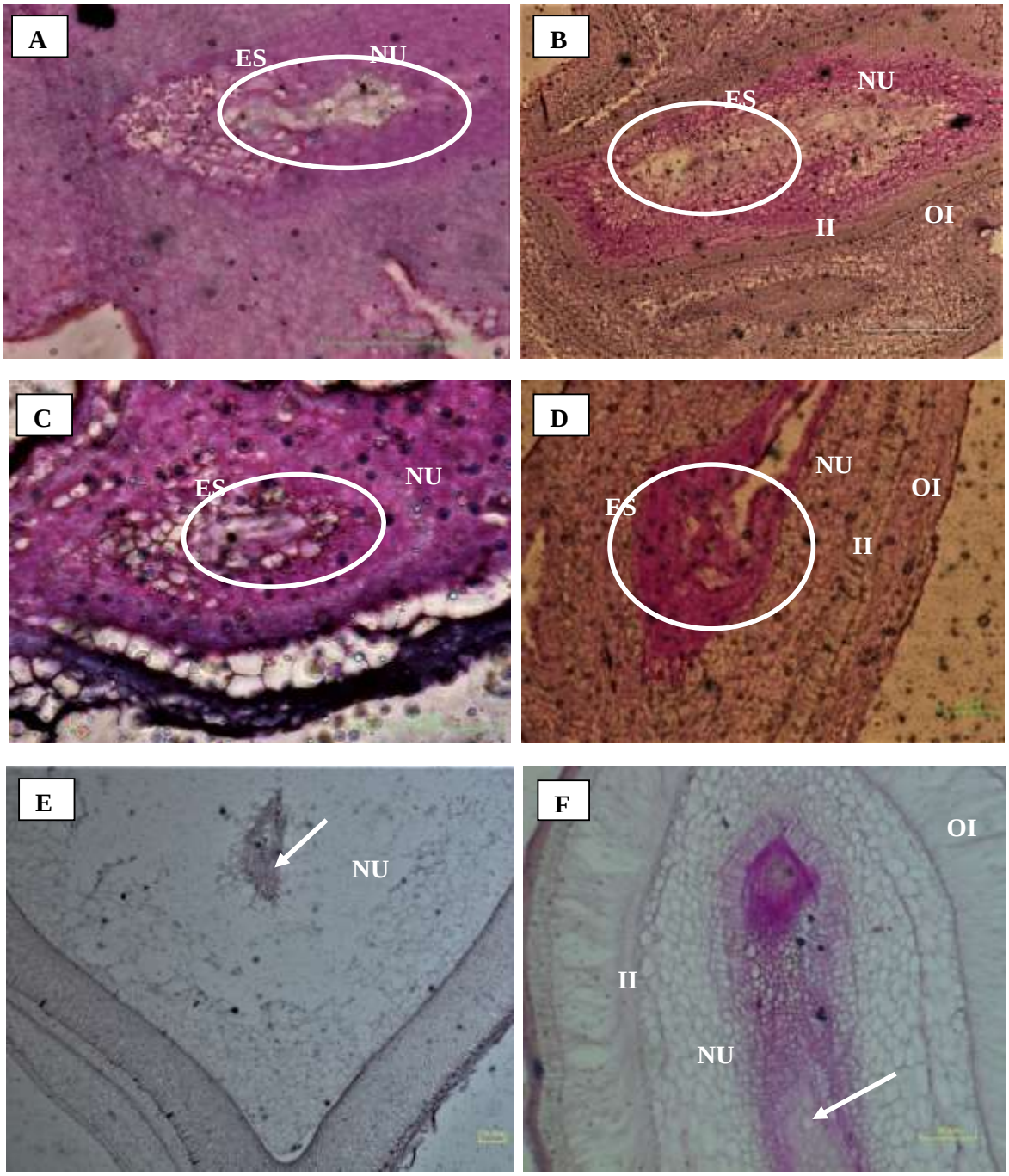

Figure 2. Comparison of developmental stages in non-pollinated and self-pollinated cassava flowers: (A) ovule at 7 DAA in a non-pollinated flower showing a degenerating egg apparatus in embryo sac; (B) ovule at 7 DAP in a self-pollinated flower showing cell proliferation in embryo sac; (C) ovule at 21 DAA in a non-pollinated flower showing a disorganizing embryo sac; (D) ovule at 21 DAP in a self-pollinated flower showing an organizing embryo sac; E) ovule at 28 DAA in a non-pollinated flower showing degenerated embryo sac (white arrow); F) ovule at 28 DAP in a self-pollinated flower showing embryo and surrounding tissues developing (white arrow).ES=embryosac; NU=nucellus; OI=outer integument; II=inner integument.

Ploidy and homozygosity level of regenerated plantlets

The diploids revealed by flow cytometry analysis of the regenerated plantlets were either as a result of embryo formation from unreduced embryo-sacs (diplospory) or embryos formed from embryo-sacs that arose from somatic cells in the ovule (apospory) or from unreduced

Table 4. Ploidy and homozygosity levels in callus and plantlets obtained from fruits of non-pollinated cassava flowers.

Sample ID	Variety	Source of DNA	Channel mean	%CV	Mean ratio of sample to diploid mother	Ploidy level	No. of heterozygous loci		No. of homozygous loci in progeny	Percentage homozygosity in progeny
							Mother sample	Progeny sample		
P1	NASE 3	Callus	101.49	7.64	1.016	2x	21	21	0	0.0
P2	NASE 14	Callus	97.7	4.86	1.000	2x	11	10	1	9.1
P4	NASE 3	Callus	98.81	5.31	0.989	2x	21	21	0	0.0
P5	NASE 3	Callus	100.17	5.24	1.003	2x	21	19	2	9.5
P6	NASE 3	Callus	95.97	5.47	0.961	2x	20	20	0	0.0
P7	NASE 3	Callus	100.63	4.72	1.007	2x	20	19	1	5.0
P8	NASE 3	Callus	100.39	5.73	1.005	2x	21	21	0	0.0
P9	NASE 3	Callus	101.78	4.67	1.019	2x	21	21	0	0.0
P10	NASE 3	Callus	99.11	4.29	0.992	2x	21	21	0	0.0
P11	NASE 3	Callus	100.29	5.23	1.004	2x	21	21	0	0.0
P12	NASE 3	Callus	99.02	5.81	0.991	2x	17	16	1	5.9
P13	NASE 3	Callus	101.01	4.21	1.011	2x	21	21	0	0.0
P15	NASE 3	Callus	97.87	5.87	0.980	2x	19	17	2	10.5
P17	NASE 3	Callus	94.53	5.55	0.946	2x	21	21	0	0.0
P18	NASE 3	Callus	100.04	4.75	1.001	2x	21	21	0	0.0
P21	UG120106	Leaf lobes	99.85	5.26	1.000	2x	17	7	10	58.8
P22	UG141658	Leaf lobes	97.06	8.50	1.003	2x	14	2	12	85.7
P24	NASE 19	Leaf lobes	96.04	4.95	0.980	2x	11	5	6	54.5
P25	NASE 19	Leaf lobes	98.77	6.33	1.008	2x	11	6	5	45.5
P26	NASE 19	Leaf lobes	97.52	6.41	0.992	2x	11	5	6	54.5
P27	UG120114	Leaf lobes	97.53	5.38	0.992	2x	13	4	9	69.2
P28	UG120114	Leaf lobes	98.21	4.84	0.999	2x	13	6	7	53.8
Mean	-	-	-	-	-	-	17.6	14.8	2.8	21.0

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. 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.

egg cells (parthenogenesis) that underwent chromosome doubling. Freitas and Nassar (2013) also reported apospory in cassava using molecular techniques. This therefore justifies the need to do high throughput genotyping so as to ascertain the exact origin of the embryos.

Apospory is by far the most common mechanism of embryo formation in higher plants (Bashaw, 1980; Barcaccia and Albertini, 2013; Lone and Lone, 2013) and has been reported in *Beta, Brachiaria, Cenchrus, Chloris, Compositae, Eriochloa, Heteropogon, Hieracium, Hyparrhenia, Hypericum, Panicum, Paspalum, Pennisetum, Ranunculus, Sorghum, Themeda,* and *Urochloa* (Barcaccia and Albertini, 2013). Diplospory has been reported in *Tripsacum, Eragrostis*, and *Taraxacum* where apomictic female gametes (2n) undergo embryogenesis autonomously (Kandemir and Saygili, 2015). In a previous review by Asker (1979) and later on by Barcaccia and Albertini (2013) it was affirmed that maternal offspring from

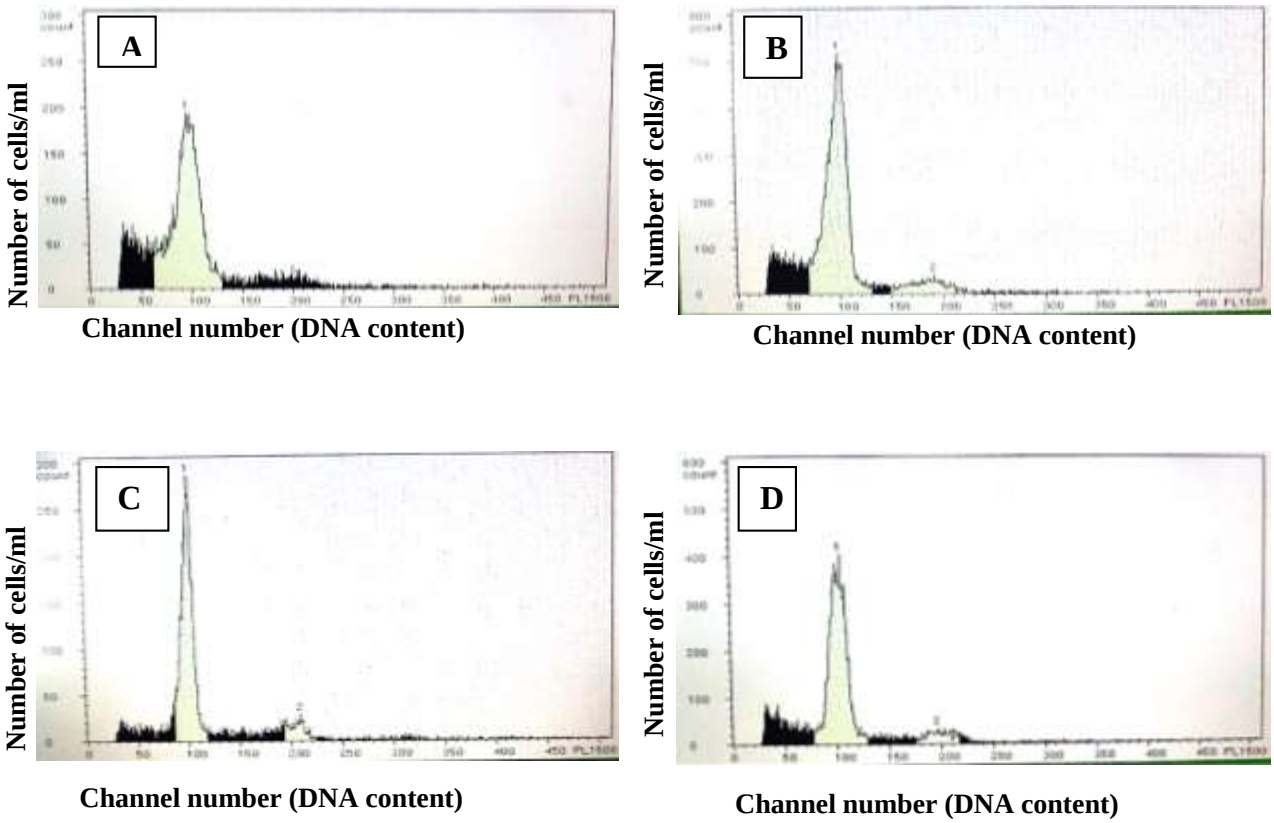

Figure 3. Distribution of DNA content of nuclei isolated from some regenerated plantlets and callus: (A) diploid mother sample of variety UG141658 (*2n=36*) used as a standard; (B) plantlet generated from non-pollinated flowers of variety UG141658 mixed with UG141658 nuclei of mother sample as a standard; (C) NASE 3 diploid mother sample used as another standard and (D) Callus generated from non-pollinated flowers of NASE 3. A peak at channel 50 would represent *1n* or *1C* DNA content in a haploid. In all cases the peaks indicated diploids in which peak 1 represents *G1* nuclei with *2C* DNA and peak 2 represents *G2* DNA.

asexually produced seeds usually result from parthenogenetic development of unreduced egg cells, however, such offspring may also be formed by nucellar embryony, or from parthenogenetic development of reduced egg cells followed by chromosome doubling. Various environmental factors are known to influence the balance between apomictic and sexual reproduction, such as light and temperature regimes. However, these were not measured in this study.

According to the theory of "wound-hormone or necrohormone" (Asker and Jerling, 1992) and programmed cell death (Bell, 1996), an egg cell can be stimulated to develop into an apomictic embryo by necrohormones released by surrounding dying cells or tissues. So, it can be concluded that the rescued embryos in this study developed through a similar stimulation due to dead or degenerated embryo sacs since no endosperm tissue was formed to support their survival.

The diploidy observed in the calli suggest that they 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 calluses with *4C* DNA content must have contained cells at the *G2* phase of the cell cycle. On the other hand, the observed deviations in peak positions 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; Doležel and Bartoš, 2005).

Further still, the diploid nature and increased homozygosity revealed by SNP genotyping in some samples confirmed doubled chromosome numbers, since all the loci had paired alleles. It is likely that automictic parthenogenesis (automixis) occurred. In Mogie (1986) and Lone and Lone (2013), automixis is defined 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).

Plant regeneration from young ovules

No embryo and/or plantlet was regenerated from the calli derived from young ovules. This failure could be attributed to the general slow response of cassava or the effect of several media components on the regeneration (not measured in this study) or probably the calluses initially isolated were not embryogenic. In normal routine tissue culture it takes one to four months to regenerate plantlets from calluses. This is dependent on the explant used (Chen et al., 2011; Mishra and Goswami, 2014). However, plantlets of gynogenic origin regenerate directly from an embryo or an embryoid and rarely from callus (Chand and Basu, 1998; Mishra and Goswami, 2014). It is known that i*n vitro* parthenogenesis where egg cells are triggered to form a sporophyte, and *in vitro* apogamy where the other cells of the embryo sac are induced to form the embryo are the two main origins of gynogenic haploids (Chand and Basu, 1998; Lone and Lone, 2013). Thus, the diploid calli as revealed by results of flow cytometry analyses in this study is an implication that the calluses isolated may not have been embryogenic and/or they originated from somatic cells within the ovules.

Gynogenesis efficiency in plants is highly dependent on the variety used and the quality of the donor material. Cassava seems to be not exceptional. The variety factor proved to be one of the most important factors affecting *in vitro* gynogenesis in squash in which the percentage of gynogenic ovules ranged from 0 to 48.8% (Chen et al., 2011). This is true with the results of this study in which out of 32 varieties used, none has regenerated plantlets from the several callus lines obtained. Other varieties need to be sought for future studies. Though the stage of ovule development at time of inoculation has not been studied extensively, its influence on embryo formation and/or plant regeneration from callus cannot be ruled out. The review in Chen et al. (2011) indicates that the most responsive ovules are those with nearly mature or fully mature embryo sacs.

Conclusion

Results from this study reveal the possibility of embryo formation in cassava when pollination is prevented. Spontaneous diploidization and increased homozygosity in cassava embryos following no pollination also provide further evidence of parthenogenic fruit and seed set in cassava. Besides, it was observed that the rescue of embryos at an advanced period after anthesis, rather than ovule culture at an earlier period is a better strategy, since efforts of plant regeneration from callus were futile. The knowledge generated is a significant contribution towards understanding flowering biology of cassava and thus contributing to on-going efforts towards developing protocols for generation of cassava DH. All in all, this study opens up more opportunities to explore gynogenesis and/or other techniques of DH breeding in cassava. The allocation of more resources into this kind of work is crucial for a rapid breakthrough.

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

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