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OPTIMISATION OF A SOMATIC EMBRYOGENESIS AND TRANSFORMATION PROTOCOL FOR FARMER-PREFERRED CASSAVA CULTIVARS IN KENYA

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ABSTRACT

Cassava (*Manihot esculenta Crantz*) is a major food crop in developing countries, and holds potential for industrial use. It is, however, affected by various biotic and abiotic stresses that greatly affect its production. The existing regeneration and transformation protocols are not compatible with all cassava cultivars, thus efficient and robust transformation and regeneration protocols for farmer-preferred cultivars need to be optimised for ease of transfer of novel genes. The objective of this study was to develop an efficient transformation and regeneration protocol for a farmer-preferred Kenyan cassava cultivar. We cultured immature leaf lobe and stem explants on Murashige and Skoog (MS) medium, supplemented with varying concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D), Picloram and á-naphthalene acetic acid (NAA). Plants were recovered on media with 6-Benzylaminopurine (BAP) and GA_3 under a 16 hour light/8 hour darkness photoperiod regime. Results showed high regeneration and transformation frequencies for both cultivars. High frequencies of callus induction (>98%) for both cultivars, were obtained when 2,4-D and Picloram were used. Similarly, both auxins initiated somatic embryogenesis, with Picloram producing the highest frequency of somatic embryos (>92%) in TMS 60444, using stem explants. Gus assays revealed high frequencies of transformation of >77% (TMS 60444) and 60% (Kibanda meno mkubwa). This protocol offers promising perspectives for rapid improvement of these cultivars and, therefore, provides a platform for cleaning planting materials, as well as cassava genetic improvement programmes such as control of viral diseases.

Key Words: Cytokinin, *Manihot esculenta,* regeneration protocol

RÉSUMÉ

Le manioc (*Manihot esculenta Crantz*) est une culture vivrière de grande importance pour les pays en développement, avec un potentiel pour l'usage industriel. Néanmoins, le manioc est sujet à des stress d'origines biotique et abiotique, affectant sa production. Les protocoles de régénération et de transformation existante ne sont pas compatibles avec toutes les accessions de manioc. Ainsi, il est nécessaire de développer des protocoles de régénération et de transformation efficaces pour les accessions adoptées par les paysans, afin de faciliter les transferts de gènes d'intérêt. L'objectif de cet etude était de développer un protocole de régénération et de

transformation adapte a la variété de manioc. Des feuilles et tiges immatures ont été cultivées sur des media Murashige et Skoog (MS), auxquels différentes concentrations de 2,4-dichlorophenoxyacetic acide (2,4-D), Picloram et á-naphtalène acétique acide (NAA). Les plantes ont été recouvertes de 6-Benzylaminopurine (BAP) et GA3 sous une photopériode de 16h jour/8h nuit. Les résultats ont montré des fréquences élevées de régénération et de transformation pour les deux cultivars Kibanda meno mkubwa et TMS 60444. Des fréquences élevées d'induction de callosités (>98%) ont été obtenues pour les deux cultivars, lorsque 2,4-D et Picloram ont été utilisés. De la même façon, les deux embryogenèses somatiques initiées a l'auxine, avec des explants de tiges et du Picloram ont exhibe la fréquence la plus élevée d'embryon somatique (>92%) en TMS 60444. Des essais de Gus ont révélés des fréquences élevées de transformation >77% (TMS 60444) et 60% (Kibanda meno mkubwa). Ce protocole offre des perspectives pour l'amélioration rapide de ces cultivars, et par conséquent, fournit une plateforme pour la production de matériels de culture propres, mais aussi servira d'outil dans les programmes d'amélioration génétique visant la lutte contre les maladies virales.

Mots Clés: Cytokinine, *Manihot esculenta,* protocole de génération

INTRODUCTION

Cassava (*Manihot esculenta* Crantz), a woody perennial shrub, is a vital food crop for over a billion people, especially in developing countries. Also, it holds potential for production of industrial starch and bioethanol (Chetty *et al*., 2013). Despite these values, the production and productivity of the crop is constrained by cyanogenic glycosides, post-harvest among other factors, pests and diseases (Legg and Raya, 1998; Fan *et al*., 2011). It is estimated that over 77,502 ha of land in Kenya are currently under cassava cultivation (FAO, 2011), yet the crop is threatened by two major diseases: cassava mosaic disease, caused by single stranded DNA geminiviruses; and the cassava brown streak disease, caused by a single stranded RNA ipomivirus (Chetty *et al*., 2013). Strategies to control these diseases have greatly relied upon conventional improvement programmes, which have encountered massive limitations (Ceballos *et al*., 2004).

Cassava improvement through conventional breeding, further remains a challenge due to poor seedset, inconsistent flowering and heterozygosity that hinder backcrossing (Opabode *et al*., 2013). Biotechnology, through genetic engineering, holds great potential for long-term improvement of crops for enhanced production towards food security. Recent reports of success in generation of disease-free plants, such as transgenic papaya against ringspot virus (Gonsalves, 1998) and CMD-free cassava (Vanderschuren *et al*., 2007) offer great promise towards other stresses not tried out so far. Cassava genetic engineering could allow rapid development of CMD and CBSD resistance, if efficient and robust transformation and regeneration technology for farmer-preferred cultivars is provided. This requires optimisation of existing protocols for ease of transfer of novel genes, since existing regeneration and transformation protocols may not be compatible with all the cultivars (Hankoua *et al*., 2006; Saelim *et al*., 2006; Elibariki *et al*., 2014).

Success in transformation and regeneration of cassava has been previously reported, with protocols employing different phytohormones and explants, including apical meristems, zygotic embryos, immature leaf lobes and stems (Medina *et al*., 2007; Fletcher *et al*., 2011; Rossin and Rey, 2011). While the majority of studies are biased towards use of immature leaf lobes as explants, their occurrence (immature leaf lobes) per parent stock is limited; hence, necessitating the possibility of exploring other plant parts such as stems.

This study aimed at regenerating Kenyan coastal cassava cultivars using different types of explants under the influence of a range of growth hormones.

MATERIALS AND METHODS

Plant materials. Cassava cultivar, *Kibanda meno mkubwa,* from the Coastal Province of Kenya was selected for the study since it is preferred by farmers because of its high starch levels that enables it fetch high prices in the market

(Mwango'mbe *et al.,* 2013). For comparison, the model variety, TMS 60444, was used due to its wide use in tissue culture.

***In vitro* cultures.** Parent stocks of the cultivar were obtained from Kenya Agricultural and Livestock Research Organisation (KARLO), Mtwapa, Mombasa, located in the coastal region of Kenya. Parent plants were propagated in a glasshouse at the Plant Transformation Laboratory at Kenyatta University. *In vitro* plantlets were established on a cassava micro-propagation medium comprising 4.4 g l^{-1} Murashige and Skoog (MS) salts with vitamins (Murashige and Skoog, 1962), 30 g l^{-1} of sucrose and 8 g l^{-1} phyto agar. Explants were then prepared by poking of immature leaf lobes (ILL) and longitudinal dissection for stems; and cultured on callus induction media. The media comprised of MS salts with Gamborg B5 vitamins, supplemented with varying concentrations of Picloram and 2,4-D, 100 mg l^{-1} myoinositol, 0.5 mg l^{-1} $CuSO_4$, 50 mg l^{-1} case in hydrolysate and 30 g l^{-1} sucrose. Auxin concentrations were 4, 6, 8 and 10 mg l^{-1} and were added individually to the media prior to sterilisation, by autoclaving at 121 ºC for 15 minutes. To induce calli, ten explants were cultured per petri-plate, across the four treatments and the cultures incubated at 28 ºC in a growth chamber. The lighting in the growth chamber followed a photoperiodic regime of 16 hours of light provided by fluorescent lamps (100 mEs^{-1} m^{-2}) and 8 hours of darkness.

For somatic embryogenesis, 30 calli (replicated 3 times) were transferred on a similar medium (as in callus induction experiments), and the cultures maintained there for 28 days. Emerging somatic embryos were matured on a medium comprising of MS salts with Gamborg vitamins, 30 g l^{-1} sucrose, 8 g l^{-1} agar and varying combination ratios of BAP, NAA and GA_3 (Table 1). Germination and plantlet recovery were achieved on maturation media, supplemented with 0.8% w/v activated charcoal. Recovered plantlets were then rooted on a hormone-free MS medium, with 30 g l^{-1} of sucrose and 8 g l^{-1} phyto-agar. These plantlets were later acclimatised on peat moss and hardened in the glasshouse in potted soil. Surviving plants were transferred to bigger pots with soil for subsequent evaluation.

Transformation. Transformability of the cassava cultivars in this study was assessed by co-cultivating immature leaf lobes with *Agrobacterium tumefaciens* strain EHA 101, harboring binary vector PTF 102 (Fig. 1) (Frame *et al*., 2002). The vector contains a *Gus* reporter gene, driven by 35S promoter and terminator. It also has the bar gene that confers resistance to the herbicide basta and, therefore, is used as the plant selectable marker for positively transformed tissues (Fig. 1). A total of ninety immature leaf lobe explants were co-cultivated with EHA 101 for generation of putative transformants,

TABLE 1. Phyto-hormone combination ratios used for somatic embryo maturation and germination of cotyledonary embryos

Media	Combination ratios (mg l^{-1})		
	BAP	NAA	GA_3
M	3	0.02	0.5
M1	3	0.01	1
M2	2	0.03	0.5
M3	2	0.01	1.5
M4	1	0.02	1.5
M5	1	0.03	1

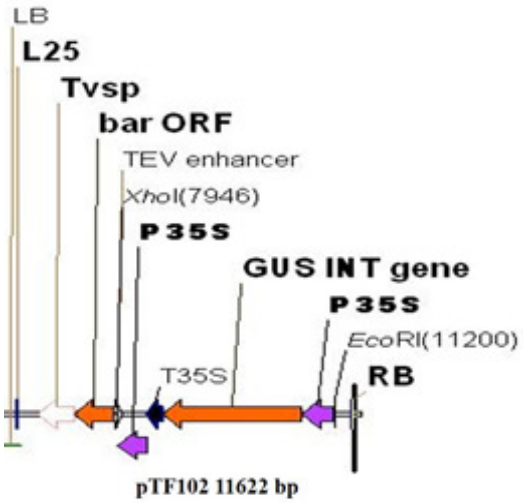

Figure 1. T-DNA region of standard binary vector pTF102. LB, Left border; RB, right border; bar, phosphinothricin acetyltransferase gene; gus-int, -glucuronidase gene containing an intron; P35S, CaMV 35S promoter; Tvsp, soybean vegetative storage protein terminator; T35S, CaMV 35S terminator.

according to Chetty *et al*. (2013). Histochemical Gus assays were performed on ten putatively transformed calli (replicated 3 times), as described by Jefferson (1987).

Data collection and analyses. All callus induction experiments were performed using ten explants per plate and five plates per treatment, for both cultivars under study. Callus induction, somatic embryo and transformation frequencies were recorded. Callus induction frequency was calculated by counting the number of explants forming a callus, as a percentage of the total explants cultured. Somatic embryo frequency was calculated by taking the number of calli showing presence of somatic embryo, as a percentage of total calli. Transformation frequency was computed by taking the number of calli showing a blue colouration, as a percentage of total calli following Gus assay.

Data were analysed by Multivariate analysis of variance (MANOVA), with statistically significant variables computed according to Tukey's HSD test, at a confidence level of 95% ($P<0.05$), using Statistical Analysis System (SAS) version 9.1 (SAS, 2004).

RESULTS

The two cultivars showed callus formation within 14 days (Fig. 2A and B). The highest frequency of callus induction using 2,4-D (98.59%) for KMM was achieved following culture of stem explants on media supplemented with 4 mg l^{-1} of the auxin. The lowest was recorded using ILL on media with 10 mg l^{-1} 2,4-D (Table 2). The model cultivar

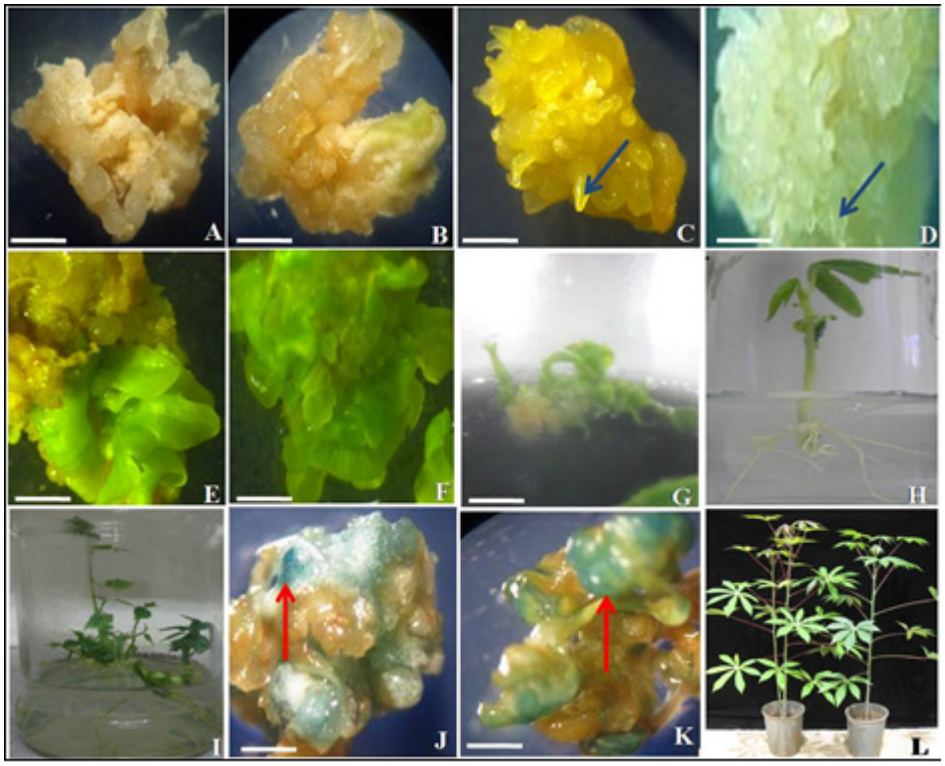

Figure 2. Callus induction, somatic embryogenesis and transformation profiles. **A.** Callus development in cultivar KMM. **B.** Appearance of callus from model cultivar TMS 60444 after 3 weeks of culture on callus induction media. **C.** Immature somatic embryos from cultivar KMM (indicated by the arrow) **D.** Cultivar TMS 60444 on immature somatic embryos CIM media. **E** and **F** Green cotyledons in KMM and TMS 60444 respectively on callus maturation media. **G.** Germination of mature somatic embryos on media supplemented with activated charcoal. Shoot elongation and rooting in TMS 60444 (**H**) and KMM (**I**). **J**. GUS assay staining of KMM and **K** for TMS 60444. **L.** Fully developed regenerants in the glasshouse Scale bar represents 0.1mm.

recorded a callus induction frequency of (98.37%) under this auxin. When explants were cultured on media with Picloram, the highest frequency of callus induction was observed in ILL (88.97%). Relatively higher frequencies of callus induction were recorded in model cultivar TMS 60444 under similar conditions (Table 3). Calli for TMS 60444 developed faster into somatic embryos (SE) (after five weeks) compared to KMM which required seven weeks to obtain high quality primary SE (Fig. 1C and D).

The frequencies of somatic embryo formation are presented in Figure 3. Somatic embryos resulted in green cotyledons (Figs. 2E and F) for both cultivars, albeit at varying frequencies. Cotyledons were observed in both cultivars, under all maturation media formulations.

The frequencies of maturing somatic embryos across the media formulations are shown in Figure 4. The matured somatic embryos (cotyledonary embryos) later germinated on media with activated charcoal. Interestingly, KMM produced a slightly higher frequency of cotyledons than the model cultivar (Table 4).

Shoots obtained from cotyledons were successfully recovered on medium supplemented with activated charcoal (Fig. 2G). The efficiencies of shoot formation for KMM and TMS 60444 are shown in Table 4. For TMS 60444, 11 out of the 39 cotyledons (28%) formed shoots. A slightly lower frequency of shooting (19%) was achieved in KMM. Relatively high frequencies of transformation were recorded for both cultivars according to the Gus assay result (Figs. 2 J and K). Here, the highest frequency of tissues, showing a blue coloration after Gus staining (75.3%), was produced by the model cultivar TMS 60444; while KMM produced the lowest frequency (60.5%) (Table 4). The regenerated cassava plants grew normally in soil (Fig. 2 L).

TABLE 2. Callus induction frequency (%) of two types of explants of two cassava cultivars cultured on MS basal medium supplemented with different concentrations of 2,4-D

2,4-D conc.(mg l^{-1})	Kibanda meno mkubwa		TMS 60444	
	Immature leaf lobes	Stem	Immature leaf lobes	Stems
4	82.26±0.58^{b*}	98.59±1.15^{a}	91.25±1.00^{ab}	98.37±0.58^{a}
6	92.11±0.58^{a}	92.06±1.15^{a}	86.12±3.06^{bc}	80.05±1.73^{c}
8	85.32±1.15^{b}	94.17±2.88^{a}	84.20±1.73^{bc}	96.72±2.31^{a}
10	74.00±1.15^{c}	80.38±1.15b^{c}	82. 00±0.58^{c}	96.41±0.58^{a}

*Values in the same column followed by the same letter are not significantly different (P<0.05, n=50). Means separated by Turkey's HDS test at P<0.05

TABLE 3. Callus induction frequency (%) of two types of explants of two cassava cultivars cultured on MS basal medium supplemented with different concentrations of Picloram

Picloram conc.(mg l^{-1})	Kibanda meno mkubwa		TMS 60444	
	Immature leaf lobes	Stems	Immature leaf lobes	Stems
4	65.33±1.16^{d*}	80.20±1.16^{b}	70.67±1.15^{f}	92.49±1.73^{ab}
6	32.31±1.17^{c}	84.33±1.73^{ab}	48.33±2.31^{e}	99.31±2.31^{a}
8	44.14±1.73^{d}	68.19±1.73^{c}	55.67±1.15^{d}	81.11±1.15^{cd}
10	88.97±1.73^{a}	82. 02±1.73^{b}	99.00±1.73^{a}	88.33±1.15^{bc}

*Values in the same column followed by the same letter are not significantly different (P<0.05). Means separated by Turkey's HDS test at P<0.05

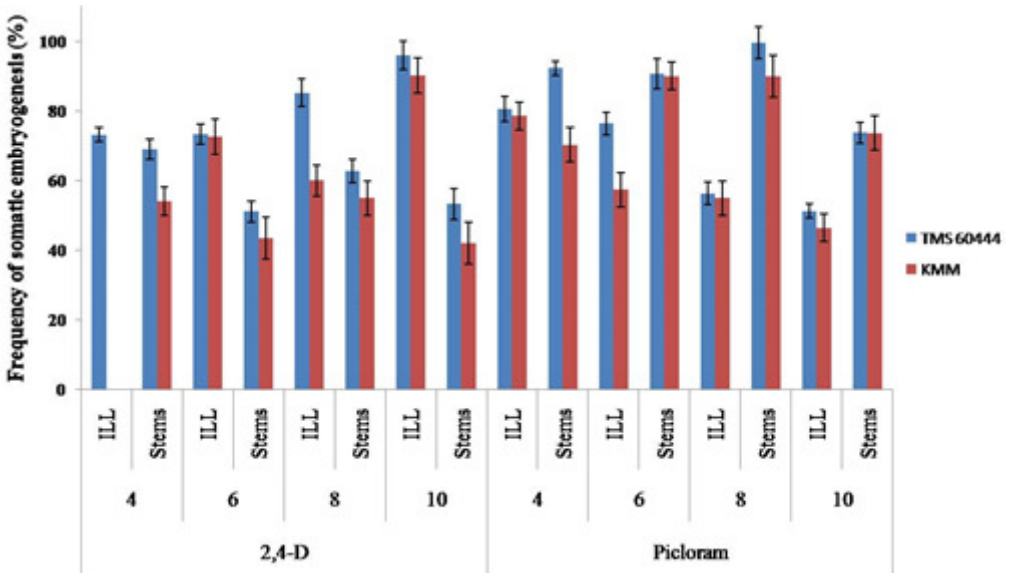

Figure 3. Effect of varying concentrations of 2,4-D/Picloram and explant types on frequency of somatic embryogenesis in a Kenyan cassava cultivar under a 16 hour light and 8 hour darkness photoperiod regime. Error bars represent standard errors of the mean (P<0.05).

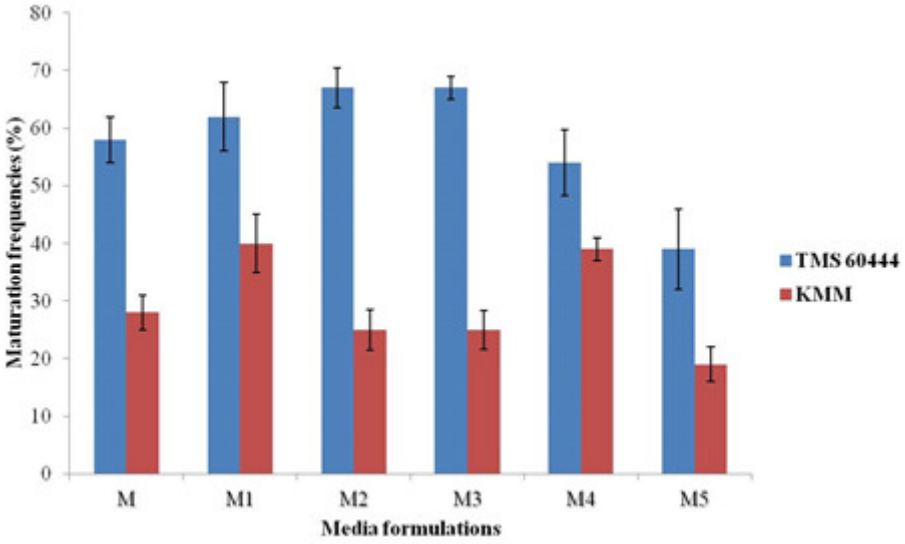

Figure 4. Effect of phyto-hormone combination ratios on frequency of maturation of somatic embryos from immature leaf lobes of Kibanda meno mkubwa (Kenya cassava cultivar) alongside TMS 60444. Error bars represent standard errors of the mean at (P<0.05).

DISCUSSION

Success was achieved in transforming and regenerating a farmer-preferred Kenyan cassava cultivar Kibanda meno mkubwa (KMM) (Fig. 2). Results on callus induction and somatic embryogenesis indicate that formation of callus in cultivar KMM varies with the type and concentration of auxins used, which is in line with findings of other studies. For instance, Rossin

TABLE 4. Somatic embryo germination, shooting and transformation frequencies of Kibanda meno mkubwa along side TMS 60444

Cultivar	Number of germinating cotyledons	Shooting frequency (%)	Transient transformation frequency (%)
Kibanda meno Mkubwa	42	19.23±3.12	60.15±2.33
TMS 60444	39	28.13±2.33	75.33±3.45

and Rey (2011) reported similar findings in a range of cassava cultivars, where Picloram was superior to 2,4-D in generation of somatic embryos. Similarly, Saelim *et al*. (2006) found 2,4-D to be a superior auxin to Picloram while comparing somatic embryogenesis using different explants in the Asian cultivar KU50.

Recalcitrance of cassava to transformation and regeneration is one of the greatest challenges facing genetic modification of this crop due to the difficulties encountered in recovering whole plants following transformation (Li *et al*., 1998). It is, therefore, imperative that protocols for each cultivar be optimised so as to allow for a more reliable system of cassava improvement. This study achieved in regeneration and transformation of KMM, which provides a platform for cassava genetic improvement programmes.

Primary somatic embryos (SE) were observed after five weeks for TMS 60444 (Fig. 3), while it took KMM explants a further two weeks to obtain high quality SE. Combining auxins and cytokinins, during somatic embryo maturation, is essential during cassava regeneration, as these mediate whole plant recovery (Hankoua *et al*., 2005; 2006; Medina *et al*., 2007). For instance, Fan *et al*. (2011) demonstrated that NAA regulated organ growth; while BA facilitated cell division and elongation in cassava tuberisation. A key factor in the use of these phytohormones, is their synergistic interaction in culture media at appropriate concentrations, which ensures optimal efficiencies of regeneration. In the present study, optimisation of these phytohormones through variation of concentrations in the maturation medium, was key in ensuring growth. Here, medium M3 comprising 2 mg l^{-1} BAP in combination with 0.01 mg l^{-1} NAA and 1.5 mg l^{-1} GA_3, was the best for maturation of somatic embryos.

The varying trend in findings observed using ILL and stem explants (Figs. 3 and 4) was as a result of genotype-explant-phytohormone interactions. In general, it was observed that somatic embryo cultures from both cultivars, induced using ILL and transferred onto maturation medium comprising 1 mg L^{-1} BAP, 0.03 mg L^{-1} NAA and 1 mg L^{-1} GA_3, matured and formed cotyledons albeit with varying frequencies (Fig. 4). Despite the high number of somatic embryos obtained from stem explants (Fig. 3), none of these matured into cotyledonary embryos and, hence, no plants were recovered from them. This could be as a result of differences in the pathways of somatic embryogenesis and organogenesis as revealed by Hankoua *et al*. (2006).

Activated charcoal was vital in plant recovery in this study, just like in other plant tissue culture efforts where it has been used to improve cell growth and development (Teixeria *et al*., 1994; Pan and Van Staden, 1998). The use of activated charcoal is based on its ability to adsorb inhibitory chemical compounds, mainly phenols (Liu, 1993; Teixeria *et al*., 1994). Cassava has been shown to produce these compounds, including coniferaldehyde and isovanillin, which are toxic, and, therefore, eliminating them from culture media is imperative for somatic embryogenesis and regeneration (Taylor *et al*., 2001).

Activated charcoal provides a dark environment in medium, thus promoting soil-like conditions. Thomas (2008) suggested the gradual release of adsorbed products such as nutrients and PGRs in culture media, in addition to substances naturally present in activated charcoal that promote plant growth. Transformed calli

showed a moderate blue colouration, which is an indication that these tissues had picked up the construct during transformation.

Data on transient expression of *GUS* reporter gene revealed differences in frequencies of transformation between KMM and the model cultivar TMS 60444 (Fig. 2, Table 4). This shows that cassava transformation remains genotype dependent, and variability in transformation efficiencies between independent procedures cannot be totally eliminated as previously reported (Koehorst-van Putten *et al*., 2012).

CONCLUSION

A reproducible *in vitro* protocol for regeneration of a Kenyan cassava cultivar Kibanda meno mkubwa has been successful using immature leaf lobe and stem explants. This sets up a platform for further studies on this cultivar, aimed at countering the various production constraints that the crop suffers such as Cassava Mosaic and Cassava Brown Streak diseases. Of the two explants evaluated, immature leaf lobes showed better response to callus induction, somatic embryogenesis and regeneration; therefore are the best explants for regeneration of these cultivars *via* somatic embryogenesis. This study has also demonstrated that the cassava cultivar is transformable through *Agrobacterium*-mediated gene transfer. The protocol for callus induction, somatic embryogenesis and whole plant recovery optimised in this study, is suitable for regenerating KMM and therefore is recommended. Using the best performing plant growth regulators and their combinations, as discussed above, it will be easy to regenerate this cultivar during any improvement programme, so long as the appropriate explants are selected and the ideal photoperiodic regime is followed. Further molecular work is needed to ascertain stability of the transgene in transgenic plants. Studies such as southern blot and reverse transcriptase polymerase chain reaction are recommended.

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