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Evaluation of *Agrobacterium*-mediated Transformation of Two Nigerian Cassava (*Manihot esculenta* Crantz) Cultivars TME 419 and "Okwuoto"

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Authors' contributions

This work was carried out in collaboration between all authors. Authors EEEO and NJT designed and supervised various aspects of the study. Author FAN conducted the experiments while authors ICO and SSK offered technical assistance. Author SOA performed the statistical analysis. Author FAN wrote the protocol whereas authors ICO and SSK supported author FAN to write the first draft of the manuscript. Authors FAN and SOA managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

For efficient transformation of cassava to enhance the productivity of the crop, developing effective protocol for the genetic transformation is necessary. Ten Nigerian cassava cultivars were screened *in vitro* for production of friable embryogenic callus (FEC) and transformation using *Agrobacterium tumefaciens*. All the ten cassava genotypes screened produced organized embryogenic structures (OES) on Driver and Kuniyuki Walnut (DKW) medium supplemented with 50 µM picloram between

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two to four weeks after culture using immature leaf lobes as explants. However, the percentage of OES formation was variety dependent. TMS 96/1632 gave the highest percentage of OES (66%) in comparison to the TMS 60444 that served as control which produced 80% of OES. Conversely, friable embryogenic callus (FEC) production was achieved only in four cultivars – two improved varieties (TMS 96/1632 and TME 419) and two local land races ('Okwuoto' and 'Nwugo') in comparison to that produced by TMS 60444. In cassava somatic embryogenesis, generation of FEC is very important because they are the target tissues for transgene insertion. The friable embryogenic calli generated by TME 419, 'Okwuoto' and the control TMS 60444 were selected and further screened for transformation via *Agrobacterium* mediated transformation. The *Agrobacterium tumefaciens* used carried the green fluorescent protein (GFP) as the marker gene. At the end of the transformation process, transgenic calli expressing the GFP gene were recovered from the three genotypes transformed. Regeneration of the transgenic calli into transgenic cotyledons were also achieved among the three genotypes screened but recovery of transgenic plantlets from the cotyledons were only actualized from those of cv. TMS 60444.

Keywords: Agrobacterium tumefaciens; friable embryogenic callus; green fluorescent protein; transgenic.

1. INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a root crop mostly cultivated in the tropics for its starchy storage roots. It is the second most important staple food in Africa [1], where it is mostly grown by low-income farmers on marginal soils. Although Africa is the largest producer of cassava in the world, the average yield per hectare is significantly below the global average [2]. Many varieties of cassava that combined good agronomic and food qualities have been developed over the years through traditional breeding methods because of its food and industrial importance. However, the increasing focus on the use of staple food crops towards mitigating malnutrition, and the increasing interest in the use of cassava as industrial raw material in the food, feed and energy industries (bioethanol) means that specific genes have to be introduced into cassava for the production of varieties that meet such target end uses, where traditional breeding may be ineffective [3,4]. Difficulties in cross-pollination in cassava resulting from the differences in timing of male and female flowers, low/no flowering ability among genotypes and low seed set are some of the problems that make conventional cassava breeding ineffective [3,4].

Biotechnology is a veritable tool that can help solve the problems confronting cassava improvement especially in the areas of quick gene incorporation through transformation. The expression of the various inserted genes into the cassava genome demonstrates that biotechnology is a potential tool for actualizing the dream of breeders towards improvement of cassava. Cassava transformation has been reported via *Agrobacterium*-mediated transformation [5,6,7,8,9,10,11] and particle bombardment [12,13,14]. The former is favoured over the latter because of the greater stability of transformants. *Agrobacterium*-mediated transformation usually results in the transfer of 1-3 copies of the transgene to plants compared to particle gun which has been known to deliver up to 12 copies of a transgene [12,15]. Also, the introduction of multiple transgene copies can lead to gene silencing [16,17,18]. Transgene expression has been observed in the leaves, roots and other parts of cassava, depending on the promoter used, such as tissue-specific patatin promoter that targets expression to the root, or constitutive like cauliflower mosaic virus 35 S promoter (CaMV 35-S) which targets expression to all parts of the plant.

Production of friable embryogenic callus (FEC) has been described as the most popular target tissues for transformation by biolistics or *Agrobacterium*-mediated methods [11]. The quality of FEC produced is critical to cassava transformation efficiency. The production of organised embryogenic structure (OES) is the first step in the production of FEC. Cassava genotype has been known to exert influence on the development of OES and FEC quality [10,19,20]. The objective of this study was to investigate the potentiality of ten Nigerian cassava genotypes in FEC production and their transformability via *Agrobacterium*-mediation.

2. MATERIALS AND METHODS

2.1 Materials

The cassava cultivars TME 419 and 'Okwuoto' were obtained from National Root Crops Research Institute (NRCRI), Umudike, Nigeria. The model cultivar TMS 60444, *Agrobacterium* strain LBA4404 and a GFP construct p8116 were obtained from International Institute for Crop Improvement (IICI) in Donald Danforth Plant Science Centre, St. Louis Missouri, USA.

2.2 Production of Transgenic Callus, Somatic Embryo and Plantlet for TME 419, 'Okwuoto' and TMS 60444

Cassava transformation via *Agrobacterium*-mediated transformation was divided into six phases which included:

Phase I: Production of organized embryogenic structures (OES); phase II: Production of friable embryogenic callus (FEC); phase III: *Agrobacterium*-mediated transformation; phase IV: Selection and regeneration of transgenic plantlets; phase V: Screening and molecular analysis of transgenic plantlets. Table 1 summarizes the various stages leading to the production and recovery of transgenic cassava.

Evaluation studies on the potential of ten Nigerian cassava genotypes – TMS 98/0581, TMS 98/0505, TMS 98/ 0510, TMS 97/2205, TMS 96/1632, TME 419, NR 87184, 'NWUGO', 'OKWUOTO' and 'NWIBIBI' to generate OES as well as production of FEC have been carried out [20]. Out of these ten cassava genotypes, the two genotypes (TME 419 and 'Okwuoto) that showed good potential for FEC production were selected for *Agrobacterium*-mediated transformation and the model cultivar TMS 60444 used as basis for comparison.

2.3 Agrobacterium–mediated Transformation of TMS 60444, TME 419 and 'Okwuoto'

Glycerol stock of *Agrobacterium tumefasciens* strain LBA4404 transformed with the p8116 a GFP construct based on pCAMBIA 2300 (www.cambia.org/daisy/cambia/home/html) carrying green fluorescent protein (GFP) driven by Cauliflower mosaic virus promoter (35S) and *neomycin phosphotransferase gene (npt*II) selection marker driven by the same promoter were streaked on Luria Broth (LB) agar plate containing 50 mg/l kanamycin, 30 mg/l rifampicin and 30 mg/l streptomycin (LB/RSK). A single colony from the selection plate was used to initiate 2 ml LB medium starter cultures. After eight hours of shaking at 150 rpm at 28°C, this suspension was used to inoculate a 20 ml Yeast Malt (YM) medium culture containing the same antibiotics, and grown overnight on a shaking platform at 150 rpm to reach an OD_{600} of 0.75 to 1.0. Bacteria cultures were transferred to 50 ml sterile tubes and centrifuged at 5000 rpm for 5 minutes at room temperature and the supernatant discarded. The suspended *Agrobacterium* at the bottom of the tubes were washed twice and re-suspended using liquid GD2 50P (Gresshoff and Doy medium containing 20 g sucrose and 50 µM picloram) medium supplemented with 200 µM acetosyringone (Sigma Chemical Co. St. Louis, MO) to generate a suspension with an OD_{600} of 0.5.

2.4 Transformation and Recovery of Transgenic Lines from TMS 60444, TME 419 and 'Okwuoto'

Transformation of the friable embryogenic callus (FEC) - the target tissues for transgene insertion recovered from these cultivars was carried out after 18 days of culture on the third cycle of GD2 50P medium, following the protocol developed by ILTAB cassava transformation pipeline [11]. The FEC tissues generated by each of these cultivars were separately transferred into an empty petri dish and mixed gently to obtain a mass of homogenous target tissues. This was followed by placing the FEC samples into sterile 12 – well plates (Coming Incorporated, Coming, NY) at amounts equivalent to settled cell volumes (SCV) of 0.5 to 0.8 cm^3 per well. Two milliliter aliquots of *Agrobacterium* suspension carrying p8116 were added to each well and mixed vigorously to disaggregate the callus tissues using a pipette tip. After an inoculation period of 30 min at room temperature, the tissue and *Agrobacterium* suspension were drawn off using a 10 ml wide bore pipette and transferred onto a 16 cm^2 plastic 100 µm mesh placed on an empty petri dish. The FEC was then spread to generate a monolayer of tissue and the mesh plus tissue transferred onto petri dishes containing semi-solid GD2 50P medium supplemented with 100 µM acetosyringone. Plates carrying one mesh together with the tissue were cultured for two days at 22ºC under continuous light at 100 µMs / m^2. After the co-culture period, FEC was removed from the mesh using fine forceps, and

each sample transferred to a graduated 15 ml tube and washed twice with GD2 50P liquid medium containing 500 mg/l carbenicillin (Phyto-Technology Laboratories, Shawnee Mission, KS), with the tubes vortexed vigorously for 30 seconds at each washing step. After the second wash, the tissues were allowed to settle for 15 min and the SCV determined to allow subsequent quantification of the transformation efficiency. Tissues were then re-suspended in 8 ml of liquid GD2 50P medium and pipetted onto a fresh 100 µm plastic mesh placed on top of sterile filter paper in an empty petri dish. This position allowed excess fluid to be drawn away, thereby, allowing the tissue to be spread forming a monolayer. Mesh and FEC samples were then placed on semi-solid GD2 50P medium supplemented with 500 mg/l carbenicillin, transferred to a growth chamber and cultured for seven days at 28ºC under 16 hours light and 8 hours darkness and a low light level of 10 µMs/m^2. At the end of the seven-day resting period, FEC was removed from the mesh and each sample placed into a 15 ml sterile tube containing 8 ml of liquid GD2 50P medium, supplemented with 500 mg/l carbenicillin. Tubes were vortexed vigorously for 1 min to encourage disaggregation of the tissues and FEC removed with a wide bore 10 ml pipette and spread onto selection medium consisting of GD2 50P medium supplemented with 500 mg/l carbenicillin and 27.5 µM paramomycin (Sigma Chemical Co.). Each FEC sample was evenly dispensed onto five such selection plates and excess liquid drawn off using a 200 µl micropipette tip. Plates were cultured at 28ºC under a light level of 10 µMs/m^2.

2.5 Regeneration of Transgenic Tissues and Plantlets

Ten to fourteen days after spreading onto selection medium, actively growing FEC clusters larger than 2-3 mm in diameter were identified under the dissection microscope and sub-cultured, using fine forceps, onto stage 1 regeneration medium (Murashige and Skoog medium containing 20 g sucrose, supplemented with 5 µM NAA, 250 mg/l carbenicillin and 45 µM paramomycin) with 10 to 12 calli placed onto each petri dish. This process was repeated 10 and 20 days later, with growing FEC units removed from the GD2 50P selection plates, at each time, and transferred to stage 1 regeneration medium. After 21 days of culture on stage 1 medium, actively growing FEC colonies were sub-cultured onto stage 2 regeneration medium (Murashige and Skoog containing 20 g sucrose, supplemented with 0.5 µM NAA and 45 µM paramomycin). At this stage, FEC tissues were assigned a line number to facilitate subsequent tracking of the transformed lines, and spread evenly onto the agar with a maximum of four putative transgenic lines contained in each petri dish. After 21 days of culture on stage 2 regeneration medium, green cotyledonary-stage embryos were selected from each putative transgenic line and five to six embryos individually transferred to a petri dish containing MS2 medium modified with 2 µM BAP (MS2 2BAP). Somatic embryos formed were then inoculated on the medium such that the undersides of the cotyledons were in contact with the agar surface. Germination of somatic embryos to produce a distinct stem and tri-lobed leaves occurred in an asynchronous manner over the following three to four weeks. Those embryos that failed to germinate by this time were removed, green foliose tissues excised using a scalpel blade and the embryo axis placed onto fresh MS2 2BAP medium to induce a second flush of germination. Once distinct shoots were formed, they were excised from the juvenile cotyledon tissues and transferred to MS2 basal medium (MS2 agar) to obtain stem elongation and plantlet establishment. A maximum of two healthy, growing shoots were recovered and maintained for each putative transgenic line. After three to four weeks of culture on MS2 medium, the more robust plant was retained and micropropagated and the lesser one discarded to ensure that only one transgenic line was retained per putative transformation event. Only such recovered transgenic plants were subjected to molecular analysis.

Green florescent protein (GFP) FEC and regenerating somatic embryos were visualized with the aid of a Nikon SMZ 1500 stereo-microscope, UV illumination and a HQ-FTIC-Long Pass filter set (Nikon Instruments Inc., Melville, NY) present at the microscopy facilities unit of the Donald Danforth Plant Science Center, St. Louis, MO, USA..

2.6 Molecular Analysis of Regenerated Transgenic Plants for GFP Expression

Polymerase chain reaction (PCR) was used to confirm the presence of GFP in the recovered putative transgenic lines. For DNA extraction, two leaves were detached from newly regenerated *in vitro* plantlets two to three weeks after their transfer to MS2 medium and placed in a 2 ml screw capped tube.

Table 1. Processes and duration of steps for the production of transgenic cassava plants cv – TMS 60444 (Modified from Taylor et al. [11])

	Process	Medium	Duration	Scale	Result
1.	**Production of target tissues** Production of mother plants	MS2	6 – 8weeks	20 – 30 plates	Generation of healthy plants as source of immature explants
	Production of OES from immature leaf explants	MS2 50P	4 weeks	20 -25 plates	Generation of OES
	Proliferation and maintenance of OES	MS2 50P	3 weekly subculture cycle	15 – 20 plates	Multiplication of OES
	Production of FEC from OES	GD2 50P	3 x 3 weekly subculture cycles	20 - 25 plates	Generation of homogenous FEC
2.	**Transformation** Inoculation with LBA4404	GD2 50P(liquid)+200 µM acetosyringone	30 mins	7 – 15 samples	Efficient *Agrobacterium* contact on callus cells
	Co- culture with LBA4404	GD2 50P(liquid)+100 µM acetosyringone	2 days	7 – 15 samples	Insertion of T -DNA
3.	**Recovery of transgenic events** Resting phase	GD2 50P + 500 µ mg /L carbenicillin	7 days	7 – 15 samples	Initiation of cell division by transgenic events
	FEC selection	GD2 50P + 500 µ mg /L carbenicillin + 2.75 µM paramomycin	3 -4 weeks	5 plates per sample	selection and recovery of transgenic EFC
	Embryo regeneration (stage 1)	MS2 5NAA + 250 mg/L carbenicillin + 45 µM paramomycin	3 weeks	12 -14 putative FEC lines per petri dish	Selection of transgenic events & regeneration of embryo o torpedo stage
	Embryo regeneration (stage 2)	MS2 0. 5 NAA + 250 mg/L carbenicillin + 45 µM paramomycin	3 weeks	2 – 4 12 -14 putative FEC lines per petri dish	Selection and maturation of embryos to cotyledon
	Embryo regeneration (first cycle)	MS2 2BAP	3 – 4 weeks	Six embryos per petri dish	development and shoot regeneration
	Embryo regeneration (second cycle)	MS2 2BAP	3 – 4 weeks	Six embryos per petri dish	Shoot regeneration if not achieved within the first BAP cycle
	Plant maintenance	MS2	6 weekly cycles indefinitely	One event per plate	Clonal maintenance and propagation of putative transgenic plant lines.

MS2 Murashige and skoog basal medium supplemented with 20 g/l sucrose; MS2 50P; MS2 medium supplemented with 50 µM picloram; GD2 50P: Grassholf and Doy basal medium supplemented with 20 g/l sucrose and 50 µM picloram; MS2 5NAA; MS2 medium supplemented with 5 µM naphthalene acetic acid; MS2 0.5NAA: MS2 medium supplemented with 0.5 µM naphthalene acetic acid ;MS2 2BAP; MS2 meduim supplemented with 2 µM benzylaminopurine; OES organized embryogenic structures; FEC; friable embryogenic callus

The tubes were then placed in Styrofoam box containing either dry ice or liquid nitrogen and stored frozen in -70ºC freezer, until when needed. Leaf tissue was pulverized using a ceramic bead and the MP Fast prep machine for 40 sec at 4.0 m/s, and the DNA extracted using the Qiagen DNAeasy Plant Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. The concentration and quality of the extracted DNA were determined using 2 µl of each of the DNA samples on Thermo Scientific Nano Drop equipment (Nano Drop Products, Wilmington, DE) and running 1 µl of each on 1% agarose gel, respectively. Specific sense and anti-sense GFP primers: TTGTTTGTCTGCCGT GATGT and GGTGATAATGGGCACAA respectively were employed for the PCR analysis.

The reaction was performed in a total volume of 25 µL consisting of 22 µL of Taq polymerase master mix (Invitrogen (Life Technologies, Grand Island, NY), 1 µL each of the GFP forward and reverse primers and 1 µL of 50 ng DNA template. The PCR conditions (profile) were as follows: initial denaturation at 94ºC for 4 min, 30 cycles consisting of denaturation at 94ºC for 30 sec, 30 sec at 48ºC for annealing temperature, 72ºC for 30 sec extension and final extension step at 72ºC for 10 min. The reaction was held at 4ºC. The PCR products were detected by electrophoresis in 1.0% agarose gel, stained with 5 µL ethidium bromide, and photographed on an AlphaImager Gel Documentation System UV transilluminator (San Jose, CA, USA).

3. RESULTS

3.1 Production of Transgenic TME 419, 'Okwuoto' and TMS 60444

Transgenic TME 419, 'Okwuoto' and TMS 60444 were produced via *Agrobacterium*-mediated transformation of cassava FEC derived from these cultivars. TMS 60444 served as a control for this experiment. Detailed information on plant tissue culture media used, their function and the duration of each step required for the production of transgenic plants were summarized in Table 1 whereas Figs. 1 (a-i) show the various stages involved in the production of transgenic cassava from putting leaf lobes on DKW medium to observing the expression of the GFP gene in the plantlet.

Data collected at the end of the transformation process showed that the total transgenic callus lines transferred from the three cultivars to stage 2 regeneration medium were as follows – 102 callus lines from TME 419, 133 callus lines from 'Okwuoto' and 1328 callus lines from TMS 60444 (Table 2). Out of this number of callus lines transferred to stage 2 medium, TME 419 gave a total of 6 transgenic cotyledons at BAP stage of the regeneration medium. A total of 16 transgenic cotyledons were recovered from 'Okwuoto' at BAP stage whereas a total of 968 transgenic cotyledons were recovered from TMS 60444 at BAP stage. None of the transgenic tissues recovered from TME 419 and 'Okwuoto' cultivars developed into plantlet stage whereas for TMS 60444 cultivar a total of 199 transgenic plantlets were recovered on MS agar medium (Table 2).

The transgenic tissues produced by the *Agrobacterium* - mediated transformation of FEC from TME 419 and ''Okwuoto'' cultivar are as shown in Fig. 2 alongside those produced by the model cultivar TMS 60444. A total of one hundred and two GFP expressing callus lines were recovered from TME 419 transformation with only 5.8% (6/102) developing into embryos but none regenerated into transgenic plantlet (Figs. 2a and d). Similarly, 'Okwuoto' cultivar produced 133 GFP expressing callus lines with only 12% (16/133) of the recovered callus lines developing into GFP expressing embryo lines. There was no plantlet recovered from this cultivar (Figs. 2b and e). Transgenic tissues produced by TMS 60444 had lines that regenerated into transgenic plantlets with established roots on MS agar medium (Figs. 2 c, f and g).

Table 2. Summary of the experiments performed on *Agrobacterium*-meditated transformation using two Nigerian cassava cultivars with potential for FEC generation and TMS 60444 FEC that served as control

Variety transformed	Optical density (OD) used	Total transgenic callus lines recovered on stage 2 medium	Total no. of transgenic cotyledons recovered at BAP stage	Total no.of transgenic plantlets recovered on MS agar
TME 419	0.79	102	6	None
'OKWUOTO'	0.79	133	16	None
TMS 60444	0.79	1328	968	199

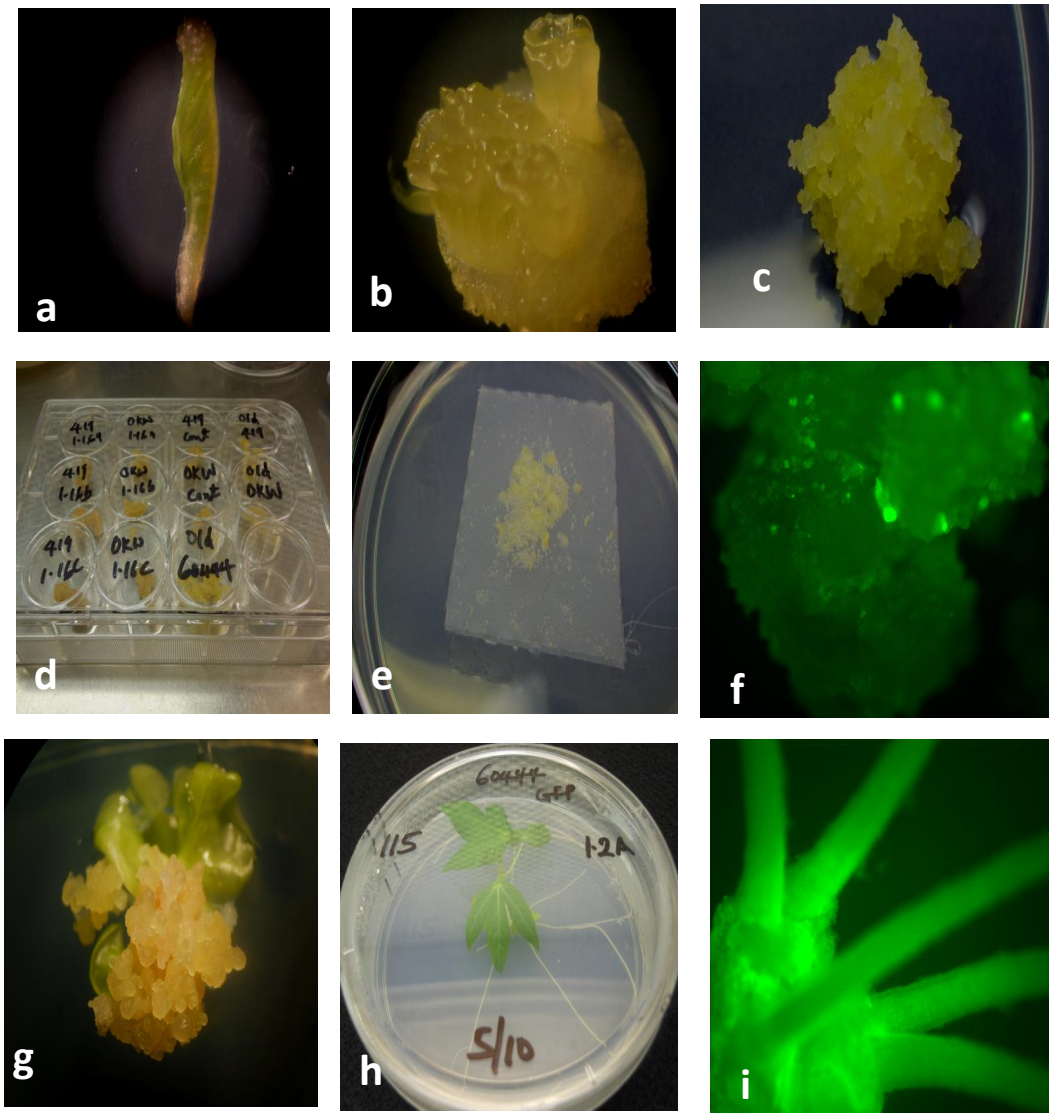

Fig. 1. Stages for transgenic cassava production – (a) Immature leaf lobe on DKW, (b) Somatic embryos (OES) on DKW, (c) FEC on GD2 50P for *Agrobacterium* inoculation, (d) FEC-Agro coculture on 12 well plate, (e) FEC spread on mesh on GD + 500 carb., (f) Transformed FEC showing GFP expression, (g) Developing cotyledon on MS2 BAP., (h) Regenerated transgenic 60444 on MS2 agar and (i) Roots of transgenic 60444 showing GFP expression under UV microscope

3.2 Molecular Analysis of Regenerated Transgenic Plants for GFP Expression

PCR analysis was carried out for the confirmation of green fluorescent protein (GFP) gene expression and integration among the regenerated transgenic TMS 60444 plantlets (Fig. 3). L (1 kb plus DNA ladder), GFP positive control is shown in lane 1 (430 bp) while the negative controls are shown in lanes 2 and 3 (wild type and water control). Result of negative GFP amplification from transgenic events recovered from TMS 60444 using specific GFP primers are shown in lanes 4 to 12 and 19 while the GFP positive amplification are shown in lanes 13, 14, 15 and 18.

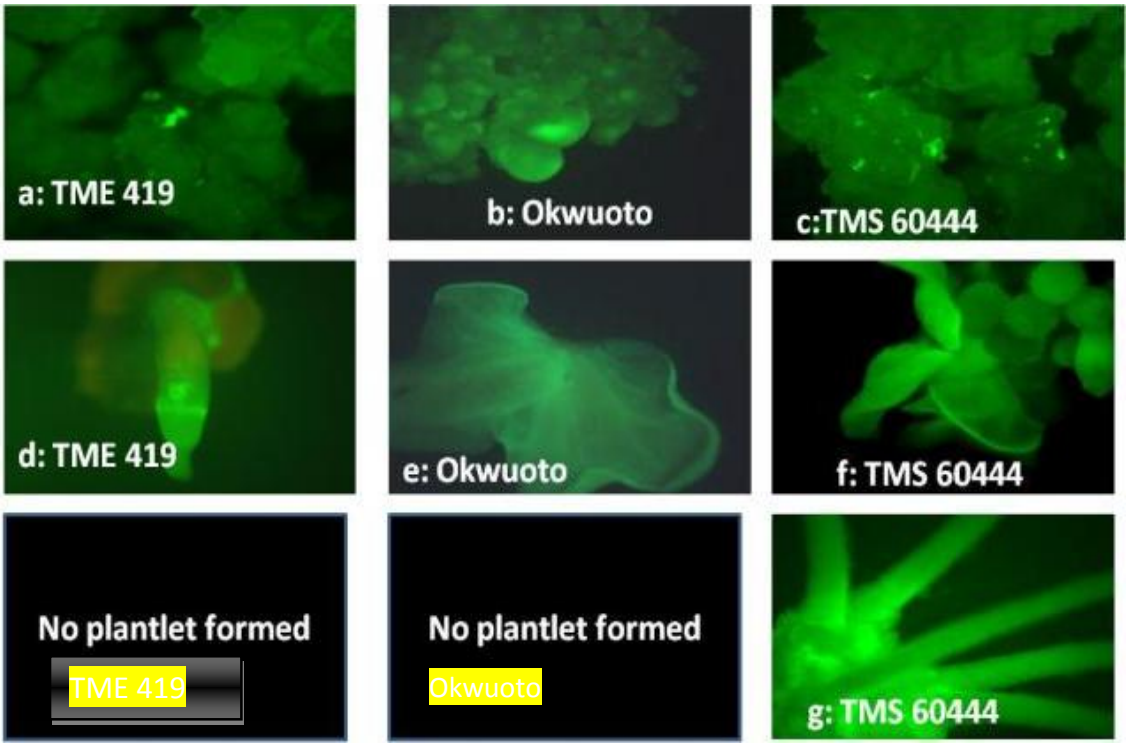

Fig. 2 (a-g). Transgenic callus lines (a, b & c), cotyledons (d, e & f) and root (g) recovered from FEC of TME 419, Okwuoto and TMS 60444 showing GFP expression

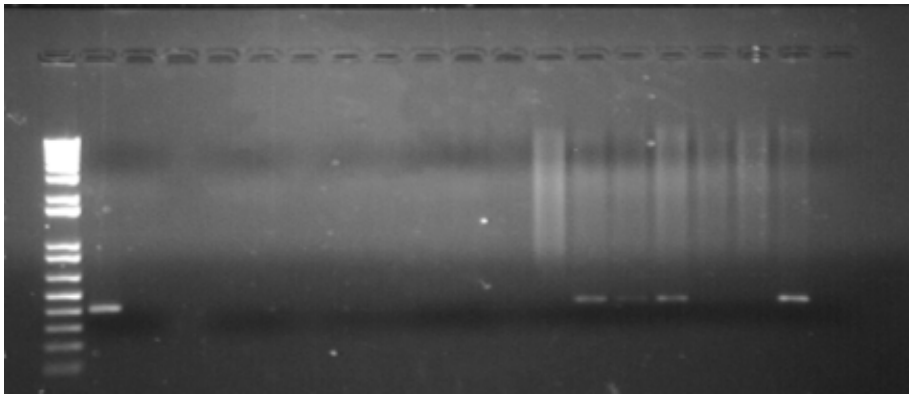

Fig. 3. PCR confirmation of GFP integration in the recovered transgenic events - L (1kb plus DNA ladder), lane 1 (GFP positive control), lanes 2 and 3 (wild type and water negative control), lanes 4 – 12, 16, 17 and 19 (samples without GFP expression) while lanes 13, 14,15 and 18 (samples with positive GFP expression)

4. DISCUSSION

The transformation protocol employed in this study is according to Taylor et al. [11]. Transgenic cassava lines were produced from TMS 60444, TME 419 and 'Okwuoto' using *Agrobacterium*-mediated transformation of cassava FEC derived from these cultivars. Transformation protocol for TMS 60444 has been developed and established by [21] and has been modified severally [11]. Even though the transformation protocol of TMS 60444 has been established, it cannot be translated to other cultivars due to recalcitrant nature of cassava genotypes [22]. In this study, we developed protocol for transformation of TME 419 and

'Okwuoto' (farmer's preferred cultivar) using GFP as a scorable marker. GFP expressing callus lines obtained from TME 419 and 'Okwuoto' were similar but differed significantly with that obtained from TMS 60444. The difference in transformation efficiency at this stage could be attributed to the quality of the target FEC used in the production of the transgenic plants. Subsequently, the number of GFP expressing embryo lines derived from TME 419 (6/102) and 'Okwuoto' (16/133) were significantly less compared to the embryo lines derived from TMS 60444 (968/1328). This shows that the protocols for organogenesis in these cultivars under investigation need to be modified to enhance the development of embryo and subsequent plantlet regeneration [10,23]. Recovered transgenic plants from TMS 60444 have been shown to perform well under greenhouse and field conditions [24,11,25].

PCR analysis using ten randomly selected putative GFP expressing TMS 60444 regenerated plantlets confirmed the presence and absence of GFP in these lines (Fig. 3). The use of PCR method to screen for presence of transgenes in a putative transgenic plant was effective in identifying transgenic lines in cassava. The method was also used by [11,26] to identify transgenic cassava lines carrying heterologous genes.

5. CONCLUSION

The benefits of application of biotechnology in the field of Agriculture cannot be overemphasized. It has the capacity of ensuring food security through introgression of useful agronomic traits into the plants' genome via *Agrobacterium* mediation. Further studies are needed to develop protocols suitable for production of quality FEC for each cassava genotype since successful transformation of cassava relies on the quality of FEC produced by each cassava genotype.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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