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In vitro Regeneration by Indirect Organogenesis of Selected Kenyan Maize Genotypes using Shoot Apices

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Abstract: The study reports a reliable and reproducible regeneration system of two open pollinated varieties-OPV's (Katumani KAT and dry land cultivar DLC1), a hybrid (DH01) and an inbred line (TL08) using shoot apices as explants via organogenesis. The shoot apices were cultured on Murashige and Skoog (MS) basal media supplemented with 9 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 8.88, 17.75, 26.64, 35.52 or 44.40 μM N^6-benzylaminopurine (BAP) with (+) or without (-) 296 μM adenine for calli induction. The most effective combination for calli induction was modified MS media containing 26.64 μM BAP and 296 μM adenine. Calli was maintained on MS media with 9 μM 2, 4-D and 4.44 μM BAP for calli proliferation. Calli of TL08 genotype directly formed shoots on the media containing 9 μM 2, 4-D and 26.64 μM BAP, while the KAT, DLC1 and DHO1 formed a mixture of embryogenic and organogenic calli on the media supplemented with 9 μM 2, 4-D and 4.44 μM BAP. The frequency of callus formation was genotype dependant with KAT 55%, DLC1 35%, DH01 47% and TL08 44%. The number of shoot formed by the selected varieties ranged from 4.9 to 5.7 shoots depending on the genotypes. The number of shoots formed on the media supplemented with 296 μM adenine was higher than that on media without adenine. Shoots were regenerated from organogenic calli after 4-6 weeks depending on the genotype and the presence or absence of adenine, with plant regeneration varying from between 29-55%. Root induction was promoted using MS media supplemented with 1.97 and 2.95 μM Indole-3-butyric acid (IBA). Seeds from *in vitro* regenerated plants (R_0) produced normal plant (R_1) in the field trial and were comparable to the plants grown with the mother seeds.

Key words: Adenine, *in vitro* regeneration, organogenesis, shoot apices, *Zea mays*

INTRODUCTION

Maize is a staple food crop for most of the population in the sub- Saharan Africa. It is an important crop as a source of income, animal feed, manure and industrial uses. Despite its importance, maize yield and production is often inadequate due to abiotic stress such as drought, aluminum toxicity or scarcity of nutrients and biotic stress such as pests, weeds and diseases (Ajanga and Hillocks, 2000; Kanampiu *et al*., 2002; Schechert *et al*., 1999). Various strategies including conventional breeding have been employed to reverse the trend. However, limited success has been achieved in attempts to overcome these constraints hence the need to use molecular breeding and genetic transformation that hold great potential to overcome these constraints. A reliable *in vitro* regeneration of normal and fertile plant from single cell, tissue and organ is the basic prerequisite for the production of genetically modified plants. Regeneration from embryogenic type II calli derived from immature embryos has been the most reproducible transformation/ regeneration system described for maize (Fromm *et al*., 1990; Gordon-Kamm *et al*., 1990; Pescitelli and Sukhapinda, 1995; Sidorenko *et al*., 2000; Srivastava and Ow, 2001). However, type II calli can only be generated from a small number of maize genotypes, such as the A188 line (Armstrong and Green, 1985), possibly because the capacity to produce them is under genetic control (Armstrong *et al*., 1992). Restriction in the formation of type II calli by immature zygotic embryos as a result of genotype specificity has led to difficulties in maintaining totipotency for extended periods of time, low frequencies of callus induction and plant regeneration in a number of temperate maize varieties (Lu *et al*., 1983; Tomes and Smith, 1985; Vasil and Vasil, 1987; Vasil *et al*., 1985). When using immature zygotic embryos most tropical inbred varieties tend to form type I callus which is non friable, grows slowly and is not readily transformable, limiting the suitability of the use of immature zygotic embryos as explants for efficient production of

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transgenic tropical lines. Shoot apices have successfully been used to regenerate and transform maize with focus on temperate and subtropical, yellow endosperm maize (Sánchez *et al*., 2002; Sairam *et al*., 2002) with less focus on tropical inbred and hybrid lines. As a complimentary system to the use of immature zygotic embryos, we report the use of apical shoot apices as an explant for regeneration and a likely candidate for transformation of selected Kenyan lines by organogenesis at Kenyatta University Plant Transformation Laboratory (PTL).

MATERIALS AND METHODS

Maize genotypes: Mature seeds of four Kenyan tropical genotypes consisting of two open pollinated varieties-OPV's (Katumani KAT, dry land cultivar DLC1) and local hybrid (DH01) obtained from a local retail seed company and the inbred line TL08 provided by Kenya Agricultural Research Institute (KARI) were planted in the Kenyatta University PTL green house. The seeds were allowed to grow to maturity, dehusked and left to dry in the tropical sun.

Induction of organogenesis, calli maintenance and plant regeneration: The mature seeds were surface-sterilized in 80% ethanol for 3 min, transferred to 2.5% sodium hypochloride solution containing 0.04 ml L^{-1} tween 20 (v/v) for 30 min and then rinsed 6 times in sterile distilled water. Disinfected seeds were soaked in double-distilled water for 24 h to ease the shedding off of the pericarp in culture. The sterilized seeds of the four maize genotypes (KAT, DLC1, DH 01 and TL08) were then germinated on MS basal media (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose and solidified with 2.9 g L^{-1} (w/v). The pH of culture media was adjusted to 5.8 before autoclaving and 50 to 100 seeds of each genotype were germinated on hormone free MS media for shoot tip formation. To facilitate germination, seeds were placed with the embryo side up on the germination media. After 7-10 days in culture (Fig. 1a), shoot tips (15 to 17 mm long sections) of the mesocotyl-coleoptile were excised and sub-cultured on fresh medium supplemented with plant growth regulators; 2,4-D and BAP in combination with ±296 μM adenine a purine whose derivatives have growth regulatory properties. The MS media supplemented with 9 μM 2,4-D and various BAP concentrations 0, 8.88, 17.75, 26.64, 35.52 or 44.40 μM with or without 296 μM adenine were used for organogenic calli induction of the selected genotypes. All media used were also supplemented with 3% (w/v) sucrose and solidified with 8 g L^{-1} agar (w/v). The pH of culture media was adjusted to 5.8 before autoclaving. Cultures were maintained at a temperature of 27°C with a 16 h photoperiod provided by cool-white light fluorescent lamps. Organogenic calli was maintained on MS medium supplemented with 9 μM 2, 4-D and 4.44 μM BAP. MS basal medium without growth regulators was used for multiple shoot induction. Shoot were then asceptically separated with a sterile surgical blade and each shoot transferred to MS media supplemented with 0-4.92 μM IBA for rooting. *In vitro* plantlets were acclimatized by transplanting into pots containing peat moss. Pots were covered with polythene paper for 3 days to reduce transpiration rate before transplanting into soil. The transplanted plants were grown to maturity, self pollinated and the seeds harvested and planted in pots to study the field performance of the R_1 generation. Data on callus induction and regeneration of the R_0 generation was statistically analyzed using ANOVA. Weight of seeds, height and highly heritable phenological traits in R_1 plants were compared to maize plants derived from non-regenerated seeds (controls) using T test at ($p<0.01$).

RESULTS

Explant preparation: After 5-8 days on MS basal media without hormone, the germinating seeds of the four genotypes formed shoots. The shoots were excised 2 days after their formation and the outer foliage removed to remain with shoot apex or removing as much foliage as possible without damaging the shoot meristem cells. The apices were then placed on calli induction media.

Induction of organogenic calli: In this step, the optimal concentration for induction of organogenic calli (Fig. 1b) for the selected maize varieties was tested upon titration of BAP in presence of 2, 4-D with or without adenine. The effect of BAP was consistent across 0-44.40 μM BAP with 296 μM or without adenine for KAT with an increase in shoot apices forming calli peaking at 26.64 μM BAP (54 and 44%, with or without adenine, respectively) and then decreasing with further increase in BAP concentration (Table 1). Adenine addition was most effective at 17.75, 26.64 or 35.52 μM BAP resulting in up to 13% increase in organogenic calli formation induced at 9 μM 2,4-D and 35.52 μM BAP with adenine. In case of DLC1 the other OPV, a similar pattern to the one seen in KAT was observed with calli formation peaking at 26.64 μM (34.5 and 29% with or without adenine, respectively) with a drastic drop in the calli formation at 35.52 or 44.40 μM BAP. Addition of adenine in DLC1 for the formation of calli was more effective at 8.88, 17.75 or 26.64 μM BAP concentrations though the impact was

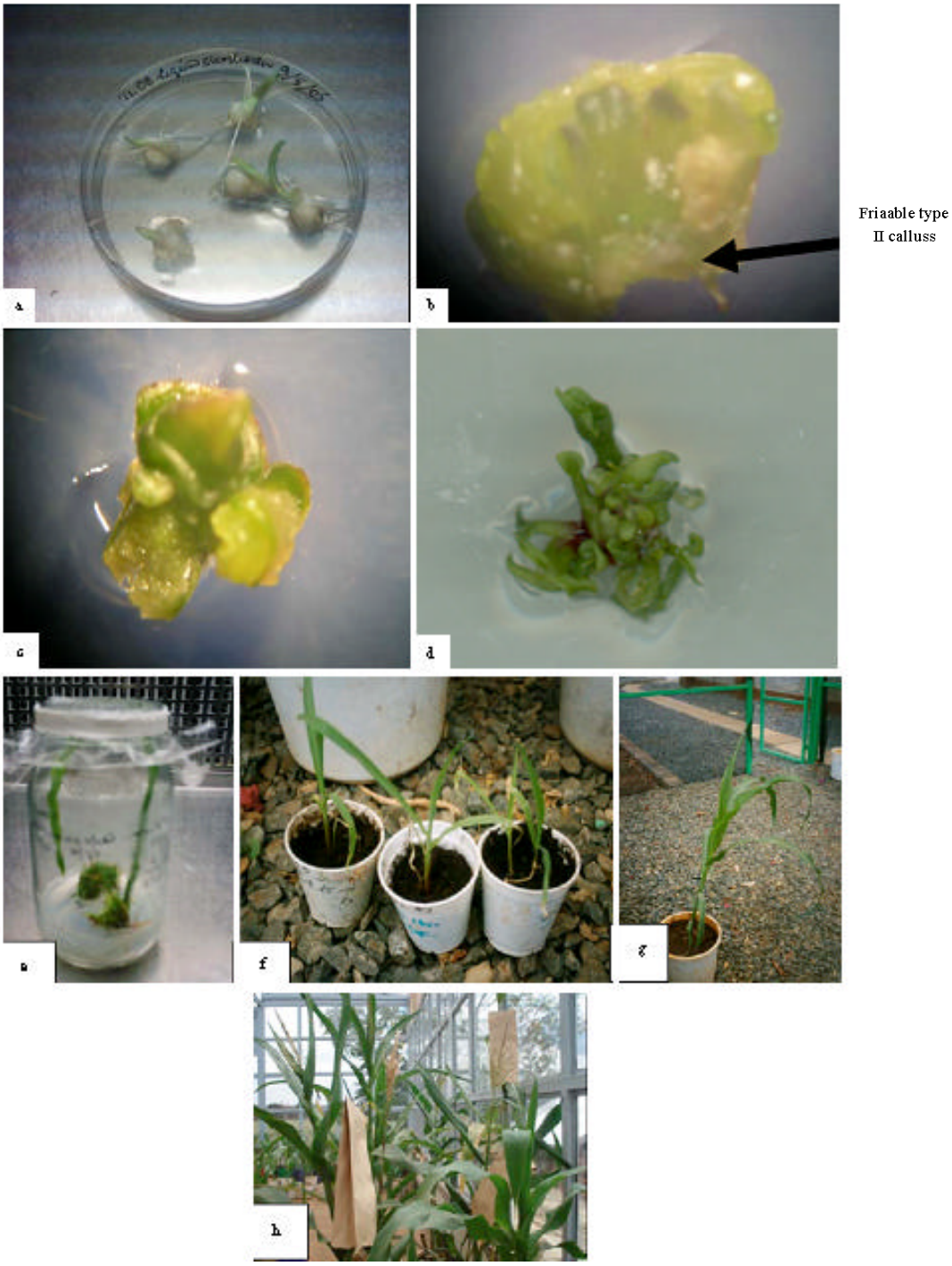

Fig. 1: Plant regeneration from shoot meristem, (a) seeds after 7 days on planting on MSO ready for excision of shoot meristem, (b) organogenic calli maintained at 9 μM 2-4 D and 8.88 μM BAP, (c) green shoot primordial appears after 10 days on shoot induction media, (d) multiple shoots formed on shoot induction media, (e) shoots replanted in MSO supplemented with IBA for root induction, (f) shoots with roots replanted in peat moss for acclimatization, (g) the acclimatized plants 1 month after replanted in loam soil and (h) R_1 regenerants

Table 1: Shoot apices producing organogenic calli as a function of genotype and BAP concentration (± adenine) on MS based medium

	Genotypes (%)											
	KAT			DLC1			DHO1			TL08		
BAP conc. (μM)	ᵃMean of apices forming calli (-A)	(+A)	(+A)-(-A) =Effect of (A) on calli	ᵃMean of apices forming calli (-A)	(+A)	(+A)-(-A) =Effect of (A) on calli	ᵃMean % of apices forming calli (-A)	(+A)	(+A)-(-A) =Effect of (A) on calli	ᵃMean of apices forming calli (-A)	(+A)	(+A)-(-A) =Effect of (A) on calli
0	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0
8.88	28.7±0.8b	35.6±1.6b	+6.90	18.3±2.3b	23.4±1.9b	+5.1	27.0±1.3b	31.6±2.1b	+4.6	34.0±0.6c	44.0±1.2d	+10.0*
17.75	32.0±1.1b	41.5±2.7d	+9.50*	26.7±1.1bc	30.5±1.4bc	+3.8	31.5±1.6c	38.4±3.3bc	+7.0*	17.9±2.0b	27.4±1.7bc	+11.5*
26.64	44.5±1.2d	54.5±2.9e	+10.0*	29.0±1.7bc	34.5±1.2bc	+5.5	36.5±2.1cd	47.0±0.4d	+10.5*	13.3±1.4b	21.9±0.7bc	+8.6*
35.52	40.3±1.5d	53.2±1.1e	+12.9*	41.3±0.9d	41.5±1.7d	0.2	34.6±0.6cd	46.1±1.1d	+11.5*	6.0±2.4ab	10.0±1.1ab	+4.0
44.40	37.5±1.9bc	39.5±3.2bc	+2.0	18.5±3.1b	18.5±2.2b	0	22.5±2.1b	24.5±0.5b	+2.0	1.5±0.2a	0.5±0.1a	-1.0
Genotype mean**	30.5±1.1b	37.4±1.9bc		22.3±1.5b	24.7±1.4b		25.3±1.3b	31.3±1.2b		12.1±1.1ab	17.3±0.8b	

Adenine-(A), (+ A)- Supplementation with adenine, (-A)- Without adenine supplementation, ᵃ Mean (percentage of callus induction was evaluated by counting the number of shoot apices that formed callus out of the total number of apices cultured (-) or (+) Adenine X 100), *Values show that there was a significant effect on supplementation with adenine on calli induction using Tukey's HSD at 5% level. **Values followed by the same letter (s) within a row of the same genotype show that there was no significant difference according to Tukey's HSD at 5% level

not significant. In KAT, there was a significant increase in calli formation at 26.64 μM in presence of adenine with up to 10 % increase at ($p<0.05$). The versatility of OPVs' shoot apices to form organogenic calli between 8.88 or 44.40 μM BAP was notable explaining the genotypic diversity expected within the seeds as a result of segregation. DH01 had a steady increase in organogenic calli formation from 8.88 to 35.52 μM BAP with a maximum at 35.52 μM (46.1 and 36.5%, with or without adenine, respectively). There was no significant difference between 26.64 and 35.52 μM BAP. The effect of adenine on the formation of organogenic calli showed a steady increase from 8.88 to 35.52 μM but had almost no effect at 44.40 μM BAP. As for TL08 the addition of adenine was effective at 8.88 or 17.75 μM BAP resulting in 10 and 11.5% increase in calli formation with a decrease in calli formation on further increase in BAP. The formation of calli reached maximum at 8.88 μM BAP (44 and 34 %, with or without adenine, respectively) in TL08.

Plant regeneration: From the *in vitro* cultured shoot apices green shoot primordial appeared within 10 days (Fig. 1c) and formed shoots within 4-6 weeks depending on the genotype (Fig. 1d). Thus, plantlet regeneration in four genotypes of maize was observed after 4-6 weeks of culture. Plants which had not formed shoots after 3 weeks were sub-cultured on fresh MS without hormones until they produced shoots. The shoots were then separated and transferred to hormone free MS media where, they produced plantlets within 3-5 weeks (Fig. 1e) depending on the genotype.

The number of shoots produced by the four genotypes ranged between 4.5 to 5.5 and 5.3 to 5.7 in media without adenine and with adenine, respectively (Table 2, 3). There was no significant effect on the addition of adenine on the mean number of shoots formed per callus in all the four genotypes tested.

Root induction: The separated shoots regenerated from organogenic calli when challenged on MS without hormones formed roots with difficulties or did not form any roots at all. This was very evident in TL08 which formed roots an average number of 0.85 roots per plant (Table 4). To improve rooting, indole-3-butyric acid (IBA) was required to induce rooting from the shoots (Table 4). The addition of IBA to MS media was most effective at 1.97 μM IBA with all the four varieties of maize selected for the study forming an average of 2-3 main roots per shoot. The effect of addition of 2.95 μM IBA on MS media to induce rooting was equally high in the four varieties with no significant difference in the use of 1.97 or 2.95 μM IBA in KAT, DLC1 and more so in the inbred line TL08 where both 1.97 or 2.95 μM IBA concentrations resulted in an average of 1.8 roots. There was though a significant difference in the use of the two concentrations in DHO1. There was an overall general increase in the number of regenerated shoots forming roots by the four maize genotypes with an increase in IBA concentration from 0-1.97 μM and a decrease in the number of roots with a further increase of IBA concentration between 2.95- 4.92 μM.

Agronomical trait: Seeds (R_0) from the selfed plants of the *in vitro* regenerants appeared normal. Seeds were planted and the R_1 plants (test entries) were compared against controls (plants from seeds used to provide the shoot apices) in the field trials conducted between March-June 2006. Mean difference of the four genotypes and their respective controls were compared using paired

Table 2: No. of organogenic calli forming shoots (-Adenine) and shoot per calli in different tropical lines cultured on MS containing 9 μM 2-4 D and 26.64 μM BAP (-Adenine)

Maize genotype	No. of shoot apices cultured	No. of apices producing organogenic calli	No. of calli forming shoots (-Adenine)	Mean No. of shoots produced per calli[b]
KAT (OPV)	200	88(44%)	81	4.9±0.5
DLC1 (OPV)	100	29(29%)	27	5.2±0.6
DHO1(Hybrid)	200	74(37%)	68	4.5±0.1
TL08 (Inbred)	200	68(34%)	24	5.5±0.2

[b]Mean±SE of 10 replicates. Average No. of shoots per calli = Total shoots produced (-Adenine)/Total number of calli producing shoots

Table 3: No. of organogenic calli forming shoots (+ Adenine) and shoot per calli in different tropical lines cultured on MS containing 9 μM 2-4 D and 26.64 μM BAP (+) 296 μM Adenine

Maize genotype	No. of shoot apices cultured	No. of apices producing organogenic calli	No. of calli forming shoots (+296 μM Adenine)	Mean No. of shoots produced per calli[c]
KAT (OPV)	200	110(55%)	93	5.7±0.3
DLC1 (OPV)	100	35 (35%)	32	5.3±0.4
DHO1(Hybrid)	200	94 (47%)	83	5.6±0.2
TL08 (Inbred)	200	88 (44%)	40	5.7±0.1

[c] Mean±SE of 10 replicates. Average No. of shoots per calli= Total shoots produced (+Adenine) / Total number of calli producing shoots

Table 4: Effect of 0-4.92 μM IBA supplementation on the number of roots formed by the regenerated shoot of the four genotypes

	Genotype			
IBA concentration (μM)	KAT	DLC1	DHO1	TL08
0.0	1.40±0.25a	1.35±0.21a	1.55±0.17a	0.85±0.21a
0.98 μM	1.55±0.21a	1.35±0.18a	1.65±0.22a	1.15±0.19a
1.97 μM	2.90±0.29b	2.35±0.19ab	3.05±0.28b	1.80±0.20ab
2.95 μM	2.70±0.27b	2.50±0.21ab	2.25±0.23ab	1.80±0.20ab
3.93 μM	1.45±0.21a	1.15±0.18a	1.50±0.21a	1.20±0.14a
4.92 μM	1.35±0.23a	1.05±0.17a	1.10±0.22a	1.05±0.16a
Genotype mean*	1.89±0.24ab	1.63±0.19a	1.85±0.22ab	1.31±0.19a

Values with the same letter(s) within a column had no significant difference from each other using Tukey's HSD at 5% level. *Values having the same letter(s) (s) within a row have no significant difference from each other using Tukey's HSD at 5% level

Table 5: Comparison of the plants (R_1) from the regenerated seeds (Test entries) and the plants from the mother plant seed (controls)

	Genotypes							
	KAT		DLC1		DH01		TL08	
Parameters	Test (T)	Controls (C)	Test (T)	Controls (C)	Test (T)	Controls (C)	Test (T)	Controls (C)
Time to tassel (days)	41.00±0.53	42.25±0.31	46.25±1.11	44.25±0.78	44.00±1.10	44.25±2.11	57.00±0.89	58.50±1.52
Time to physiological maturity (days)	75.00±0.60	76.75±0.22	80.25±0.57	80.50±1.50	77.75±1.27	80.00±1.45	89.25±1.17	90.00±1.67
Plant height (cm)	123.50±2.35	132.75±0.51*	139.50±7.06	159.00±4.11	153.25±1.28	166.75±4.80*	174.50±2.77	178.75±3.99
Viable pollen shedding (days)	5.75±0.38	5.25±0.38	6.75±0.44	6.00±0.35	5.75±0.27	5.50±0.33	7.25±0.25	7.75±0.17
Weight 10 of seeds (g)	3.26±0.13	3.27±0.13	2.62±0.10	2.70±.0.11	2.76±0.09	2.83±0.13	2.51±0.06	2.63±0.21

Test entries (T) - Regenerated (R_1) plants, Controls (C) - Plants from seeds (Non regenerants). Means values of each genotype followed *have a significant difference between the Test entries and control when compared using t-test at 1% significant level

sample t test. There was no significant difference in the highly heritable phenological traits like time to flowering, silking and physiological maturity $p<0.01$ (Table 5). The same was true for weight of seeds and days of viable pollen shedding time. In the case of height, the OPV (KAT) and Hybrid (DHO1) there was a significant difference ($p<0.01$) between the controls and the test entries. Significant differences were not detected within the test entries and the controls seeds in the height of the inbred TL08 and OPV (DLC1) used in this experiment.

Maintenance of the morphogenic capacity of the organogenic calli: To determine whether the organogenic calli could be sub-cultured without loosing regeneration capacity, freshly induced organogenic-like calli from the four genotypes under study were propagated on MS media supplemented with 9 μM 2,4-D and 8.88 μM BAP. Callus from each genotype was maintained on regeneration condition for 4 weeks and transferred on fresh maintenance media. Organogenic calli was propagated on this media for 4 months and during this period, it was found that at least 40-60% of the calli generated produced shoots and formed roots when their shoots were cultured on MS supplemented with IBA. Therefore using the stated protocol, calli from the selected genotypes can be propagated as stock culture.

DISCUSSION

A reliable routine method for regeneration of numerous maize genotypes with a reasonably high success rate is required for maize genomics to become a reality. This will be useful as a system for availing transformed commercial maize lines with desirable traits introduced through transformation. The most well documented system for regeneration of maize which has been repeatedly reported as workable is based on generation of type II embryogenic calli (Fromm *et al*., 1990; Gordon-Kamm *et al*., 1990; Pescitelli and Sukhapinda, 1995). Despite its successful application this system seems to work for a few genotypes such as A188 among the temperate lines and CML216 among the sub-tropical lines. The calli derived from this system is provided by excision of immature zygotic embryos from 14-16 days old cob a method which is time consuming and laborious for it requires a consisted supply of immature zygotic embryos. An alternative method for maize regeneration based on obtaining organogenic calli from shoot meristem has been reported (Zhong *et al*., 1992, 1996) preventing the need for the protracted method used for availing immature zygotic embryos. In the use of shoot apices, the media formulation used by Zhong *et al.* (1996) was effective only on temperate lines for a repeat of the same formulation by O'connor was not successful leading to the development of a new formulation for sub-tropical lines (Sánchez *et al*., 2002).

The major difference between Zhong *et al.* (1996) formulation and Sánchez *et al.* (2002) formulation which was used in this study is the addition of adenine in the media. Adenine a purine with a variety of roles in biochemistry has low cytokinin activity (Gaspar *et al*., 1996) and is a central metabolite required for the synthesis of all amino acids (Renaud *et al*., 2000). Administration of adenine in this experiment complimented the effect of other growth regulators in the induction and formation of organogenic calli (Table 3) which was consistence with findings by Sánchez *et al.* (2002). Being a first time experiment, titration of BAP complemented with 9 μM 2,4-D at the induction step was done and best concentration for calli formation by the respective genotypes was confirmed in presence or absence of adenine (Sánchez *et al.* 2002).

In absence of BAP and at lower concentration of BAP (4.44-8.88 μM), some of the calli tended to form embryogenic calli (friable type I and type II calli) (Fig. 1b). This concur with the expected commitment when using immature zygotic embryos 14-16 days old confirming the totipotency of shoot meristem cells and their use as a promising explant for maize regeneration. The callus type formed in the different concentration of BAP also elaborates the importance of hormones in the formation of either embryogenic friable type I, type II or organogenic calli. This has been exhibited by the titration of BAP and the type of calli formed at the different concentrations of BAP.

The low frequency of root formation in MS without hormones was the biggest drawback to the study a consisted phenomenon with apices induced shoots (Sánchez *et al*., 2002). This was alleviated by the use of indole-3- butyric acid (IBA), which was titrated across five concentrations with 1.97 μM giving the best rooting results (Ludwig-Müller *et al*., 2005).

Agronomical comparison of the R_1 and the controls at 1% significance (Table 4) indicated that the plants in two groups of each genotype were indistinguishable for morphological traits like grain weight, leaf size and growth pattern. Overall, all the maize lines selected for the study regenerated with varying efficiencies depending on the genotype. This was achieved by titration of the hormones and amino acids used giving different responses depending on the genotype.

CONCLUSION

The successful regeneration of the two OPV's, a hybrid and an inbred line was consistent and provides a platform for the study of regeneration of other tropical maize genotypes found in Kenya including other recalcitrant and important cereals such as sorghum and millet found in the sub-Saharan set up. The results of the study show that the shoot apices are strong candidates for the provision of regenerable and probably transformable organogenic calli. The use of shoot apices as a candidate for regeneration of selected genotypes of maize fulfilled the two important requirements of an effective regeneration system: (a) high frequency of regeneration through organogenesis (b) an average *in vitro* culture period from explant stage until whole plant development to minimize somaclonal variation.

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