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EFFECT OF SELF-POLLINATION WITH HEAT-TREATED POLLEN ON PARTHENOCARPY AND HOMOZYGOSITY IN CASSAVA

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ABSTRACT

Cassava's (*Manihot esculenta* Crantz) high heterozygosity complicates its genetic improvement *via* selective breeding. Double haploid (DH) technology can be used to improve the crop's heterozygosity, thereby improving the capacity for genetic improvement. The objective of this study was to evaluate the effect of self-pollination using heated pollen on pollen tube penetration, fruit set, seed and haploid embryo development in cassava genotypes for the production of haploid cassava. Pollen from two cassava genotypes, NASE3 and NASE14, was heated at 40, 50 and 60 °C for 0.5, 1.0 and 2.0 hr each. The heated pollen was used in six rounds of self-pollinations. Pollen tube penetration was monitored by fluorescent microscopy, followed by early embryo rescue and ovule culture. Ploidy and zygosity were assessed using flow cytometry and single-nucleotide polymorphism analysis, respectively. Pollen germinated on the stigma, grew within the style through the nucellar beak, but did not reach the embryo sac, thus achieving no fertilisation in all the 5756 self-pollinated flowers. There was a reduction in pollen germination (*in vitro* and *in vivo*), pollen tube penetration and fruit set with increasing temperature. Heat-treated pollen stimulated division of the egg cell and induced development of parthenocarpic fruits. Up to 6 embryoids per ovule were observed and all regenerated plantlets were diploid, with up to 93.0% increased homozygosity. For the first time, plant regeneration from ovules, pollinated with fresh pollen at 14 days after pollination, was achieved indicating improved speed in plant regeneration. The data generated are important for the development of protocols for cassava DH plant production.

Key Words: Double haploids, embryo rescue, *Manihot esculenta*

RÉSUMÉ

La forte hétérozygotie du manioc (*Manihot esculenta* Crantz) complique son amélioration génétique par sélection sélective. La technologie d' haploïde double (DH) peut être utilisée pour améliorer l'hétérozygotie de la culture, améliorant ainsi la capacité d'amélioration génétique. L'objectif de cette étude était d'évaluer l'effet de l'auto-pollinisation à l'aide de pollen chauffé sur la pénétration du tube pollinique, la nouaison, le développement des graines et des embryons haploïdes dans les génotypes de manioc pour la production de manioc haploïde. Le pollen de deux génotypes de manioc, NASE3 et NASE14, a été chauffé à 40, 50 et 60 °C pendant 0,5, 1,0 et 2,0 heure (s) chacun. Le pollen chauffé a été utilisé dans six cycles d'auto-pollinisation. La pénétration du tube pollinique a été surveillée par microscopie fluorescente, suivie d'un sauvetage précoce des embryons et d'une culture d'ovules. La ploïdie et la zygosité ont été évaluées à l'aide de la cytométrie en flux et de l'analyse du polymorphisme mononucléotidique, respectivement. Le pollen a germé sur le stigmate, s'est développé dans le style à travers le bec nucellaire, mais n'a pas atteint le sac embryonnaire, n'obtenant ainsi aucune fécondation dans toutes les 5756 fleurs autogames. Il y avait une réduction de la germination du pollen (*in vitro* et *in vivo*), de la pénétration du tube pollinique et de la nouaison avec l'augmentation de la température. Le pollen traité thermiquement a stimulé la division de l'ovule et induit le développement de fruits parthénocarpiques. Les 6 embryoïdes par ovule ont été observés et toutes les plantules régénérées étaient diploïdes, avec 93,0% d'augmentation d'homozygotie. Pour la première fois, la régénération des plantes à partir des ovules, pollinisées avec du pollen frais 14 jours après la pollinisation, a été réalisée, indiquant une vitesse améliorée de régénération des plantes. Les données générées sont importantes pour l'élaboration de protocoles de production de plantes de manioc de DH.

Mots Clés: haploïdes doubles, sauvetage d'embryons, *Manihot esculenta*

INTRODUCTION

Cassava (*Manihot esculenta* Crantz), a woody shrub of family Euphorbiaceae, is the third most important staple food crop sustaining millions of people in developing countries; especially in sub-Saharan Africa (Howeler *et al*., 2013). Despite the fact that the crop shows relative adaptability and good performance in marginal soils and under drought, cassava production is constrained by numerous diseases and pests, sometimes accounting for up to 100% of the losses (Obiri *et al*., 2017). To overcome these challenges, cassava breeding holds the greatest promise because of large natural genetic variability and broad genetic base, which provide opportunities for genetic improvement (Fukuda *et al*., 2002).

Cassava breeding is hampered by long breeding cycles and its high heterozygosity, that make its improvement slow and inefficient (Wang *et al.,* 2014; Ceballos *et al*., 2015). High levels of heterozygosity in cassava are preserved by frequent cross pollination, coupled with centuries of farmer-based vegetative propagation (Wang *et al*.. 2011). This makes even simple monogenic traits difficult to introgress, due to unpredictable back-crossing results.

This notwithstanding, some progress has been made in breeding towards control of cassava mosaic disease (Hahn *et al*., 1980), cassava brown streak disease (Patil *et al*., 2015), enhanced nutritional levels (Stupak, 2008; Nassar and Ortiz, 2010), yield increase (Nassar and Ortiz, 2010), increased dry matter content (Kawano, 2003) and drought resistance (Okogbenin *et al*., 2013). In such heterozygous species, the breeding schemes can be drastically improved using doubled haploid (DH) plants, which produces large numbers of homozygous lines, after a single round of pollination.

DH plants are genotypes formed as a result of spontaneous or induced chromosome

duplication of haploid cells (Germana, 2011a; Kumar and Choudhary, 2020). The main advantage of DH lines is their homozygosity and genetic purity; and therefore, is essential in basic genetics research, molecular biology, and facilitation of plant breeding (Germana, 2011b). DHs are useful in improving the speed and efficiency of the otherwise inefficient and cumbersome classical methods of plant breeding, because heterozygosis is overcome quickly (Chen *et al.,* 2011; Piosik *et al*., 2016).

There are various techniques used to induce DHs (Murovec and Bohanec, 2012). Such techniques used in different plant species include wide crosses (Wêdzony *et al*., 2009; Mishra and Goswami, 2014), pollination using a haploid inducer line (Murovec and Bohanec, 2012), pollination using irradiated pollen (Godbole and Murthy, 2012); androgenesis (Forster *et al*., 2007; Shumilina *et al*., 2020), and a culture of unfertilised ovules (Mishra and Goswami, 2014). Other techniques include ovule or ovary cultures in which DHs are produced from the female gametophyte *via* either gynogenesis or parthogenesis. In gynogenesis, unfertilised egg cells are stimulated to divide by plant *in vitro* pre-treatment, giving rise to haploid plants (San and Gelebart, 1986; Bohanec *et al*., 1995; Michalik *et al*., 2000; Bohanec *et al*., 2009). In parthenogenesis, DHs arise from stimulation by pollination with pollen that has been subjected to heat, high energy irradiation, or pollen donated from other plant taxa at a genera or species level (wide pollination) (Foroughi-Wehr and Wenzel, 1993).

DH induction in cassava was studied previously, in which calli of varied ploidy levels including haploids, were generated but no plants were regenerated (Wang *et al*., 2011; Perera *et al*., 2014). An initial step in the production of DHs *via* gynogenesis in cassava was recently described by Lentini *et al*. (2020a), involving use of unpollinated ovules from ovaries 1 day post anthesis, for the induction of embryo formation. In our earlier study, while progeny derived from self-pollination with irradiated pollen yielded frequencies of 28-55% of homozygous loci, none of the regenerated cassava plants was haploid (Buttibwa *et al*., 2015). Recently, our attempt to use castor bean pollen in wide crosses with cassava, resulted in increased homozygosity and automictic parthenogenesis, but no DH plants were regenerated (Baguma *et al*., 2019).

Pollination with heat-treated pollen is an alternative to the use of irradiated pollen and wide crosses, and has been used for the induction of haploids in Aspen (Lawson *et al*., 1968), but not in cassava. Double haploidy using heat-treated pollen involves pollination with heat-treated pollen and use of immature embryo rescue techniques under *in vitro* conditions. The key determinants in the success of this process are temperature used for the treatment, stage of embryo development, culture media formulation, composition, and growth conditions (Germana, 2011a). Heat-treated pollen can germinate on the stigma, grow through the style, but may not reach the embryo sac to stimulate the development of haploid embryos, but this is not known in cassava.

The objective of this study was to evaluate the effect of heating pollen on pollen tube penetration, fruit set, seed and haploid embryo development in cassava genotypes for the production of haploid cassava.

MATERIALS AND METHODS

Plant materials. Plants of two elite 'Ugandan' cassava genotypes, namely, NASE3 and NASE14, were grown at the National Crops Resources Research Institute (NaCRRI), Namulonge, Uganda; approx. 100 plants per plot, situated ~500 metres away from neighbouring cassava fields. National Crops Resources Research Institute, Namulonge is located at 0^{o} 321 N, 32^{o} 371 E at 1150 meters above sea level. Namulonge has an annual mean temperature of 27 ^{o}C, relative humidity of 65%, and deep loamy clay soils. The soils are weakly

acidic, with pH of 5 to 6, and organic matter levels of 2 to 3% in the top soil (Yada *et al.*, 2010). The rainfall shows a bimodal pattern, with a tropical wet and mild dry climate (Yada *et al.*, 2010). NASE3 and NASE14 were selected because they are farmer-preferred, flower profusely and are also highly responsive in tissue culture.

Collection and heat-treatment of pollen. Cassava plants were grown for 6 months in a field at Namulonge in dimensions 50 m by 50 m. Inflorescence on different plants of NASE3 and NASE14 genotypes with mature female flowers ready for pollination (~6 months after planting), were identified and bagged, 2-3 days prior to pollination to prevent unwanted pollinations (Ramos Abril *et al.*, 2019). On the day of pollination, inflorescences on mature male flower buds (about to dehisce, and containing mature pollen) were identified, collected and placed in plastic Petri dishes. Petri dishes were sealed using parafilm, to avoid hydration of the buds and the pollen; and wrapped in aluminum foil, while ensuring air-, light- and water-tight conditions inside the sealed Petri dishes.

Water baths were set up at 50 and 60 °C for the heating treatment. The male flower buds were then subjected to heat treatment in water baths, by submerging the sealed Petri dishes under water at 40, 50 and 60 °C for 0.5, 1.0 and 2.0 hours. Quantities of 200 g were placed on top of the Petri dishes to keep them submerged under hot water. Heating of pollen and pollinations were performed on the same day.

Pollination with heat-treated pollen. The heated male flower buds containing pollen were opened and used to self-pollinate mature female flowers in the field (which had been bagged 2-3 days earlier). Pollination was done by brushing the heat-treated pollen from the male flower buds on the stigma manually. Six rounds of self-pollination were carried out. Self-pollination with non-heated (fresh) pollen served as controls. After self-pollination, the inflorescences were re-bagged for at least 3 days to eliminate contamination by unwanted external pollen. Pollen tube penetration capacity into the embryo sac, for each pollination event, and *in vitro* germination capacity were monitored and quantified.

Field observations were made on the total number of self-pollinations made per temperature treatment. After pollination the experimental set up was categorised into the first and second experiment. In the first experiment, ovules were rescued 14 DAP, after which ovule rescue was done. In the second experiment, the number of surviving fruits (cyathia) per treatment was recorded up to 21 DAP, and embryos were rescued. Data were recorded on seed-set (seeded or seedless embryos), and number of embryos recovered in second experiment. Pollination with fresh pollen (pollen not heat-treated) was used as a control.

***In vitro* pollen germination tests.** Both fresh (non-heated) and heat-treated pollen were germinated on a medium, as described by Brewbaker and Kwack (1963), with modifications. These modifications included addition of 0.03% casein hydrolysate, 12.5% w/v poly ethylene glycol-600 (PEG-600), and 15Mm N-morpholino ethanesulfonic acid (MES) into the medium. The pH of the medium was adjusted to pH 5.8 and autoclaved for 30 min at 120 °C. The medium contained 100 mg L^{-1} boric acid; 300 mg L^{-1} calcium nitrate; 200 mg L^{-1} magnesium sulphate; 100 mg L^{-1} potassium nitrate; and 5% sucrose.

Following incubation in a dark humid chamber at 40 °C and 100% relative humidity for 24 hours, the pollen was examined under an inverted microscope (Nikon Alphaphot-2 YS2, Japan) as described by Kundu *et al.* (2014). Observations on pollen germinations were done on five replicates of 100 pollen grains per treatment. Pollen germination on the medium was used as an indicator for pollen viability, in which a pollen grain was scored

as germinated if a pollen tube was observed. Pollen germination frequencies were determined and comparisons made between germinated and non-germinated pollen grains, and between heat-treated and fresh pollen.

***In vivo* pollen germination analysis.** To collect the sample tissues for *in vivo* pollen germination analysis, 2 to 3 female flowers of NASE3 and NASE14, pollinated with fresh or heat-treated pollen (at 40, 50 and 60 °C for 0.5, 1.0 and 2.0 hr) were detached from the plants at 1, 2 and 3 DAP. Bracts were removed and female flowers (pistils) submerged immediately in the fixation solution in falcon tubes. The fixation solution was prepared one day in advance; and contained ethanol and acetic acid at a 3:1 v/v ratio. The volume of the fixative was 5 times higher than that of the fixated tissue to avoid dilution of the fixative by the water diffusing from the fresh tissues. Samples were carried from the field to the laboratory in the next 20 to 30 minutes, and stored in a refrigerator at 4 °C in the same fixing solution. The fixing solution was renewed every 24-hr intervals until further processing. For extended preservations, samples were stored in 70% ethanol.

Fixation and microscopic observations. To prepare samples for fixation and subsequent observations under the microscope, the old fixative solution was decanted off and 8M NaOH added to macerate pistils for 4 hr in darkness at room temperature (25 °C). This was followed by three washes, each of 5 minutes with 0.5N K_2HPO_4 at pH 10-12, to neutralise NaOH. Pistils were then washed thrice with 500 ml of 0.1% aniline blue in 0.5N K_2HPO_4 at pH 10-12, and stained in the same solution for 1 hr at 4 °C in darkness, as described by Ramos Abril *et al.* (2019). The pistils were removed from the stain and placed in a drop of glycerol on a glass slide. Stigmas were separated by cutting and the ovaries dissected to extract ovules. The ovary wall tissues were discarded and a drop of 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 fresh pollen were used as control.

Embryo rescue and ovule culture. At 14 and 21 days after pollination (DAP), cyathia (fruits) from the first and second experiment, respectively, were harvested and placed in zip-lock bags containing a refrigerant gel; and transported from the field to the laboratory. Cyathia (fruits) were surface sterilised by soaking in 70% ethanol for one minute, in 1.5% sodium hypochlorite (NaClO), with 3 drops of Tween 20 for 20 minutes. This was followed by four washes of each 5 minutes with sterile distilled water, in a laminar flow hood. Ovaries were then cut longitudinally into three sections. Individual carpels of the ovary, containing one ovule each per locus, were isolated and cultured. The isolated carpels were placed with the basal cut end on MS3 medium (Lentini *et al*., 2020b). Thirty ovary-slices were placed per Petri dish of 90 cm x 15 cm diameter and sealed with a cling film. After 4 weeks of culture, ovules were dissected and isolated from each carpel, and cultured with the adaxial side facing the MS3 medium. Cultures were then kept in the dark 12 hr-day and 12 hr-night photoperiod at 28-30 °C for 4 weeks; and then transferred to new MS3 medium.

Culture medium for induction of embryos from ovules. At 7 DAP, ovules from self-pollinated flowers with heat-treated or fresh pollen were cultured on MS3 medium as described by Lentini *et al.* (2020b). The medium contained MS macro- and micro-nutrients, supplemented with 0.8 mg L^{-1} $CuSO_4$ $5H_2O$, 2.5 mg L^{-1} nicotinic acid, 1.2 mg L^{-1} pyridoxine-HCl, 10 mg L^{-1} thiamine-HCl, 4 mg L^{-1} glycine, and 500 mg L^{-1} myo-inositol. Also

added was 0.2 mg L^{-1} biotin, 0.2 mg L^{-1} Ca-panthotenate, 0.2 mg L^{-1}ascorbic acid, 0.4 mg L^{-1} riboflavin, 200 mg L^{-1} L-proline, 2 mg L^{-1} 2,4-D, 2 mg L^{-1} BAP, 1 mg L^{-1} gibberellic acid (GA3), and 8% sucrose; and all solidified with 0.3% phytagel (Lentini *et al*., 2020b).

The pH of the medium was adjusted to 5.8 using 0.5N HCl, before autoclaving at 121 °C for 15 minutes. Ovules were cultured on this media for embryo formation. At the end of the 4-week of culture, the ovules were extracted from the carpels. The extracted ovules were transferred onto fresh MS3 medium under the same growth conditions at 28 °C under a 12/12 hr-light *versus* 12/12 dark period for 84 days. Data were collected by recording the number of ovules bulging and callus formed from ovules.

Fruits harvested at 21 DAP were dissected under a microscope and the embryos extracted. The embryos were cultured *in vitro* on modified MS basal medium (Murashige and Skoog, 1962) on deep petri dishes/glass jars with radicles pushed down into the medium. The M6 medium has half MS basal salts, supplemented with 1.0 mg L-1 gibberellic acid (GA3), 2% sucrose and 0.2% gelrite or agar as described by Yan *et al*. (2014). The embryos at 21 DAP were incubated at 28±1 ºC under a 12/12 hr light/dark period with light supplied by white fluorescent tubes (25 µmol $m^{-2}s^{-1}$) in a growth room for four weeks.

Embryo maturation and conversion into plantlets from ovules at 14 DAP. Primary embryos were sub-cultured on maturation medium consisting of MS salts and organics supplemented with 0.8 mg L^{-1} $CuSO_4 5H_2O$, 1 mg L^{-1} NAA and 2% sucrose; and solidified with 0.7% agar. Cultures were incubated at 28 °C in 12/12 hr light/dark period for 15 days, or until the cotyledons developed and turned green (Stamp and Henshaw, 1987). After 15 days of culture on a maturation medium, individual embryos or clusters of embryos with cotyledons were transferred onto MS salts, supplemented with 0.8 mg L^{-1} $CuSO_4.5H_2O$, 0.45 mg L^{-1} BAP and 12/12 hr light/dark period, for further development. The remaining early stage embryos were re-cultured on fresh maturation medium. After cotyledons expanded and roots formed, plantlets were transferred onto the medium for plant elongation for full development. This elongation medium (4E) consisted of MS salts and organics supplemented with 0.8 mg L^{-1} $CuSO_4$, 20 % sucrose solidified with 0.45% agar cultured at 28 ºC in 12 hr photoperiod.

Histology analysis on the rescued embryos. To determine how heat-treatment of pollen may affect embryo development, cell divisions and differentiation in the embryo sac region, histological analyses were done on a few ovules to track embryo developmental changes following pollination. The ovules were excised from fruits at 21 DAP, and then fixed in glacial acetic acid to 96% ethanol solution in a 1:3 (v/v) ratio, and kept in darkness at 4 °C for at least 3 hours. The ovules were prepared 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 stained with Schiff's reagent and counterstained with Naphthol Blueblack, NBB (5% w/v). Stained sections were mounted using Depex mounting medium to make permanent slides. Examination of slides was performed under an inverted light microscope and images taken using a camera head (Nikon DS-L3).

Determination of ploidy and zygosity analysis. Flow cytometry was used to determine the ploidy levels of regenerants, using young leaves from *in vitro* cultured plants. Plant tissue (approx 0.5-1.0 cm^2 size) was cut with a razor blade in 1 ml of lysis buffer [10 mM Tris, 2 mM $MgCl_2$ $6H_2O$, 50 mM sodium chloride, 1% (v/w) polyvinylpyrolidone, 0.1% (v/v) Triton X-100, pH7.0 with addition of 1 mg of 4'6-diamidino-2-phenylindoole (DAPI) (Ochatt *et al*., 2011).

The suspension was filtered through a 30-mm nylon filter and stained at room temperature for 5 minutes. The suspensions were measured for relative nuclear DNA content, using Partec CA II (Partec, Münster, Germany). Leaves of diploid (2n = 2x = 18) mother plants of cassava (NASE3 and NASE14) were used as a reference standard; while diploid *Medicago sativa* was used as an external standard. Ploidy was determined by comparing a position of the peak of the diploid standard, placed on channel 100, with the position of the peak corresponding to G1 nuclei of the regenerants.

To determine the homozygosity of the samples, genotyping was performed using single nucleotide polymorphisms (SNPs). DNA was extracted from regenerated plantlets using a Qiagen® kit, following the manufacturer's instructions. The quantity of DNA was estimated by comparing the fluorescent yield of the samples with a series of lambda (*ë*) DNA standards at varying known concentrations. The quality was checked through the digestion of 250 ng of the genomic DNA from 10 random samples, with the restriction enzyme *Eco*RI at 65 °C for two hours; and thereafter visualised on agarose gel.

DNA samples were genotyped at the Biosciences for Eastern and Central Africa (BecA) hub in Nairobi, Kenya. Genotyping by sequencing (GBS) was done using a protocol described by Elshire *et al*. (2011), wherein the DNA was digested by the enzyme *Ape*KI, a type II restriction endonuclease that recognises a 5-base degenerate sequence GCWGC, in lengths of 100 bp, as recommended by Hamblin and Rabbi (2014). The binding between *ApeKI* cleavage fragments and the adapter was performed after the digestion of samples and a 192-plex sequencing run.

In order to analyse the sequences and quality filters, the TASSEL software package, version 5.2.37 (Bradbury *et al*., 2007) was used. A total of 22,618 heterozygous and polymorphic SNPs were used to assay the DNA samples. The DNA of heterozygous mother plants, NASE3 and NASE14, was used as a control. We limited our comparisons by focusing on heterozygous alleles between mother samples and their respective progeny calluses.

Data analysis. Mean numbers of flowers, fruits, ovules, seeds, embryos, for each pollination event were computed. From these, mean numbers of flowers, fruits, ovules, seeds, for each pollination event were computed for six rounds of pollination. For *in vitro* pollen germination, mean numbers and percentages of germinated pollen grains were also computed for six rounds of pollen germination tests using R-statistics (Verzani, 2018). The results were computed using the Shapiro-Wilk normality test (Villasenor and Estrada, 2009) and by looking at the normality of the quantile-quantile plots (Q-Q plot). Following the normality test, the Kendall rank correlation coefficient (Abdi, 2007) was computed to examine the relationships between changes in heat-treated pollen and the corresponding effect on pollen viability, fruit set and regeneration. Analysis of variance (ANOVA) was used to determine the significant differences between in the treatments.

RESULTS

***In vitro* and *in vivo* viability and germination of pollen.** Pollen germination was evident across all heat treatments in the two genotypes studied (Table 1; Fig. 1). There were higher germination percentages for fresh pollen than in the heat-treated cases. Generally, pollen germination rates decreased with increased temperatures, with evidence of a negative correlation (r = - 0.72). For example, pollen heated at 50 to 60 °C registered germination percentages of 0.0 and 0.0-2.3% for NASE3 and NASE14, respectively (Table 1). Fresh (non-heated) pollen grains exhibited varying germination percentages and pollen tube growth (Fig. 1, Table 1). NASE14 fresh pollen

TABLE 1. Pollen treatment, *in vitro* pollen germination, pollinations and the results of Fruit Formation (FF), ovule Formation (OF) and calli (CF) in cassava genotypes NASE3 and NASE14. Plants pollinated with heat-treated pollen at 40 °C were not considered under experiment 2

Genotype		Experiment 1						Experiment 2				
	Heat-treatment of pollen	Flowers pollinated	Mean fruit formation	Mean ovule formation	Mean callus formation	Germination rate (%)	SD of Germination	Total fruit set	Mean fruit set	SD of fruit set	Total ovules	Number of embryos
NASE3	40°C_0.5hr	189	55.5	16	12.8	7.6	2.1					
	40°C_1.0hr	213	33.7	19.2	8	4	1					
	40°C_2.0hr	176	39.6	13.7	8.5	26.7	5.8					
	50°C_0.5hr	289	38.6	66.6	41.2	0	0	8	3.2	1	14	2
	50°C_1.0hr	270	37.9	37.7	16.2	0	0	0	0	0	0	0
	50°C_2.0hr	483	58.8	33.6	7	0.2	0.3	4	0.5	0.7	5	2
	60°C_0.5hr	284	27.8	73.3	26.2	0	0	11	2.7	3.8	20	8
	60°C_1.0hr	355	49.7	56.4	13.8	0	0	14	5.7	4.5	20	12
	60°C_2.0hr	401	15.8	50.3	3	0	0	8	1.2	1.7	10	3
	Fresh Pollen	338	59.6	59.5	37.5	60	10	95	34.7	42	203	19
		2998	μ = 40.4	μ = 42.6	μ = 17.4							
NASE14	40°C_0.5hr	226	8.4	84.7	35	12.4	8.2					
	40°C_1.0hr	226	27.4	46.8	10	3.6	3.8					
	40°C_2.0hr	345	21	79.8	1.8	62.9	5.8					
	50°C_0.5hr	275	3.4	68.8	35	0.7	1.2	2	4.4	6.2	4	2
	50°C_1.0hr	229	14.7	37.2	7.5	0.1	0.2	4	8	11.3	0	0
	50°C_2.0hr	345	12.3	49.5	2	1.3	2.3	1	1.2	1.7	1	0
	60°C_0.5hr	259	4	71.9	35	0	0	5	1.1	1.5	10	3
	60°C_1.0hr	312	17.1	55.1	9.5	0	0	2	0.4	0.5	5	1
	60°C_2.0hr	355	23.1	60.4	3.5	0	0	4	0.7	1	8	2
	Fresh Pollen	186	27.6	95	51.2	72.8	11.8	24	26	23.9	65	40
		2758	μ = 15.9	μ = 64.9	μ = 19.1							

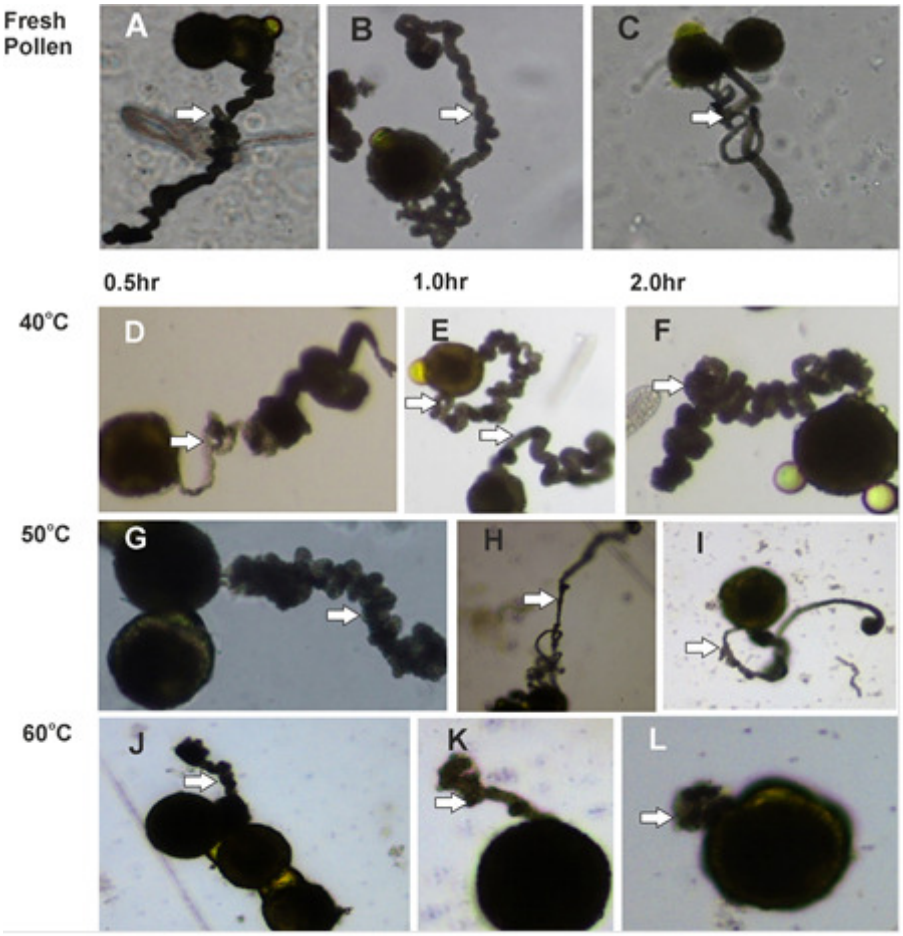

Figure 1. *In vitro* germination of pollen derived from cassava genotype NASE3. Germination of fresh pollen (A, B and C) in comparison germination of pollen treated with temperatures of 40°C, 50°C and 60°C for different durations of 0.5, 1.0 and 2.0 hrs (D to L). In all cases, the thread-like structures are the pollen tubes (white arrows) arising from the germinated pollen. Pollen germination and pollen tube development at 60°C (J, K, and L) was poor. Observations on pollen from cassava genotype NASE14 (data not presented) were similar to those of NASE3.

had the highest germination percentage (72.8%) compared to NASE3 (60.0%). A significant difference was observed in pollen germination rates of the test genotypes ($P<0.05$) (Table 1), with NASE3 responding more to the heat treatment compared to NASE14.

In vivo pollen germination was observed in fresh and heat-treated pollen in NASE3 (Fig. 2) and NASE14 (Fig. 3). Indeed, pollen heated at 60 °C for 2.0 hr allowed for the pollen tube growth to the embryo sac, without fertilisation at 3 DAP. In contrast, pollen tubes from fresh pollen grew to reach the embryo sac region within 24 hr after pollination (Fig. 2, Fig. 3). Pollen tubes from heat-treated pollen (60 °C for 0.5 hr) germinated on the stigma and formed pollen tubes, which penetrated through the cassava pistil, up to the nucellar beak (Fig. 2D1, Fig. 3D1). Histological examinations on ovules following self-pollinations with heat-treated pollen, showed degeneration of the embryo sac 14 DAP (Fig. 2E2-E4). This was evidenced by the presence of many empty

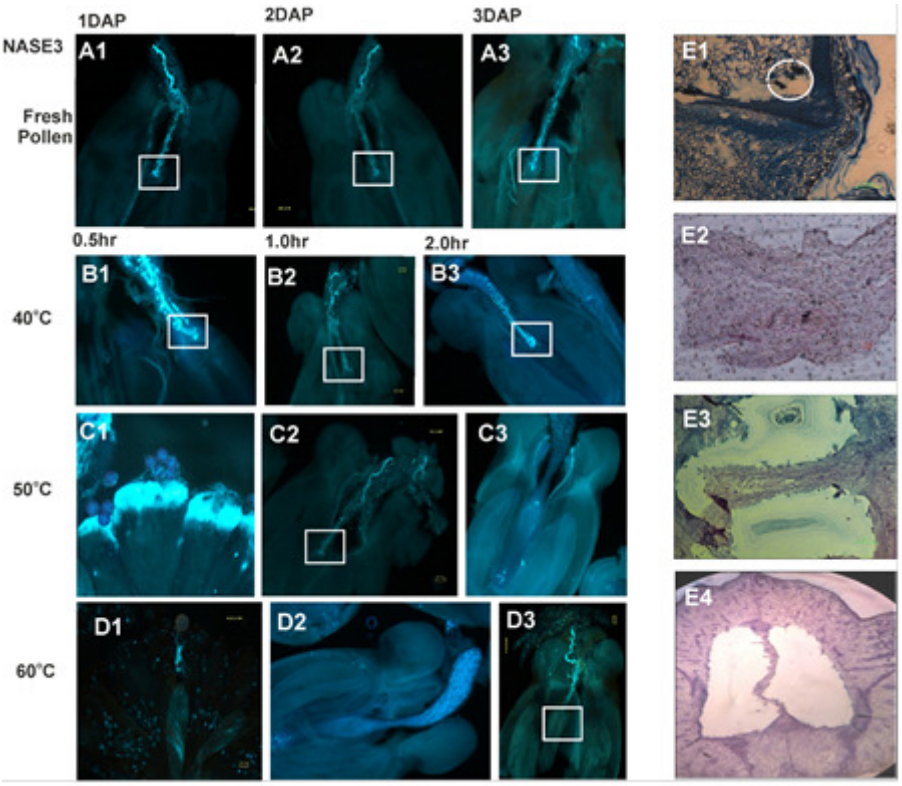

Figure 2. *In vivo* pollen germination on the stigma and pollen tube penetration at 1, 2 and 3 DAP along the style of cassava genotype, NASE3. Pollen tube from fresh pollen entered the embryo sac region (white squares in A1-A3) by 1DAP. Pollen tube penetration at 1, 2, and 3 DAP after pollination with heat-treated pollen at different temperatures (40, 50 and 60 °C) for different periods (0.5, 1.0 and 2.0 hrs) are shown in B1 to D3. Pollen tube arising from different heat-treated pollen penetrated into the embryo sac region (white squares in B1-B3, and C2). In C1, C3, D1 and D2 the pollen germinated on the stigma but there was no formation of pollen tube, while in D3 the pollen tube could not penetrate the embryo sac region (white square). E1-E4 show NASE3 embryo development 14 DAP with fresh pollen (white circle in E1), whle E2-E4 shows cell ploriferation in the embryo sac region on 21 DAP with 60 °C for 0.5 hr-treated pollen (E2), and degeneration of embryo sac region on pollination with 60 °C/2.0 hr heat-treated pollen (E3, E4).

embryo sacs with disorganised tissues (Fig. 2E3).

Seed/fruit set and embryo development. A total of 2,998 of NASE3 and 2,758 of NASE14 female flowers in both cases were self-pollinated (Table 1). In NASE3, the highest fruit retention capacity of 58.8% was observed at 50 °C for 2.0 hr, compared to 28.1% as highest in NASE14 observed at 60 °C for 2.0 hr (Fig. 4A). In both NASE3 and NASE14, fruit set was highest when fresh pollen was used in pollination, compared to the 93-100% reduction in fruit set as result of heat-treated pollen (Table 1).

Dissection and examination of approximately 500 ovules showed that the number of ovules per fruit was 42.6 for NASE3 and 64.9 for NASE14 (Table 1). Inoculation of immature ovules onto MS3 culture medium resulted into the formation of calli from several ovules, three months after initiation on culture (Fig. 5). Multiple embryos emerging from single ovules pollinated with

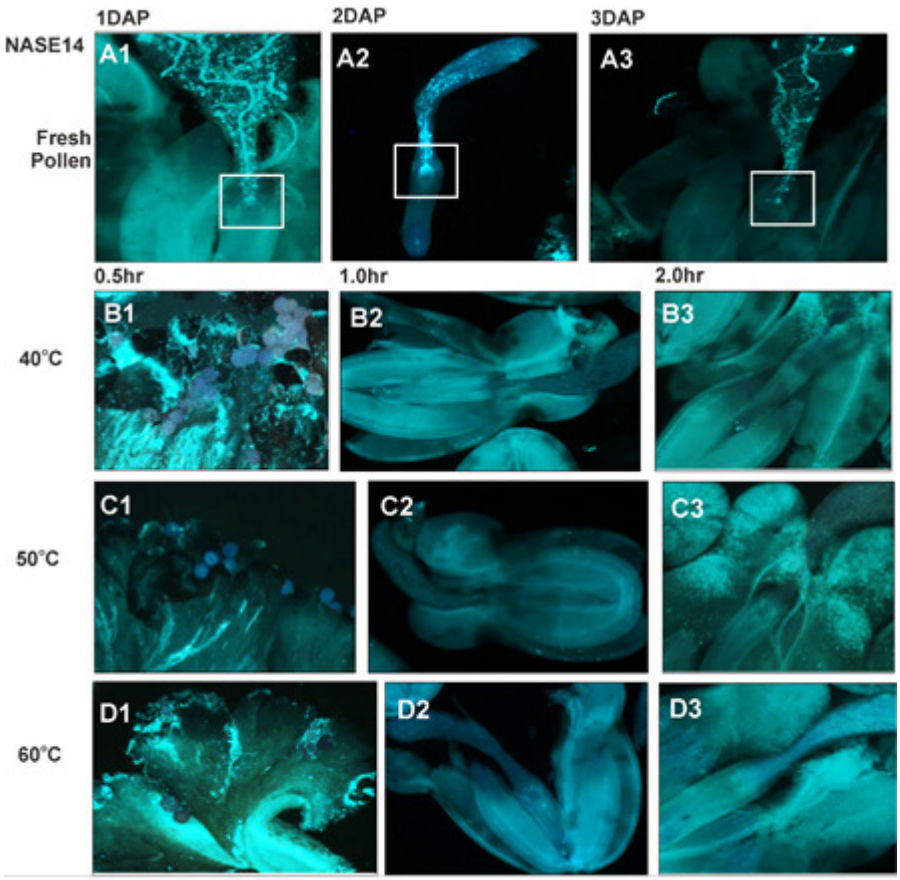

Figure 3. *In vivo* pollen germination on the stigma and pollen tube penetration at 1, 2 and 3 DAP along the style of cassava genotype, NASE14. Pollen tube from fresh pollen entered the embryo sac region (white squares in A1-A3) by 1DAP. A1 is a massive pollen tube in the nucellar beak penetrating into the embryo sac region. Pollen germination at 1, 2, and 3 DAP after pollination with heat-treated pollen at different temperatures (40ºC, 50ºC, 60ºC) for different periods (0.5, 1.0 and 2.0 hrs) are shown in B1 to D3. Pollen heated for different periods at different temperatures were able to germinate on the stigma surface without formation of pollen tube into the embryo sac region (B1-B3, C1-C3, and D1-D3).

fresh pollen at 14 DAP, were observed (Fig. 5A, 5B and 5C). Ovules obtained from fruits picked at 14 DAP increased in size and later formed callus in both genotypes. Generally, ovule formation (OF) and callus formation (CF) decreased as the temperature and time duration increased. The highest OF and CF were recorded from pollinations made using in fresh pollen of NASE14 at 95.0 and 51.2%, respectively (Table 1). In both NASE3 and NASE14, callus formed from the ovules were mucilaginous, transparent and friable; but in some cases it appeared like a compact mass of cells (Fig. 5E). For ovules subjected to heated pollen of 50 ºC for 1.0 hr, callus was observed coming from the inside of the ovules (Fig. 5H). Some of the callus formed green spots (Fig. 5K), and rooting was also observed (Fig. 5L).

Plant regeneration and characterisation. Regeneration capacity generally varied across the genotypes (Fig. 4B). Only the embryos obtained from ovules at 21 DAP heat-treated and 14 DAP non-treated; and cultured *in vitro* developed into individual plants after 3-4

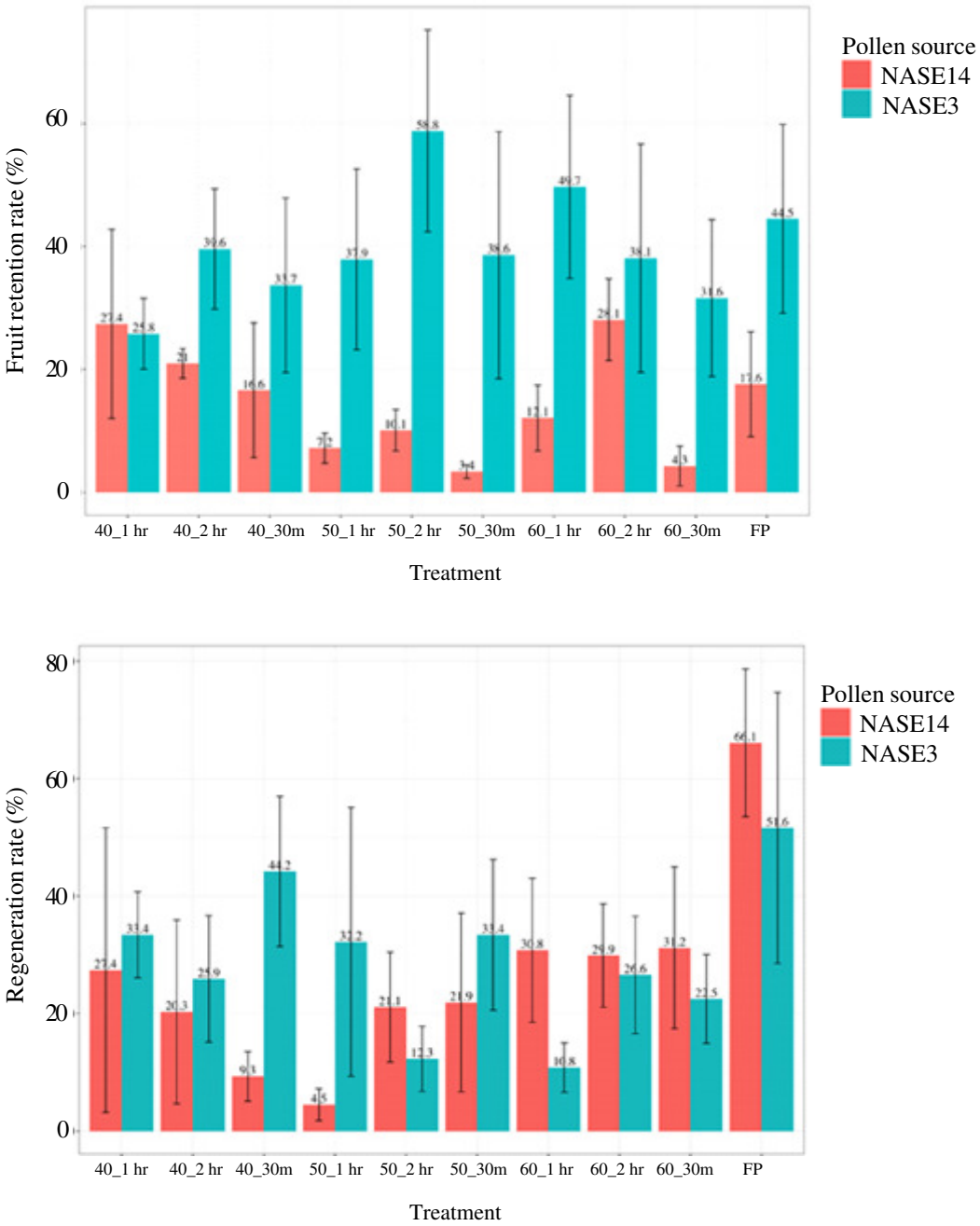

Figure 4. Fruit retention capacity (A) and plant regeneration rate (%) (B) of genotype NASE3 and NASE14. There was considerable variation in fruit set as evident across genotypes and treatments (A), although increase in temperature resulted in higher fruit abortion. Highest plant regeneration was observed when Fresh pollen was used with evidence of drastic reduction in regeneration at different temperatures (B).

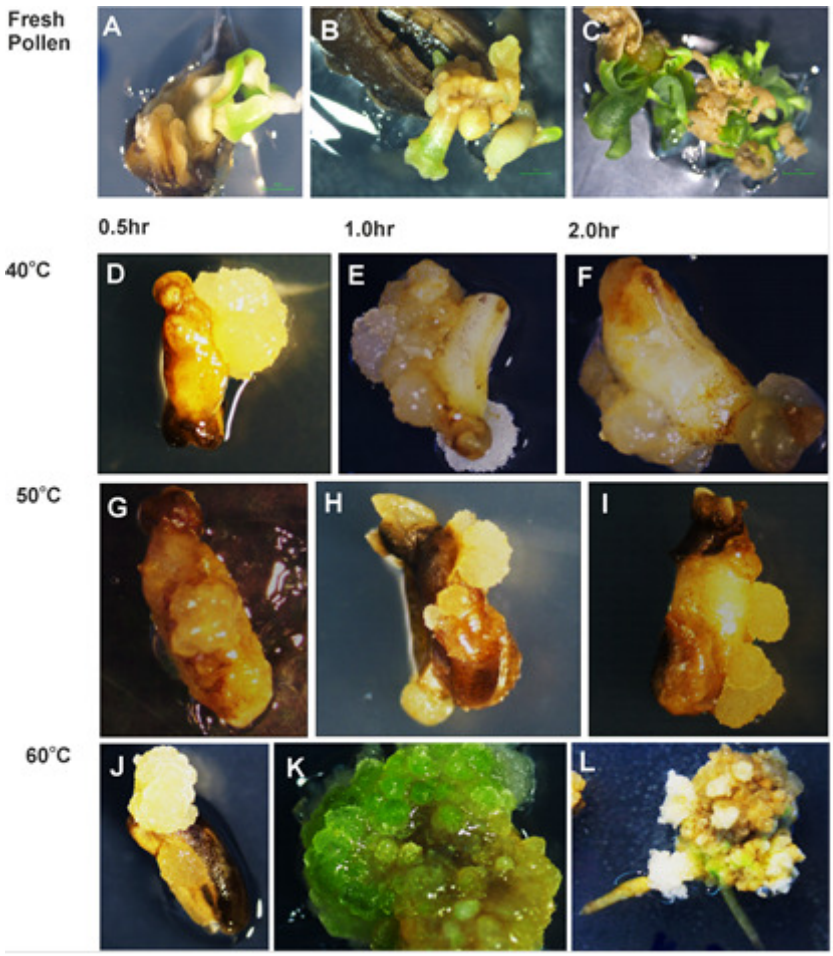

Figure 5. Callus regeneration and direct embryo formation following early ovule culture 14 days after pollination (DAP) for cassava genotype NASE3. A-C: Direct embryo formation from ovules formed from self-pollinations with fresh pollen; (A) embryo formation on culture from ovule pollinated with fresh pollen (B), polyembryos emerging from an ovule following pollinated with fresh pollen, and (C) regeneration of embryos. D to L shows calli regeneration from ovules following self-pollination with pollen treated with heat (40, 50 and 60 °C) for different durations of 0.5, 1.0 and 2.0 hr. Compared to other calli, those generated from ovules pollinated with 60 °C-treated pollen (J, K and L) show compact green calli on regeneration and formation of roots form calli transferred on rooting medium. These observations were the same for cassava genotype NASE14.

transfers onto fresh medium (Fig. 6I). Different embryo types were formed from fruits pollinated with heat-treated pollen at 60 °C for different periods. Up to 40 embryos were obtained using fresh pollen in NASE14, and this was higher than the 19 embryos in NASE3 (Table 1). Although there was no significant difference ($P>0.05$) in regeneration between the two test genotypes, recovery frequency of the embryos for the treatments was generally higher in NASE3 at 60 °C for 1.0 hr duration than at 50 °C for 1.0 hr (Table 1, Fig. 4B). Regeneration of plants (Fig. 6) was not dependent on heat treatment imposed on the pollen ($r = 0.047$, $P>0.05$). The majority of plants obtained were diploids (Table 2).

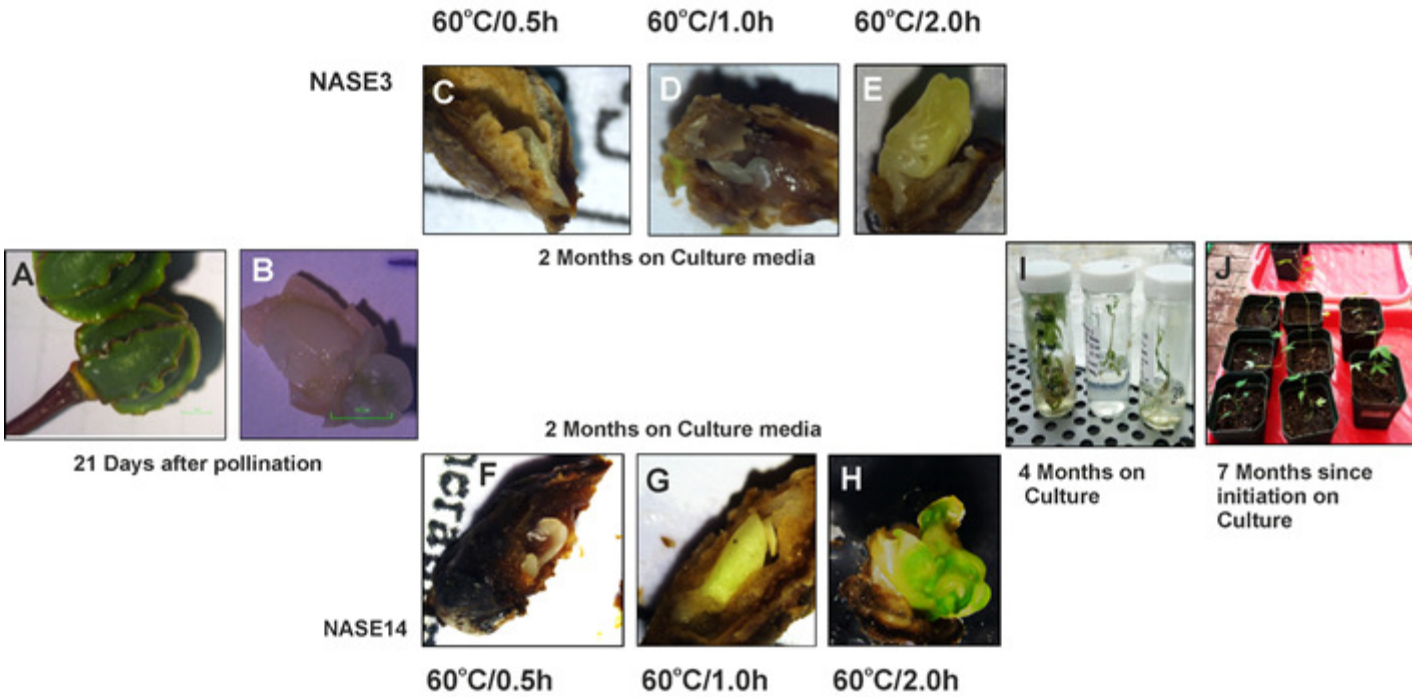

Figure 6. Embryo formation from rescued ovules. Fruits (A) of cassava genotypes NASE3 and NASE14 were picked 21 DAP; their ovules (B) excised out and inoculated on the culture medium. NASE3 (C to E) and NASE14 (F to H) embryos formed from self-pollinations with pollen heated at 60 °C for various durations. C and D: the ovules have no endosperm, E: embryos later regenerated into plants. F: ovule has no endosperm compared to G and H. I and J: Regenerated plants of NASE3 60°C/1.0hr arising from D on MS medium and weaned in the screen house 7 months after initiation on ovule culture. No plant was regenerated from NASE14 or ovules arising from self-pollinations with other heat-treated pollen.

TABLE 2. Ploidy and homozygosity levels in plantlets obtained from fruits of cassava following self-pollination of the flowers with heat-treated pollen in NASE3 and NASE14 cassava genotypes

Crosses	Sample mode	Mean	CV%	Ploidy level	Treatments	Number of heterozygous loci		Number of homozygous loci in progeny	Percentage homozygosity in progeny
						Mother sample	Progeny sample		
NASE3 X NASE3	101	99.97	4.75	2X	60°C_0.5hr	5932	390	6370	93.0
NASE3 X NASE3	100	99.13	4.79	2X	50°C_2.0hr	5932	4793	16616	19.0
NASE3XNASE3	100	98.7	5.31	2X	60°C_1.0hr	5932	461	6295	92.0
NASE 3	99	98.37	4.32	2X	Zero(0)	5932	3656	16236	38.0
NASE14 x NASE14	83	82.16	10.04	3X	60°C_2.0hr	4287	859	12876	80.0
NASE3 X NASE3	101	99.37	5.79	2X	60°C_1.0hr	5932	7381	14113	20.0
NASE3 X NASE3	102	99.48	5.78	2X	60°C_1.0hr	5932	442	1182	93.0
NASE3 X NASE3	99	98.31	5.85	2X	60°C_1.0hr	5932	3513	17272	41.0
NASE3 X NASE3	101	101	4.7	2X	60°C_2.0hr	5932	944	12732	84.0

Plants of increased homozygosity of 92.0-93.0% were recorded for NASE3 treatment of 60 °C for 0.5 and 1.0 hr duration (Table 2). Plants were validated for homozygosity by analysing 22,618 SNPs and flow cytometry analyses, which showed variable homozygosity depending on the treatment ranging from 24.0-93.0% (Table 2).

Multiple embryonic structures observed led to production of multiple plantlets per embryo, in embryos from fresh pollen, and regeneration efficiency of embryos into plants was low in NASE14 as compared to NASE3 (*data not shown*).

Changes in the colour of embryogenic structures were observed from off-white to green after transfer to 16/8 light/dark photoperiod for 56 days on half-strength basal MS medium with 0.09M sucrose (Fig. 5). After colour change, multiple embryoids were isolated (Fig. 5) and transferred to the same medium for plantlet regeneration, of which some of the regenerated plantlets developed yellow shoots (*data not shown*). These plantlets stopped growing and eventually died; while green shoots developed normally (Fig. 5) into whole plantlets and were hardened-off in a growth chamber, and transferred to the screen house (Fig. 6).

Ploidy and homozygosity of plantlets. Ploidy analyses revealed that most of the plant samples were diploid (2X), apart from NASE14 x NASE14 at 60 °C for 2 hr, which had increased level 3 of homozygosity of 80.0%; and NASE3 x NASE3 (at 60 °C at 0.5, 1.0, and 2.0 hr) with increased levels of homozygosity to 93.0% (Table 2). Zygosity analyses in the mother and progeny samples, showed increased homozygosity at varying degrees of 19.0% at 50 °C for 2.0 hr to 93% at 60 °C for 1.0 hr (Table 2).

DISCUSSION

Pollen germination and pollen tube growth. High temperature treatments in cassava reduced pollen germination and pollen tube

growth, *in vitro* and *in vivo*, and these processes were completely inhibited at 60 °C (Table 1), indicating possible inhibition of fertilisation at this or higher temperatures. Inhibition of germination and pollen tube growth was previously reported under high temperatures in almond (*Prunus dulcis*) (Sorkheh *et al*., 2018) and common hazel (*Corylus avellana*) (Çetinbaº-Genç *et al*., 2019). Accordingly, high temperatures caused destructive effects to the pollen by de-polymerisation of actin cytoskeleton by preventing production of a functional pollen tube (Çetinbaº-Genç *et al*., 2019). The development of pollen tubes is essential because they are the only passage for two sperm cells to reach the embryo sac for effecting fertilisation. As such, pollen germination and pollen tube growth analyses are critical for understanding plant reproduction (Obermeyer and Feijó, 2017). In this study, self-pollination with heat-treated and fresh pollen revealed the presence of developed pollen tubes reaching the embryo sac region at 1 DAP for fresh pollen, as was previously observed in *Manihot mill* species by de Jesus *et al.* (2015). For heat-treated pollen, germination was observed only on the stigma in both NASE3 and NASE14 genotypes, though no penetration in the nucellar beak or embryo sac region was observed, except for NASE3 60 °C at 3 DAP, whose pollen entered the nucellar beak but not the embryo sac region.

Stimulating the division of the egg cell without fertilisation, and thus inducing parthenocarpic embryo development in plants, can be achieved using heat-treated pollen (Winton and Einspahr, 1968) and this was observed in our study (Fig. 6D). A similar observation was made in pollen grains of *Brassica juncea* exposed to high temperature (45, 60 and 75 °C for 4-24 hr), in which pollen germination and pollen tube growth induced parthenocarpy (Rao *et al*., 1992). Further studies are needed to understand biochemical and/or physiological changes that occur in cassava pollen when subjected to high temperatures, leading to failure of pollen tube to reach the embryosac and archiving no fertilisation in cassava. Any impact of temperature is not expected to depend on self- versus cross-pollination because the mode of pollination in cassava was recently shown to be irrelevant for pollen tube growth and development (Ramos Abril *et al*., 2019). Failure of cassava pollen tube to reach the embryosac under this study is similar to previous observations in maize in which isolated pollen lost its fertilisation abilities when subjected to heat treatment of 40 °C for 4 h (Dupuis and Dumas, 1990). Also, heat stress treatment resulted in the reduction of pollen viability of faba bean (Kumar *et al*., 2016) and low germination rate and poor pollen tube growth of pedunculate oak (Sever *et al*., 2012). Moreover, after 6 days of heat stress, the pollen viability and germination of flax was adversely affected (Cross *et al*., 2003). Steps in pollen tube development in cassava shows a major slowdown in pollen tube entry into the embryosac (Ramos Abril *et al*., 2019) and how this development slowdown may get further decelerated due to heat stress on the pollen is unclear. Studies in *Arabidopsis thaliana* delineate four stages of pollen tube development (Kandasamy *et al.*, 1994) and a related pattern of pollen tube development occurs in cassava (Ramos Abril *et al*., 2019). Essentially, the negative effect of heat stress could be felt at any of the phases of pollen tube development, although such effects in angiosperms' sexual reproduction are generally higher in earlier than later stages of pollen tube development (Hedhly, 2011; Snider and Oosterhuis, 2011; Muller and Rieu 2016; Raja *et al*., 2019).

Embryo rescue and fruit set. We present a new approach for embryo rescue in cassava in which, instead of traditionally isolating embryos, individual ovary carpels (containing one ovule each) can be cultured. This approach makes it possible to successfully

culture immature zygotic embryos before they are fully formed, while supporting the development of the proembryos inside the ovules by exogenous supply of nutrients from the culture medium and avoiding their physical damage in the handling process. This method of culture, in combination with an optimised rich culture medium composition sequence under this study, allowed rescuing embryos at very early stages of development collected at 14 DAP. Previously, cassava embryos could only be rescued not earlier that 28 DAP (Yan *et al*., 2014), indicating 50% improved efficiency in embryo rescue under our study.

Embryo rescue techniques are essential for supporting juvenile embryos that would otherwise not progress under *in vivo* development conditions; as such, successful embryo culture technique is dependent on both stage of development of the embryo and culture medium composition (Shahzad *et al*., 2017).

The observed reduction in fruit set and high fruit abortion in the two cassava genotypes following self-pollination with heat-treated pollen, was probably due to the presence of poorly developed or incompatible endosperm and early inhibition of embryo development. This was evidenced by the histological examinations done in this study, showing high degeneration of embryo sac region. Although not confirmed in our studies, it is also likely that the aborted fruits had fragile haploid embryos because of the numerous deleterious alleles fixed by clonal propagation (Ramu *et al*., 2017). In such cases, embryo rescue techniques are helpful in inducing the development of haploid embryos, by isolating and culturing whole ovaries, ovules or embryos (Dwivedi *et al*., 2015). This procedure was initially applied in *Phaseolus vulgaris* and *Fagopyrum tataricum*, which later developed to mature plants (Schopfer and Noecker, 1943). Previously, we employed a similar technique following self-pollination with irradiated pollen in cassava (Buttibwa *et al*., 2015), which allowed the regeneration of diploid cassava plants. Embryo rescue medium provides nutrients needed for embryo development (Mobini *et al*., 2015), which together with the stage of embryo at rescue may determine the success of embryo into maturation (Sharma *et al*., 1996).

In our study, embryos with high vigour were able to develop into plantlets, but those that were white in colour did not develop further, indicating that poorly developed embryos are a dead end to plant regeneration. Similarly, Relan (2017) showed that a small and poorly developed (< 0.5 mm) durum wheat haploid embryos failed to germinate, whereas those with a defined structure and vigorous appearance; and about 2 mm in length had high germination capacity. Therefore, well developed embryos in cassava can be rescued and plants successfully regenerated earlier than usually practiced.

Polyembryony. We observed a high rate of multiple embryo formation (polyembryony) from a single ovule in cassava self-pollinated with fresh pollen, indicating that polyembryony may be a norm of cassava's sexual reproductive systems (Fig. 5A, B and C). Previously, polyembryony was found at a frequency of 1% in the hybrid of cassava with *Manihot oligantha* produced *via* outcrossing, in which the extent of polyembryony was increased by polyploidisation (Nassar, 2006a, b; 2010). In cassava, whether polyembryony is more pronounced under cross or self-pollination is not clear. In many plants with polyembryonic reproduction, all the embryos, except one are usually aborted, creating an opportunity for reproductive compensation within ovules (Sorensen, 1982; Porcher and Lande, 2005a,b). Cassava is predominantly outcrossing; however, there is also no evidence of self-incompatibility, implying that in nature, self-pollinations may yield viable botanical seed and associated inbreeding depression (Westwood, 1990; Rojas *et al*., 2009; Ramos Abril *et al*., 2019). In contrast, possible low embryo survival in self-pollinations together with observed

polyembryony, may allow post fertilisation embryo abortion and selection, which may constitute a mechanism of maintaining heterozygosity in cassava, similar to observations in conifers (Sorensen, 1982; Williams and Savolainen, 1996).

The emergence of polyembryos in cassava may also be induced or enhanced by culture conditions. For example, in grapevines (*Vitis vinifera*), 4-5 times increase in vitamin and hormone levels in the culture medium increased polyembryony from 20.0 to 85.7% (Tsolova and Atanassov, 1994). In a fern, *Pteris tripartite* Sw, BAP cytokinin concentrations at 3 mg L^{-1} in MS medium significantly induced more polyembryony than lower concentrations (Ravi, 2016). In wheat, polyembryony was attributed to high concentrations of sucrose in the medium (Altamura *et al*., 2016). In our study, we used strong auxins such as 2, 4-D at 5 mg L^{-1}, but whether or not this may account for the observed polyembryony in cassava under culture remains to be verified.

Homozygosity and ploidy. Using 22,618 SNP markers to analyse zygosity, plantlets rescued from embryos at 21 DAP indicated increased homozygosity of up to 93.0%. Increased levels of homozygosity of up to 98.0% was previously observed in maize (Asker and Jerling, 1992), and up to 68.0% in cassava progeny obtained from flowers pollinated with irradiated pollen (Buttibwa *et al*., 2015). In the current study, 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) (Munné *et al*., 1995). Either way, increased homozysgosity observed under this study implies that any deleterious alleles could be eliminated from cassava during double haploid breeding. Cassava is predominantly outcrossing in nature, yet frequently propagated by vegetative means, which implies that the norm of heterozygosity has indeed fixed deleterious alleles and shifted the mutational burden toward common variants (Ramu *et al*., 2017). Indeed, in cassava, deleterious mutations are the main inherent constraint associated with inbreeding depression (Ceballos *et al*., 2016; de Freitas *et al*., 2016; Ramu *et al*., 2017).

Even in elite cassava accessions, the effects of inbreeding depression are extremely severe in which a single generation of inbreeding may lead to over 60.0% decrease in fresh root yield hence the need for the homozygous lines in cassava breeding (de Freitas *et al*., 2016). Therefore, the observed increased homozygosis should facilitate purging of deleterious alleles that for long are masked in by heterozygosity and facilitate pure line breeding in cassava (Ramu *et al*., 2017).

CONCLUSION

This study highlights the effect of heat-treated pollen from cassava in self-pollinations and the associated high incidence of polyembryony and parthenocarpy in the development of doubled haploids. Self-pollination with heat-treated pollen and the technique of embryo rescue allowed for recovery of plants in cassava that can be further developed into haploids, which provides opportunities for double-haploid (DH) plants.

Results of this study indicate successful fruit set, induced by heated cassava pollen, and increased homozygosity in cassava embryos, following pollination of cassava female flowers with heat-treated pollen. For the first time, cassava proembryos were regenerated from ovules rescued 14 DAP. Until now, this was possible only earliest 28 DAP, indicating improved efficiency of our embryo rescue procedure. Limited studies show a general recalcitrance in respect to development of DH protocols *via* both gynogenesis and androgenesis in cassava in comparison to other crops whose DH protocols have undergone rigorous optimisation over decades in numerous laboratories. Increased homozygosity in cassava observed under this

study can be exploited in eliminating disadvantageous alleles that have long been hidden in their heterozygous stare. Overall, the 'unit of success' reported in this study contributes to efforts towards development of DH cassava for the enhancement of cassava breeding.

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