

In vitro Culture of Heat-treated Anthers Induces Embryogenic Callus in Cassava (*Manihot esculenta* Crantz)

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ABSTRACT

Microspore embryogenesis is the developmental plasticity of juvenile male gametophytes to switch from pollen to embryo development upon exposure to *in vitro* stress. It is a common method for obtaining haploid and doubled haploid plants in breeding programs for development of superior varieties via haploid-diploidization which allows fast development of homozygous lines from heterozygous parents. In cassava, obtaining haploidization through traditional methods of successive selfing is difficult because of cassava's long reproductive cycle, high heterozygosity, and inbreeding depression. As a first step towards microspore embryogenesis, callus induction following heat treatment has not been investigated in cassava. We used two elite Ugandan cassava varieties, hereafter called "genotypes", NASE3 and NASE14, for the study of callus induction. Heat stress of 40 °C for 0, 6, 12, 18 and 24hrs and Murashige and Skoog medium supplemented with 2-9% sucrose, and 2,4-dichlorophenoxy acetic acid were used. Heating anthers of NASE3 at 40 °C for 6 hrs resulted in a significantly higher percentage of callus induction on MS medium supplemented with 2% sucrose. Callus emerged from inside of the anthers with production influenced by genotype, sucrose concentration, anther density and duration in culture ($P \leq 0.001$). Limited *in vitro* callus differentiation was observed on auxin-and cytokinin-supplemented media. In both genotypes, embryo genic callus was obtained in liquid medium, while green callus was achieved on solid medium. This is a significant step upstream of double haploid plant production pathway in cassava: rigorous optimization of protocols downstream of callus induction are needed for regeneration of microspore-derived embryos and haploid plants.

Keywords: Anther culture; Cassava (*Manihot esculenta*); Microspore embryogenesis; Doubled haploids; Sporophytic development

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) can grow and perform on marginal soils with minimum farm inputs and still yield up to 40-50 tons ha⁻¹ year⁻¹ [1]. In addition, high tolerance to climatic changes [2] and suitability for piece-meal harvesting make cassava a crop of choice in the fight against hunger in sub-Saharan Africa. Moreover, cassava is an important source of starch contained in the storage roots for many downstream industrial applications

[3]. The increasing demand of cassava roots, therefore, requires faster means of generating new genotypes amenable to food/feed or industrial needs. Cassava breeding holds a great promise in providing improved genotypes suitable for various uses and environments [4].

Naturally, cassava is an out-crossing monoecious species, a sexual reproduction strategy that is associated with high heterozygosity. High heterozygosity in cassava is a constraint to the genetic

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improvement of cassava through conventional breeding methods because many rounds of backcrossing must be undertaken prior to overcome unwanted traits [5]. Under such circumstances, production of homozygous cassava lines would enhance genetic improvement and allow faster production of new genotypes for diverse needs [4]. One of the means through which homozygous cassava lines can be produced, is the use of isolated microspore culture (IMC). IMC is a unique system in plants during cell development, where a particular stress treatment results in reprogramming of microspores by switching from the default developmental gametophytic pathway into embryogenesis [6]. In this process, named androgenesis, plant regeneration is archived from microspore culture under *in vitro* conditions in which the normal development into pollen cells is stopped and instead force their development directly into a complete plant [7,8]. This system can promote the production of homozygous lines in cassava and significantly accelerate the production of cassava hybrids because in a single generation, doubled haploid (DH) techniques can be used to produce homozygous lines. Apart from use in production of hybrids, homozygous lines have numerous applications such as the ease to generate induced mutations to study gene function, chromosome reduction of polyploid species, gene or quantitative trait loci mapping, and other genomics studies [9].

Usually, development of inbred-lines requires several cycles of inbreeding. However, using anther culture, the process can be reduced to a single generation, which can be equivalent to a year of cassava growth under field conditions. The first bottleneck is in the ability to regenerate haploid plants from microspores in the anthers which determines the effectiveness of the anther culture technique to be employed. The second bottleneck is the capacity to convert the haploids to doubled haploids.

Microspore culture results in true-breeding lines in the subsequent generation. Like other haploid-inducing techniques, androgenesis is genotype-dependent, and can be influenced by the physiological state and growth conditions of donor plants. The growth stage of male gametes, pretreatment of flower buds or anthers also determine the success of this method. The composition of the culture medium, and physical factors during culture together with their interactions also determines the success of the method [10]. Physiochemical factors cause stress responses that halt microspore development or young pollen grains in their gametophytic pathway. Such factors include temperature pre-treatment, sucrose and nitrogen starvation, and osmotic stress [11].

In cassava, the limited reports on anther culture show that it is possible to produce callus [12] and the difficulty in generating haploid plants. However, whereas no plant regeneration was achieved, the induction of cassava microspores towards sporophytic development is possible [13]. The aim of this study, therefore, was to develop a reproducible protocol to induce callus from the microspores enclosed in anther lobes as a first step towards development of doubled haploid plants in cassava via anther culture.

MATERIALS AND METHODS

Cassava plants and growth conditions

Two elite 'Ugandan' cassava genotypes, NASE3 and NASE14 (used as the donor plants for the anthers) were grown at National Crops Resources Research Institute (NaCRRI), Namulonge, Uganda. These genotypes were selected because of their ability to flower profusely and also being preferred by farmers. To have flowers all year round, plants were established in a staggered fashion at 15-day intervals. Fields were generally managed according to local cassava production practices with weeding done using a hand-held hoe. No application of agrochemicals was done [14].

Flower bud sterilization

To keep fresh, inflorescences harboring male flower buds were collected from the field early in the morning before sunrise (06:00-07:30 hours) and transferred to the laboratory. In the laboratory, male flower buds of 2.0-2.8 mm in diameter containing developmental stages of tetrads were isolated according to Perera et al. (2013). The isolated flower buds were subsequently washed under running tap water and soap and then dried on filter papers for 10 min. To surface sterilize the buds, dried flower buds were placed in a laminar flow hood and placed in 70% ethanol for 1 min, followed by 10% (v/v) sodium hypochlorite (NaOCl) solution (Jik) supplemented with 60 µl/L Tween 20 for 20 minutes. Using sterile water, the disinfected flower buds were then washed four times for 2 minutes each.

Heat stress treatment and anther culture

To subject the male flower buds to heat shock treatment, the flower buds were sealed in the Petri dishes using parafilm and wrapped in aluminum foil while ensuring air-, light-, and water-tight conditions inside the sealed Petri dishes. A water bath was set up at 40 °C into which sealed Petri dishes containing the male flower buds were submerged for 6, 12, 18 and 24 hours. Metallic weights of 200g were placed on top of the Petri dishes to keep them submerged in under hot water. Untreated buds (0 hours) were used as controls.

Thereafter, buds were dissected to release the anthers of different densities of 20, 40, 60, 80 and 100 anthers per plate (equivalent to 2, 4, 6, 8, 10 male flower buds in each case). The anthers were cultured on a modified Murashige and Skoog (MS) medium [15] similar to that used by Lentini et al. 1995 [16]. MS medium was supplemented with 2.5mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 800 mg/L glutamine and varied concentrations of sucrose 2-9% (w/v) with the pH adjusted to 5.8 before autoclaving for 15 min, at 120 °C. After autoclaving, 100 mg/L 2-(N-morpholino) ethanesulfonic acid was added to the culture medium and used for culturing the anthers. The rates of callus formation were calculated by counting the number of anthers that became enlarged, formed callus and later embryos relative to the starting number of cultured anthers.

Five replicates were considered per treatment for each experiment. Anthers were cultured on a 60 × 15cm plate, using MS medium supplemented with 2-5 mg/L 2,4-D, 800 mg/L glutamine and different concentrations of sucrose. Cultures were kept in the dark at 28°C until enlargement, and callus formation were monitored. Observations of callus development and growth were made using Nikon light microscope and corresponding images of callus captured using Nikon digital sights DS-Fi1c camera (Nikon, 1300 Walt Whitman Road Melville, NY 11747-3064, USA). Different experiments were carried out to study the effect of anther density, sucrose concentration, and duration in culture on callus response.

Callus regeneration

To determine the frequency of callus formation following culture in the dark at 28°C, the number of callus-bearing anthers were recorded. This best temperature shock pre-treatment (40°C for 6 hours) was selected for pre-treatment. Callus was then transferred to the regeneration medium containing MS basal salts supplemented with 3% sucrose and phytigel 3.5% (w/v) and 1 mg/L 6-Benzyl amino purine (BAP) and 0.5 mg/L 1-Naphthalene acetic acid (NAA) for one month. Embryogenic callus formed were further cultured onto the same medium containing only 0.5 mg/L BAP at 1-month intervals until embryos were formed.

Histological analysis of callus derived from anthers

Anthers were sampled 2, 8, and 16 weeks after culture initiation. Histological analysis was performed on ten cultured anthers subjected to heat treatments. Twenty (20) anther-derived structures were sampled at different stages of culture ranging friable calli to globular embryos for the aim of observing embryogenic callus for evidences of various stages of differentiation. Thus, callus was sampled and fixed in Formalin-acetic acid-alcohol (FAA) (composed of 50% ethanol, 10% formaldehyde, and glacial acetic acid, 18:1:1) for 72 hours. The callus was then dehydrated through a series of graded ethanol of 30, 50, 70, 95, and 100% (v/v), followed by 100% butanol for 48 hours. Impregnation was followed by embedding the samples in Technovit 8100 resin (Heraeus Kulzer GmbH, Wehrheim, Germany).

Sample sections of 3.5-5.0µm thick were made using a rotary microtome (Histostat, Reichert Scientific Instruments) and thereafter were double stained with periodic acid Schiff's reagent (Sigma-Aldrich) for starch- and protein-specific Naphthol Blue Black. All sections were studied under a light microscope (DM500, Leica GmbH, Wetzlar, Germany) and photographed with an attached camera (ICC50HD; Leica GmbH, Wetzlar, Germany). Observations for meristematic cells and vascular elements were using an inverted microscope (Nikon Alphaphot-2 YS2, Japan) equipped with a 100W mercury lamp and a UV light excitation as done previously by Kundu et al. 2014 [17].

Data collection and statistical analysis

Statistical analysis was performed using R-statistical package (R Core Team 2014) on five experiments consisting of five replicates

per treatment. The data collected included the undeveloped anthers, swollen anthers, anthers producing callus and anthers producing embryos recorded for each Petri dish. Analysis of variance (ANOVA) was used to determine significant differences between responses in the treatments at P=0.05.

RESULTS

Androgenesis induction and development

At the time of inoculation, microspores were observable through a thin wall of anthers in NASE14 (Figure 1A) and NASE3 (Figure 1M). Cultured anthers for both genotypes became swollen in 28 days on MS culture medium and induced callus (Figure 1B and Figure 1N). Callus was observed forming inside the anthers (Figure 1D and Figure 1O). Anthers from both genotypes contained highly friable and fragile callus.

At 45 days of culture, callus development through the anther wall was observable (Figure 1F and 1G, Figure 1P and 1Q). At 45 days, embryogenic callus was also observed (Figure 1H and Figure 1S). Globular-embryos were induced from embryogenic callus after 20 days on MS medium containing BAP and NAA (Figure 1F). Torpedo embryos were also observed as shown in Figure 1I. A line of weakness on the anther wall open up and callus was coming from the inside separated lines (Figure 1P and Figure 1Q).

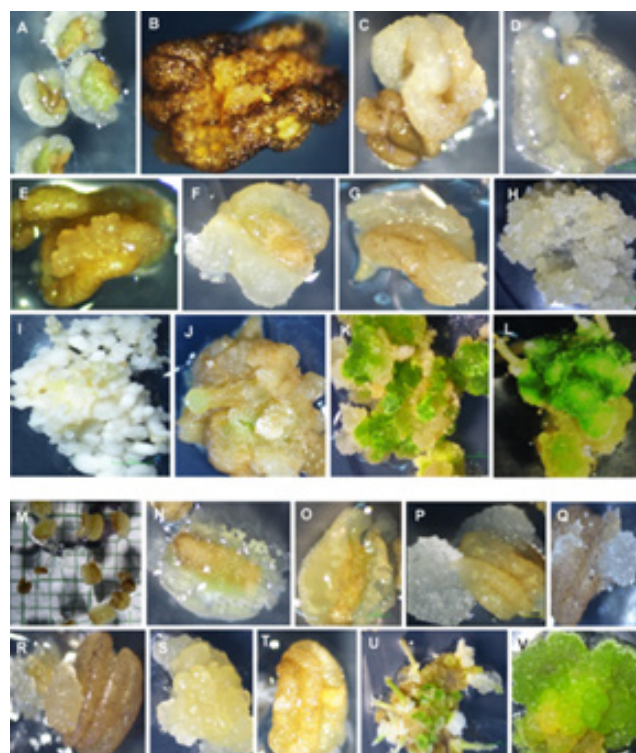


Figure 1: Callus induction and gametic embryogenesis of cassava genotypes (*M. esculenta* Crantz). NASE14 (A to L) and NASE3 (M to V). NASE14: (A) Fresh anthers on induction; (B) Callus wall dissolving exposing the microspores; (C) Bulging anthers on

induction medium; (D) Anther becomes transparent exposing the callus inside the anther; (E) Callus proliferation emerging from the interior of the anthers; (F-G) Calli proliferation coming out from inside the anther; (G) Embryogenic callus on multiplication medium; (I) Globular embryos forming on MS basal salts medium; (H) Shoot formation; (J-L) Chlorophyll pigmentation in the secondary embryos after exposing to the light (556 μm); (L) Adventitious root development on the callus placed on rooting medium. NASE3: (M) Fresh anthers on induction; (N) Calli wall dissolving exposing the microspores; (O) Swollen anther and callus inside the anthers on induction medium; (P-Q) Different stages of callus proliferation emerging from the interior of the anthers; (R) Calli coming out from anther line of weakness; (S) Globular-embryos forming on MS basal salts medium; (T) Callus coming out from the anther wall; (V) Chlorophyll pigmentation in the secondary embryos after exposing to light at 556 μm ; (U) Adventitious root development on the callus on rooting medium.

The anthers without heat treatment formed callus following culture. Callus proliferated, filling the petri dish on both liquid and solid medium within 30 days (data not shown). The highest percentage of enlarged anthers (43.75 ± 1.0) and callus formation (32.00 ± 0.9) was recorded in NASE14 on solid media (Table S1). However, on liquid medium, the highest percentage of enlarged anthers (35.50 ± 0.9) and callus formation rate (6.80 ± 1.0) was observed in NASE3 (Table S1).

Table 1: Percent of callus and pro-embryo formation from cassava genotypes, NASE14 and NASE3.

Genotype/Medium	Undeveloped anthers	Enlarged anthers	Mean	Calli formed	Mean	Pro-embryos	Mean	Mean
Solid medium								
NASE3	66.50 ± 1.3	13.3	33.25 ± 0.9	6.65	12.50 ± 0.5	2.5	3.75 ± 0.2	0.75
NASE14	55.00 ± 1.1	11.0	43.75 ± 1.0	8.75	32.00 ± 0.9	6.4	9.75 ± 0.5	1.95
Liquid medium								
NASE3	65.50 ± 0.9	13.1	34.50 ± 0.9	6.9	6.80 ± 1.0	6.8	3.70 ± 0.2	0.7
NASE14	-	-	-	-	-	-	-	-

The differences between media and genotypes were significant ($P < 0.05$). Upon continuous presence of anthers in culture medium, the anther wall turned brown (Figure 1B). However, small globular structures emerged through ruptured anther wall (Figure 1T) and proliferated (Figure 1S) increasing in volume in the basal medium (Figure 1H). MS media containing 1mg/l BAP and 0.5mg/l NAA and 4% phytigel triggered compaction of the callus which led to induction of embryogenic callus (Figure 1U). Moreover in both genotypes, callus developed green spots on regeneration media under light conditions (Figure 1K-L and Figure 1V). However, conversion of the embryos into plants was not observed, under these culture conditions, although low frequency of roots (Figure 1L and Figure 1U) and abnormal shoot (Figure 1J) development were observed. For NASE3, dissolving of the anther wall was observed (Figure 1N), leading to exposure of the microspores in the liquid medium.

Callus production from cassava anthers under variable culture conditions

Callus production was significantly influenced ($P < 0.0001$) by the sucrose concentrations 2, 9, 12, 15 and 20% (w/v), with genotype NASE3 having a higher mean callus production (75.0, 43.8, 29.6, 13.4 and 3.6) compared to NASE4 (65.0, 36.0, 26.0, 11.0 and 0.8) (Figure 2).

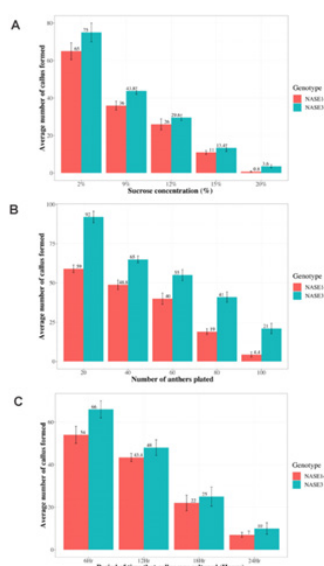


Figure 2: Effect of sucrose concentration (A), anther density (B), and period on culture (C) on callus formation, in cassava (*M. esculenta*) genotypes NASE14 and NASE3. Callus formation decreased with increasing concentration of sucrose, anther density and period on culture in both genotypes (A-C), and in all these cases, callus formation was consistently higher in NASE3 than NASE14.

- Salix integra* Linn. in haploid poplar (*Populus simonii* × *P. nigra*). *J Forest Res.* 2018;29:321-330.
21. Yutaka H. Callus and embryoid induction by anther culture of Apple. *Bull Fac Educ Hirosaki Univ.* 1982;48:69-74.
22. Perera PIP, Quintero M, Dedicova B, Kularatne J, Ceballos H. Comparative morphology, biology and histology of reproductive development in three lines of *Manihot esculenta* Crantz (Euphorbiaceae: Crotonoideae). 2013;5:1-7.
23. Sabbadini S, Ricci A, Limera C, Baldoni D, Capriotti L, Mezzetti B. Factors affecting the regeneration, via organogenesis, and the selection of transgenic calli in the peach rootstock hansen 536 (*Prunus persica* × *Prunus amygdalus*) to express an RNAi construct against PPV Virus. *Plants.* 2019;8:178.
24. Yildiz M. The prerequisite of the success in plant tissue culture: high frequency shoot regeneration. Leva M and Rinaldi L.M.R (eds), *Recent Adv Plant InVitr Cult.* 2012;63-90.
25. Erland LA, Shukla MR, Glover WB, Saxena PK (2017) A simple and efficient method for analysis of plant growth regulators: a new tool in the chest to combat recalcitrance in plant tissue culture. *Plant Cell, Tiss Org Cult.* 2017;131:459-470.
26. Abdollahi MR, Rashidi S. Production and conversion of haploid embryos in chickpea (*Cicer arietinum* L.) anther cultures using high 2, 4-D and silver nitrate containing media. *Plant Cell Tiss Organ Cult.* 2018;133:39-49.
27. Abiri R, Maziah M, Shaharuddin NA, Yusof Z, Atabaki N, Hanafi M, Sahebi M, Azizi P, Kalthori N, Valdiani A. Enhancing somatic embryogenesis of Malaysian rice cultivar MR219 using adjuvant materials in a high-efficiency protocol. *Intern J Environ Sci Tech.* 2017;14:1091-1108.
28. Leena T, Tripathi JN. High frequency shoot regeneration of various cultivars of banana (*Musa* sp). *J Crop Improv.* 2008;22:171-181.
29. Foroughi-Wehr B, Friedt W, Wenzel G. On the genetic improvement of androgenetic haploid formation in *Hordeum vulgare* L. *Theor Appl Genet.* 1982;62:233-239.
30. Silva TD (2010) Indica rice anther culture: can the impasse be surpassed? *Plant Cell, Tiss. Organ Cult.* 2010;100:1-11.
31. Germana MA. Gametic embryogenesis and haploid technology as valuable support to plant breeding. *Plant Cell Rep.* 2011;30:839-857.
32. Bogunia H, Przywara L. Rola cukrowców w roślinnych kulturach *in vitro*. *Wiadomości Botaniczne.* 1999;43(1/2):25-36.
33. Alina T, Magdalena J, Andrzej T. The effect of carbon source on callus induction and regeneration ability in *Pharbitis nil*. *Acta Physiol Plantarum.* 2006;28:619-626.
34. Warchoń M, Czyżyło-Mysza I, Marcińska I, Dziurka K, Noga A, Skrzypek E. The effect of genotype, media composition, pH and sugar concentrations on oat (*Avena sativa* L.) doubled haploid production through oat × maize crosses. *Acta Physiol Plantarum.* 2018;40:93.
35. Bogunia H, Przywara L. Effect of carbohydrates on callus induction and regeneration ability in *Brassica napus* L. *Acta Biolog. Cracoviensia, Series Botanica.* 2000;42:79-86.
36. Tripathy SK, Swain D, Mohapatra PM, Prusti AM, Sahoo B, Panda S, et al. Exploring factors affecting anther culture in rice (*Oryza sativa* L.). *J Appl Biol Biotech.* 2019;7:87-92.