



Somatic embryo production and *GFP* genetic transformation in elite Ugandan cassava genotypes

Stephen Magambo^{a,c}, Alice Nabatanzi^{b,*}, Titus Alicai^c, Enoch Wembabazi^c, Ketra Oketcho^{c,d}, Immaculate Nakalembe^a, Henry Wagaba^c

^a College of Veterinary Medicine, Animal Resources and Biosecurity, Makerere University, P.O. Box 7062, Kampala, Uganda

^b College of Natural Sciences, Makerere University, P.O. Box 7062, Kampala, Uganda

^c National Crops Resources Research Institute, P.O. Box 7084, Kampala, Uganda

^d Department of Biology, University of Missouri-St. Louis, USA

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ABSTRACT

Friable embryogenic callus (FEC) production is a prerequisite for the genetic transformation of cassava (*Manihot esculenta* Crantz). This process necessitates the development of organized embryogenic structures (OES) as a precursor. However, the production of OES and FECs in cassava is dependent on the genotype. This study aimed to optimize somatic embryo production in elite Ugandan cassava genotypes (NASE13, NASE19, and NAROCASS1) and demonstrate genetic transformation using the enhanced green fluorescent protein (eGFP) gene. OES were induced by initiating leaf-lobe explants on Murashige and Skoog (MS) media at various concentrations of 24D and picloram followed by friable embryogenic callus (FEC) production and regeneration. There were highly significant differences among the different media compositions in the percentage response to OES formation ($p < 0.001$). NASE 13 exhibited the highest regeneration frequency of 38.4%; while NAROCASS1 had the lowest regeneration frequency of 10.5% recorded. The presence of GFP gene in NAROCASS1 was confirmed by polymerase chain reaction (PCR) analysis of the transformed lines. Compared to existing studies, this research pioneers genotype-specific optimization for somatic embryo production and successful genetic transformation in Ugandan cassava varieties. The utilization of these genotypes, already embraced by farmers, presents promising opportunities to enhance them with desirable traits, further augmenting cassava cultivation.

Introduction

Cassava (*Manihot esculenta* Crantz) is a staple food crop in many parts of the world [1]. In Uganda, recent advances in cassava research and processing techniques have revolutionised its role as a high-value industrial crop [2]. However, cassava production is constrained by several biotic factors, especially inadequate quality planting materials (Bull et al. [3]), yet its breeding and productivity are constrained by long propagation cycles [4,5]; which facilitate infection and transmission of systemic viral diseases [6]. The crop is threatened by two major diseases: cassava mosaic disease, caused by single-stranded DNA geminiviruses [7]; and the cassava brown streak disease, caused by a single-stranded RNA ipomivirus [8]. Host plant resistance offers a more sustainable approach to crop

* Corresponding author.

E-mail address: alice2nabatanzi@gmail.com (A. Nabatanzi).

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improvement against biotic stresses in the development of elite varieties [9]. Several studies using conventional breeding have been successful in generating cassava varieties with improved traits such as high yield, high dry matter content, and resistance to pests and diseases (Ceballos et al. [7,10]). However, breeding in cassava is constrained by the high heterozygosity of the crop, allopolyploidy, long breeding cycle, poor seed set, and irregular flowering in some genotypes [11]. Recently, efforts have been made to transfer genes from related or other species into the crop *via* genetic engineering or transformation [6,12]. Cassava improvement through genetic engineering helps to overcome the challenge of high heterozygosity and trait separation [13] and would also allow rapid development of CMD and CBSD resistance if efficient and robust transformation and regeneration technology for farmer-preferred cultivars is provided. The generation of friable embryogenic callus (FEC) is a key step in the genetic engineering of cassava. *Agrobacterium*-mediated gene transfer has been used as a standard and reproducible method for the transformation of cassava. Cassava genotype 60,444 is used as a model cassava cultivar in cassava transformation research due to its good capacity in OES and FEC formation. However, this genotype is not cultivated by local farmers in Africa due to low yield, low nutritional quality, and high sensitivity to viral and bacterial disease [14]. Somatic embryogenesis in cassava has previously been reported [15–20]. These studies have shown that the frequency and efficiency of OES and FEC production is genotype-dependent, and that not all cassava cultivars are responsive to somatic embryogenesis, regeneration, and/or transformation. It therefore becomes necessary to optimise the production of embryogenic structures for each cassava genotype. Previous studies towards FEC production among Ugandan farmer preferred cassava genotypes have been reported by Apio et al. [21] using picloram as the auxin of choice. Picloram has been reported as the best growth regulator used for inducing somatic embryo in cassava besides 2,4-dichlorophenoxyacetic acid (2,4-D) [16,18,20]. The effect of plant growth regulators on *in vitro* morphogenic response in different genotypes must be investigated for optimal somatic embryo production Yelli et al. [22]. Therefore, the objective of this study was to optimise conditions suitable for somatic embryo production in the Ugandan elite cassava genotypes; NASE13, NASE19, and NAROCASS1, and to demonstrate genetic transformation

Materials and methods

Plant materials

Three elite cassava genotypes commonly preferred by farmers in Uganda (NASE13, NAROCASS1, and NASE19) were used in this study. Plant stem cuttings from these cassava cultivars were collected from experimental fields at the National Crops Resources Research Institute (NaCRRI) and established in plastic pots in the screen house. After sprouting, the stem cuttings were introduced to the tissue culture laboratory on basic MS media [23].

Initiation of nodal cuttings

At least 10 stem cuttings from new stem growth, each carrying approximately 10 nodes, were picked from soil-grown plants of cultivars NASE13, NAROCASS1 and NASE19 in the screen house and introduced into the tissue culture laboratory. The explants were excised from one or two nodes and cleaned using running soapy water. Individual nodes were placed in sterile beakers under a laminar flow hood, with approximately 20 explants in each beaker. A 70% solution of ethanol was added to the explants for one minute followed by a 20% v/v solution of sodium hypochlorite containing two drops of Tween-20 for ten minutes. The explants were rinsed four to five times with sterile distilled water, ensuring that all soap and sodium hypochlorite had been completely washed off. The edges of each nodal cutting were cut off using sterile forceps and a scalpel to remove the necrotic tissues. The excised nodal cuttings were placed on MS basal media supplemented with 20 g/L sucrose (MS2) and solidified with 2.5 g L⁻¹ of phytigel at pH 6.12. The cultures were transferred to the growth-room and maintained for approximately four weeks with a 16 to 8 hour photoperiod at 26 ± 2 °C.

Induction of organised embryogenic structures (OES)

Twenty leaf-lobe explants ranging in length from 2 to 5 mm were excised from *in vitro* mother plants using a hypodermic needle and placed on five different media types in a manner described by (Taylor et al. [24]). The media types used included full-strength MS supplemented with 2,4-dichlorophenoxyacetic acid (2,4D) at concentrations of (5, 8, and 11) mg L⁻¹, MS half-strength supplemented with 50 µM picloram, and MS full strength supplemented with 50 µM picloram as the control media. The MS full-strength media was considered control media because it's commonly used for OES induction in cassava. The plates were incubated in the dark at 26 ± 2 °C for 21 days and observed through this period. Production of OES was assessed as the percentage of leaf explants seen to produce these structures (percentage response) and as the amount of OES produced per responding explant for each genotype.

This experiment consisted of 100 explants per cultivar (five plates with twenty explants per plate) used for each treatment. A scale of 1–5 described an area of the explant surface covered by the embryogenic structures where: 1 = 1–20%, 2 = 21–40%, 3 = 41–60%, 4 = 61–80%, and 5 = 81–100% [21]. Mean values of surface area coverage of OES on leaf lobes and frequencies of OES produced were subjected to an analysis of variance (ANOVA) using R statistical software (version 4.1.3) at a 5% significance level.

Friable embryogenic callus induction

The generated OES was excised from associated non-embryogenic callus using hypodermic needle and fine-tip forceps with the aid of a dissecting stereomicroscope [25]. The excised embryogenic structures were collected and crushed through a 1–2 mm pore size stainless-steel wire mesh using a stainless steel spatula. The crushed tissue was spread on 15 × 100 cm Petri dishes containing Gresshoff and Doy (GD) media supplemented with 20 g L⁻¹ sucrose, 8 g L⁻¹ noble agar, 50 µM picloram, and 250 mM tyrosine.

Five Petri dishes, each containing six 1 mm² OES clusters, were established for each genotype. The tissue was cultured in the dark at

26 ± 2 °C for FEC induction, with regular sub-culturing every after 21 days on the same medium for a maximum of four cycles.

Regeneration of plantlets from friable embryogenic callus

The FECs of NASE13, NAROCASS1, and NASE19 were regenerated following the method described by [25]. The FECs were placed on stage 1 regeneration media containing MS, supplemented with 5 μ M 1-naphthaleneacetic acid (NAA). The cultures were transferred to the growth room and maintained under a 16 to 8 photoperiod at 26 ± 2 °C. After one-month emerging cotyledons from the FECs were transferred onto MS media supplemented with 2 μ M 6-benzylaminopurine (BAP) media; while the remaining FECs were moved to stage 2 regeneration media containing MS2 0.5 μ M NAA. The regeneration frequency for each genotype was determined by the number of emerging plantlets from the FECs after a period of 3months.

Binary vector and *Agrobacterium* preparation

Agrobacterium tumefaciens (strain LBA4404), harboring the binary vector pCambia2300 carrying the enhanced green fluorescent protein (eGFP), was used in transformation. Plasmid DNA was provided by the Donald Danforth Plant Science Center located in St Louis Mo. USA. The T-DNA of the plasmid contains the green fluorescent protein (GFP) gene driven by the Cauliflower Mosaic Virus promoter (CaMV35S) and neomycin phosphotransferase II (*nptII*) as a plant cell selectable marker gene.

Agrobacterium suspensions were prepared as described by Taylor et al. [25]. A single *Agrobacterium* colony carrying plasmid DNA was transferred from Yeast Mannitol (YM) plates containing kanamycin (50 mg L⁻¹), rifampicin (50 mg L⁻¹), and streptomycin (30 mg L⁻¹) and used to inoculate 5 ml liquid Luria broth (LB) containing the same antibiotics (day culture) the day culture was grown at 28 °C on a shaker at 200 rpm for at least eight hours.

About 1 ml of the starter culture was used to inoculate 20 ml of LB culture containing antibiotics and grown overnight at 28 °C and 200 rpm until OD₆₀₀ = 0.7–1. Suspensions were centrifuged at 4000 g for 10 min at room temperature; the supernatant discarded and the pellet washed with GD media containing 200 μ M acetosyringone. This process was repeated, and the bacterial pellet re-suspended in GD medium and diluted to a final concentration of OD₆₀₀ = 0.05.

Plant transformation and regeneration

For this study, transformation was only done for NAROCASS1 genotype. NAROCASS1 FEC tissue obtained after the fourth 21-day cycle on GD2 50P media, supplemented with tyrosine and moxalactam, was used as a target tissue for transformation. The FEC tissue measuring 0.5–0.8 cc of settled cell volume (SCV) were placed in 12-well plates, and each sample inoculated with 2 ml of *Agrobacterium* suspension for 45 min. After inoculation, the FECs were transferred to sterile nylon mesh (110 μ m pore size), placed on co-culture media containing acetosyringone, and incubated at 22 °C for three days under low light. After coculture, the FECs were washed with liquid GD2 50P containing 125 mg L⁻¹ cefotaxime, and the settled cell volume (SCV) was determined. The FECs were moved to sterile nylon mesh and placed on GD2 50P supplemented with 125 mg L⁻¹ cefotaxime. Ten days later, tissues were re-suspended by vortexing in liquid GD2 50P containing 125 mg L⁻¹ cefotaxime and spread evenly on a Petri dish containing GD2 50P supplemented with 125 mg L⁻¹ cefotaxime and 20 μ M paromomycin sulphate (Duchefa Biochemie, Netherland), and cultured in low light. After 21 days, actively growing FEC units were sub-cultured and placed on to Stage 1 embryo regeneration media containing MS media with 2% sucrose (MS2) supplemented with 5 μ M NAA and 45 μ M paromomycin sulphate. FEC that had not formed cotyledons after 21 days was transferred to Stage 2 embryo regeneration media containing MS2 supplemented with 0.05 μ M NAA and 45 μ M paromomycin sulphate. The emerging cotyledons from stage 1 and 2 embryo regeneration media were transferred to MS2 media supplemented with 2 μ M Benzyl Amino Purine (BAP) to aid shoot formation.

Tracking green fluorescing cells

Following three days of co-culture with *Agrobacterium*, the tissues were scored for GFP expression using a Zeiss Lumar.v12 epi-fluorescence stereoscope attached to a QImaging color camera. The transient transformation was assessed by scoring each FEC sample at the end of the co-culture period using a scale of 0–5 as described by [26] where the number of visible GFP-expressing cells was: 0 = no visible signal; 1 = 1–10 GFP-expressing cells; 2 = 11–50 cells; 3 = 51–100 cells; 4 = 101–500 cells, and 5 = greater than 500 GFP-expressing cells visible. GFP expression was scored again 10 days after subculture onto a selection-free medium (GD2 50P supplemented with 125 mg/l cefotaxime) using the 0–5 scale as described.

Molecular characterisation of transgenic lines

The DNA was extracted following the Cetyl trimethyl ammonium bromide (CTAB) method [27] as modified by Ogwok et al. [6]. PCR analysis was carried out on six randomly selected putatively transgenic lines generated to confirm gene integration in NAROCASS1 regenerants using eGFP and npt II specific primers (Table 1). PCR products were resolved by electrophoresis on a 1% w/v

Table 1
Primers used for amplification in this study.

Gene	Primer sequence	Product size (bp)
eGFP	5'-AACTCAGTAGGATTCTGGTGTGTGCG-3' (Forward) 5'-CGGCCACAAGTTGGAATACAAC-3' (Reverse)	430
npt II	5'-TGTGCTCGACGTTGTCTACTGAA-3' (Forward) 5'-CACCATGATATTCGGCAAGCAG-3' (Reverse)	600

agarose gel and DNA fragments were visualised under a UV transilluminator after ethidium bromide staining. Images were recorded using the Syngene U:Genius 3 gel documentation system(Integrated Scientific Solutions, Inc., San Diego, CA). Figs. 1 and 2

Results

Induction of organised embryogenic structures (OES)

There were highly significant differences among the genotypes NASE13, NASE19 and NAROCASS1 in percentage response to OES formation ($p < 0.001$) (Table 2). There were also highly significant differences among the media types MS (5, 8, and 11) mg L⁻¹ 24D, MS half strength supplemented with 50 μ M picloram, and MS full strength supplemented with 50 μ M picloram in percentage response to OES formation ($p < 0.001$) (Table 2). The interaction between genotype and media type was also highly significant in OES response ($p < 0.001$). The mean separation for the genotypes showed that generally NASE 13 had the highest percentage response to OES formation of 66.6% while NAROCASS1 had the lowest percentage response to OES formation of 38.6% (Table 3). Maximum OES frequency for each genotype varied with media type. Maximum OES frequencies were achieved at 8 mg L⁻¹ 2,4D for NASE13, at 5 mg L⁻¹ 24D for NASE19, and in MS full strength media containing 50 μ M picloram for NAROCASS1 (Fig. 3). The mean separation for the media showed that there was high level of significance among the media types; MS containing (5 and 8) mg L⁻¹ 24D and MS half strength supplemented with 50 μ M picloram, in eliciting OES formation (Table 4). However, the MS full strength media containing picloram and MS 11 mg L⁻¹ 24D media showed no significant difference ($p < 0.05$) in percentage OES response (Table 4). Generally, the media containing 5 mg L⁻¹ 24D showed the highest percentage mean OES response at 65.6% across the three genotypes; NASE13, NASE19 and NAROCASS1 (Table 4). The MS half strength media containing picloram showed the lowest percentage OES response of 13.8% across all the genotypes.

Friable embryogenic callus production and regeneration

Friable embryogenic callus (FEC) was successfully generated in NAROCASS1, NASE19, and NASE13. NASE13 and NAROCASS1 formed FECs in the first or second cycle of subculture while NASE19, while NASE19 formed FECs in its third and fourth cycles of subculture.

Thirty clusters of FECs for each genotype were used for regeneration. NASE 13 showed the highest regeneration frequency of 38.4%; while NAROCASS1 had the lowest regeneration, frequency recorded (Fig. 4). There was no significant difference ($p < 0.05$) in regeneration frequency of NASE19 and NASE13.

Transformation efficiency

Transformation was only performed with the NAROCASS 1 genotype. After four days of *Agrobacterium* coculture with the NAROCASS1 FECs, transformation transient GFP expression was assessed, and an average score of 2 was recorded. Transformed callus lines were determined by visualizing the stable GFP after culture on stages 1 and 2 regeneration media (Fig. 5). A total of 68 callus lines were recovered, of which 23 putative transgenic plant lines were regenerated. Fig. 6

Molecular characterisation of putatively transgenic plants

PCR analysis of regenerants

Molecular characterization of putative transgenic lines was done using PCR. PCR detection using gene specific primers gave a 430 bp and 600 bp fragments for coding regions of eGFP and *npt* II genes respectively. Fifteen putatively transformed lines were randomly tested and out of these, 12 were PCR positive (Figs. 7 and 8). These lines included 1, 2, 3, 4, 5 and 6. However, lines 5 and 6 did not show any amplicons.

Discussion

Somatic embryogenesis is a very critical step towards the genetic improvement of cassava using *Agrobacterium tumefaciens* as well as particle bombardment (Schopke et al. [28,29]). Some of the factors that influence somatic embryogenesis in crops include; the origin of explant, in vitro culture condition, and culture medium (Nic-can et al., 2015). The cassava genotypes NAROCASS1, NASE19, and NASE13 were able to produce organized embryogenic structures (OES), friable embryogenic callus (FEC) and regenerate into plants using different media types. The genotypes used responded differently to each of the media types for OES formation (Fig. 3). This finding offers potential for adopting specific media types for optimal OES production in specific genotypes (NAROCASS1, NASE19, and NASE13). Additionally, as reported by Lentz et al. [30] precise adjustments of media and hormone concentrations have been shown to result in successfully transformation of recalcitrant cassava genotypes. Also, Saelim et al. [31] found 2,4-D to be a superior auxin to



Fig. 1. T-DNA region of binary vector (source: [26]).

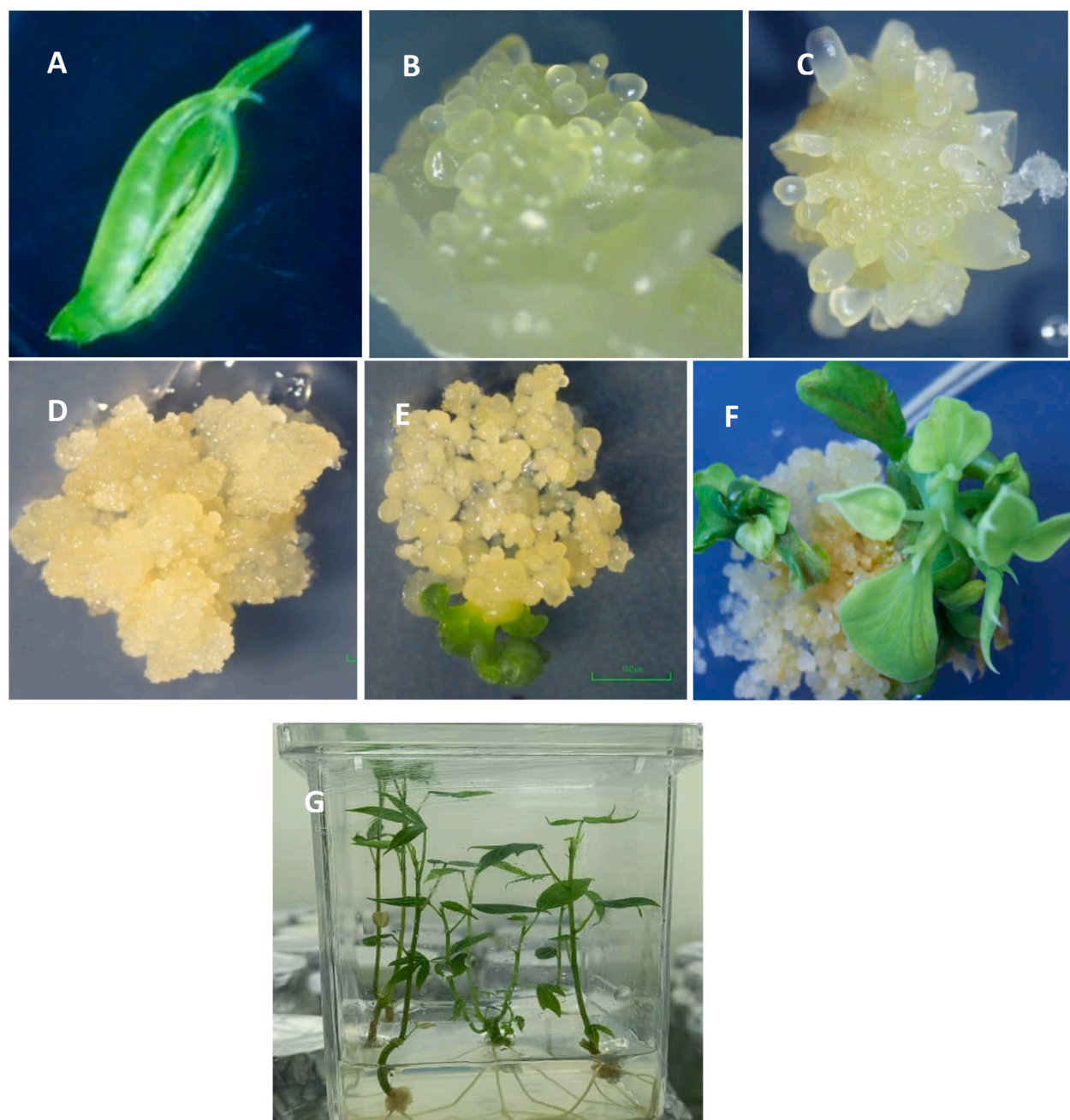


Fig. 2. Production of friable embryogenic callus (FEC) from elite Ugandan cassava cultivars and regeneration into complete plants. A) Leaf lobe established on organized embryogenic structure (OES) induction media, B) Globular organised embryogenic structures (OES) of cassava genotype NAROCASS1, C) Torpedo somatic embryos of NASE13 cassava genotype. D) FEC produced by the genotype NASE19, E) mature cotyledon embryo emerging from FECs, F) a mature cotyledon embryo germinating into a plant. G) Regenerated plantlet in culture vessel.

picloram, while comparing somatic embryogenesis of different explants in the Asian cassava cultivar KU50. In this study, half-strength MS media had the lowest OES frequency and OES score across all genotypes. This observation contrasts with findings by Groll et al. [32] who reported no significant difference in somatic embryo induction between half strength and full-strength MS media. The full strength MS media contains all the macronutrients, micronutrients, and vitamins; therefore, changing the strength or concentration of MS media concentration would directly alter the nutrient content and affect somatic embryogenesis. Therefore, the effect of different medium strengths could also affect plant organogenesis *in vitro* as reported by Rezali et al. [33]. NASE13 and NAROCASS1 formed FECs more readily than NASE19 by forming FECs in their first or second cycle of subculture; while NASE19 formed FECs in its third or fourth cycle of subculture. This observation could imply that these genotypes would be highly responsive to genetic engineering. As reported

Table 2

Effect of genotype x media type on percentage OES response in Ugandan farmer preferred cassava genotypes.

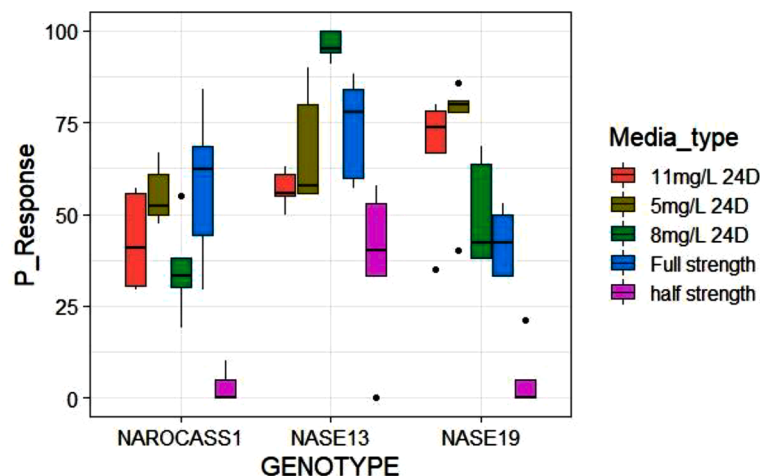
Source of Variance	Df	Sum sq	Mean sq	F Value	Pr(>F)
Genotype (G)	2	220,639.4	110,319.7	767.5289	0.0001
Media type (M)	4	565,858.9	141,464.7	984.2145	0.001
G x M	8	188,768.1	23,596.0	164.1648	0.0001
Residuals	1595	229,255.1	143.7	NA	NA

Sq: sum of squares Df: Degrees of freedom Pr: Probability value.

Table 3

Mean separation for the percentage OES response in Ugandan farmer preferred cassava genotypes.

Genotype	Response (%)	Grouping
NASE13	66.6	a
NASE19	45.8	b
NAROCASS1	38.6	c

Genotypes having different subscripts (groupings) are significantly different in percentage OES response at $P < 0.05$.**Fig. 3.** P-response is percentage OES response of cassava genotypes (NAROCASS1, NASE19 NASE13) in different media types. NASE13 showed the highest OES percentage response while NAROCASS1 showed the lowest response.**Table 4**

Mean separation for the percentage OES response across the media types; MS containing (11, 5 and 8) mg/L 2,4D, MS full strength with picloram and MS half strength with picloram.

Media type	Response (%)
5 mg L ⁻¹ 24D	65.64a
8 mg L ⁻¹ 24D	60.76b
Full strength	56.28c
11 mg L ⁻¹ 24D	55.11c
Half strength	13.86d

Media types having different subscripts (groupings) are significantly different in OES response ($p < 0.05$). Media types having same subscripts (groupings) are not significantly different in percentage OES response at $p < 0.05$.

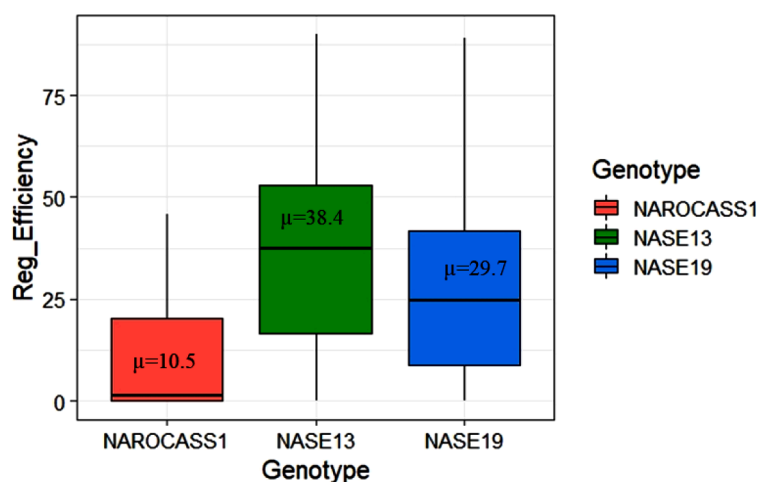


Fig. 4. Mean regeneration frequency for cassava genotypes (NAROCASS1, NASE19 NASE13) from 30 clusters of friable embryogenic callus.

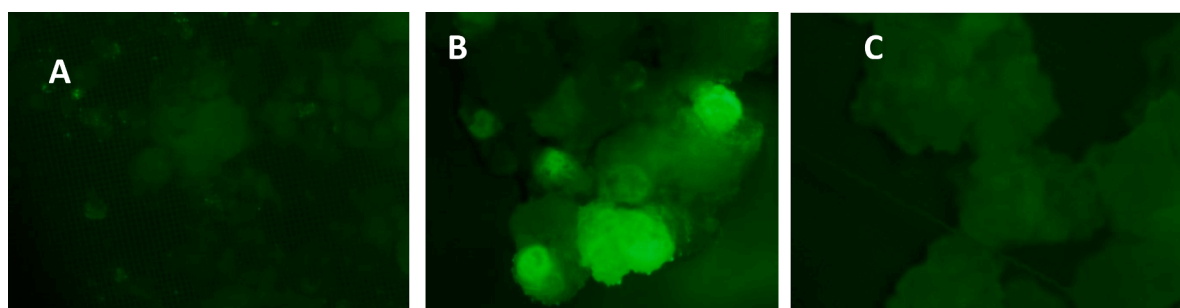


Fig. 5. Schematic presentation for the green fluorescence protein (GFP) expression in the NAROCASS1 friable embryogenic callus (FEC) using the Zeiss Lumar.v12 epifluorescence microscope. A) Transient GFP expression after 4 days of co-cultivation. B) Stable GFP expression in FEC dividing units 28 days after co-culture. C) No GFP expression in FEC after 4 days of co-cultivation of non-transformed FECs (negative control).

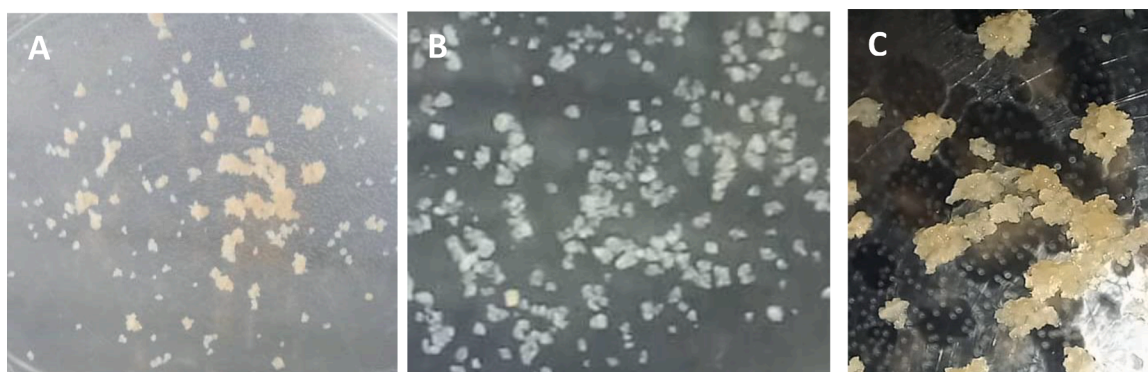


Fig. 6. Friable embryogenic callus (FEC) on selection after transformation, A) Non-transformed cells turned white and died while the transformed cells resistant to paromomycin remained cream-yellow. B) Non-transformed negative control FECs transferred to selective medium all turned white and died. C) The FECs on the non-selective media continued to grow (positive control).

by Thangaraj [19], the findings also confirm that FEC formation in cassava is genotype dependent.

Exposing NAROCASS1 FEC target tissues to *Agrobacteria* during coculture in this study showed a positive effect on the ability to transform it (Fig. 5). An average score of 3 for the transient *gfp* expression after 4 days of coculture with *Agrobacteria* was obtained. High transformation frequency was also confirmed by numerous FEC dividing units 28 days after coculture (stable transformation stage) that survived during the selection process. During selection, whitening and death of non-transformed FECs is reported to be due to necrotic effects of selective agent. This observation implies that a proportion of these FEC cell clones were not transformed. While

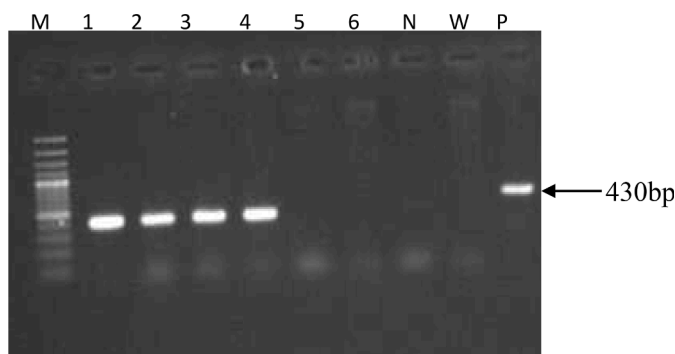


Fig. 7. PCR products for eGFP primers; M- DNA marker, Lines 1, 2, 3, 4, 5 and 6 are putatively transgenic samples, N- Negative control (non-transformed), W-dd water, P-positive control (eGFP plasmid DNA). The arrows indicate the expected PCR products of eGFP.

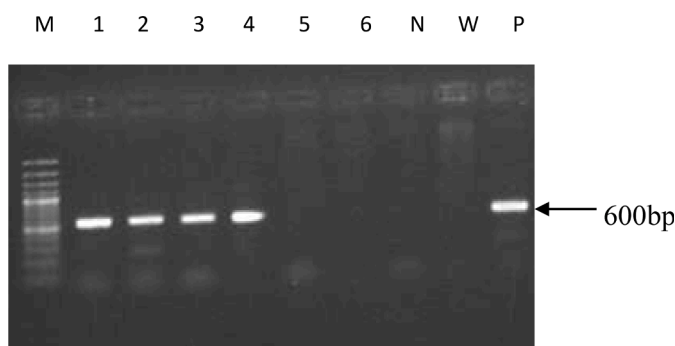


Fig. 8. PCR products for nptII primers; M- DNA marker, Lines 1, 2, 3, 4, 5 and 6 are putatively transgenic samples, N—Negative control (non-transformed), W- dd water p-positive control. The arrows indicate the expected PCR products of nptII.

monitoring *gfp* gene expression in FECs after *Agrobacterium* co-culture, there was a decrease in FEC cell clones expressing *gfp* gene from 4 days after co-cultivation to 28 days after *Agrobacterium* co-culture. This observation may be because some clones were escapes and others were not stably transformed (transiently transformed). Thus, cell clones where the gene was successfully integrated into the genome survived and subsequently developed into shoots.

Conclusion

This study demonstrates the genotype-specific responsiveness of cassava genotypes NAROCASS1, NASE19, and NASE13 to tailored tissue culture protocols. The successful induction of somatic embryos and the transformation of NAROCASS1 using the eGFP reporter gene, reveals that the results of this study could apply to other elite cassava genotypes. Consequently, this research will contribute to creation of a robust platform for advancing the genetic improvement of other farmer-preferred cassava genotypes. The ability to introduce crucial traits through optimized tissue culture techniques holds substantial promise for enhancing crop productivity, resilience, and quality in diverse cassava cultivars, specifically addressing food security (SDG 2), sustainable agriculture (SDG 15), and poverty reduction (Agenda 2063, SDG 1) contributing significantly to sustainable agricultural practices and addressing food security challenges.

CRediT authorship contribution statement

Stephen Magambo: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Alice Nabatanzi:** Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization, Supervision. **Titus Alicai:** Conceptualization, Methodology, Investigation, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Enoch Wembabazi:** Formal analysis, Data curation, Writing – review & editing, Visualization. **Ketra Oketcho:** Writing – review & editing. **Immaculate Nakalembe:** Writing – review & editing, Supervision. **Henry Wagaba:** Methodology, Investigation, Writing – original draft, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Data availability

The data presented in this study are available on request from the corresponding author.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.sciaf.2023.e02039](https://doi.org/10.1016/j.sciaf.2023.e02039).

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