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Full Length Research Paper

In vitro regeneration of selected commercial Tanzanian open pollinated maize varieties

Miccah Songelael Seth[1,2]*, Leta Tulu Bedada[1], Emmarold E. Mneney[2], Richard O. Oduor[1] and Jesse S. Machuka[1]

[1]Department of Biochemistry and Biotechnology, Kenyatta University, P.O. Box 43844, 00100 Nairobi, Kenya.
[2]Mikocheni Agricultural Research Institute, P. O. Box 6226, Dar Es Salaam, Tanzania.

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Four Tanzanian open pollinated maize varieties namely; Kito, Situka M-1, Staha and TMV-1 were regenerated *in vitro* using immature zygotic embryos as ex-plants. Callus induction was achieved using Murashige and Skoog (MS) basal medium supplemented with 1, 1.5, 2 or 2.5 mg/l of 2, 4-D. Callus induction was significantly affected by the genotype of the varieties. Among the varieties tested, Kito, Situka M-1 and Staha had significantly higher callus induction frequency of 84.4, 92.7 and 88.7%, respectively compared to TMV-1 (43.3%). Embryogenic callus induction percentage was significantly influenced by the genotype, 2, 4-D concentrations and their interaction effect implying differential response of the genotypes to 2, 4-D concentrations. Among the genotypes tested, Kito gave significantly ($p<0.05$) the highest embryogenic callus frequency of 69.5%. Great variations were observed among the genotypes with regard to regeneration frequencies. Staha was comparatively the most regenerable variety with RF of 32.6% than even Kito which had the highest callus induction and embryogenic callus formation. MS medium amended with 1 mg/l 2, 4-D was observed to be the optimum formulation for the induction of embryogenic calli for genotypes tested in this study. Using this regeneration system Situka M-1, Staha and TMV-1 can now be improved against various production constraints through genetic engineering.

Key words: 2, 4-dichlorophexyacetic acid, embryogenic callus, immature zygotic embryos, regeneration frequency, somatic embryos, Tanzanian open pollinated maize.

INTRODUCTION

Maize (*Zea mays* L.) is the third most important staple crop in the world and is a main source of food for more than 300 million people in the sub-Saharan Africa (Edmeades, 2008). In Tanzania, maize is the major and most preferred cereal and is grown for both food and source of income (Moshi et al., 1997; RATES, 2003). The popularity of maize is evidenced by the fact that it is grown in all agro-ecological zones in the country. Maize accounts for 31% of the total food production and constitutes more than 75% of the cereal consumption in the country. Despite the importance of maize as the main staple crop, average yields in farmers' fields are relatively low averaging 1-2 metric tons per hectare compared to estimated potential yields of 4-5 metric tons per hectare (AATF, 2010). The grain yield has remained low due to biotic and abiotic constraints. Drought is a major threat to maize production in many parts of Tanzania. Research has been going on in Tanzania under the national research program to coordinate key aspects of maize research including developing maize hybrids and open pollinated varieties (OPVs) with improved quality and quantity (Hash et al., 2003).

Chromosome-mediated gene transfer through sexual hybridization has resulted into genetic improvement of both hybrids and OPVs. However, this method is time consuming and presents other limitations (Repellin et al.,

*Corresponding author. E-mail: micahgunah@yahoo.co.uk.

Table 1. Culture media used for callus induction, maintenance and regeneration.

Media	Composition
CIM	4.43 g/l MS premix, 3% sucrose, 2.9 g/l Proline, 0.1 g/l Casein hydrolysate, 0.8% agar, supplemented with either 1, 1.5, 2 or 2.5 mg/l 2, 4-D, pH 5.8
CMM	4.43 g/l MS premix, 3% sucrose, 2.9 g/l Proline, 0.1 g/l Casein hydrolysate, 0.8% agar, supplemented with either 1, 1.5, 2 or 2.5 mg/l 2, 4-D, pH 5.8
EMM	4.43 g/l MS premix, 6% sucrose, 1 mg/l NAA, and 0.8% agar, pH 5.8
SM	4.43 g/l MS premix, 3% sucrose, and 0.8% agar, pH 5.8
RM	2.2 g/l MS premix, 1.5% sucrose, and 0.8% agar, pH 5.8

CIM: Callus induction medium, CMM: Callus maintenance medium, EMM: Embryo maturation medium, SM: Shoot induction medium, RM: Rooting medium.

2001). Advances in modern biotechnology has enabled direct transfer of foreign genes with potential for improvement of cereal crops (Li et al., 2003).

A major drawback to genetic transformation of recalcitrant cereal crops such as maize is the lack of reliable and efficient *in vitro* regeneration system (El-Itriby et al., 2003; Satyavathi et al., 2004; Oduor et al., 2006). Because regeneration has been reported to be genotypic dependent (Oduor et al., 2006), it is important to establish efficient regeneration procedures for particular maize OPVs. This report describes the development of regeneration protocol for selected commercially important Tanzanian maize OPVs using immature zygotic embryos as source of explants through somatic embryogenesis. This was also aimed at identifying the most regenerable varieties that would be used for drought tolerance improvement through genetic engineering.

MATERIALS AND METHODS

Source of ex-plants, establishment and seed bulking

Maize seeds of Tanzanian commercial OPV; Kito, Situka M-1, Staha and TMV-1 were collected from East African Seed (T) Ltd (EASEED), Arusha, Tanzania. These varieties were selected based on farmers' preference for yield, maturity, tolerance to diseases, pounding quality and potential for genetic improvement. Maize plants were grown in pots and bulked in the green house at Plant Transformation Laboratory (PTL), Kenyatta University, Nairobi, Kenya. The research was carried out from November, 2009 to October, 2010. The planting time and days to silking and tasseling of the varieties were recorded to determine the anthesis-silking intervals.

Surface sterilization and embryo excision

Ears were harvested between 12 to 16 days post pollination depending on weather and variety. Ears were dehusked and surface sterilized using 3% commercial bleach (JIK) (PY-REX East Africa Ltd, Nairobi, Kenya) supplemented with 1 to 2 drops of Tween 20® for 20 min under sterile conditions prior to rinsing 3 times with sterile distilled water. Embryos of approximately 1 to 1.5 mm were aseptically excised using scalpel blade by superficially trimming the top edge of kernels to release the embryos using a spatula.

Callus induction, maintenance and maturation

Calli were initiated by placing 35 embryos per plate in triplicates on callus induction media (CIM) amended with varying concentrations (1, 1.5, 2 or 2.5 mg/l) of 2, 4-D (Table 1). The embryos were incubated on CIM for 14 days in darkness at a temperature of 26±2°C. Primary calli were transferred to callus maintenance medium (CMM) and further incubated for 28 days in dark at 26±2ºC following a fortnight sub-culturing regime (Ombori et al., 2008). Calli from CMM were transferred to embryo maturation medium (EMM) (Binott et al., 2005). All media were prepared using MS premix basal salts (Murashige and Skoog, 1962) enriched with relevant supplements as presented in Table 1. The pH of all media used in this study was adjusted to 5.8 before autoclaving at a temperature of 121°C for 20 min with a pressure of 15psi.

Regeneration

Embryogenic calli (EC) with mature somatic embryos were transferred from EMM medium to shoot induction medium (SM) and incubated under cool white fluorescent tubes (60 to 90 µmol photons $m^{-2}s^{-1}$) photoperiod of 16 h light and 8 h darkness at a temperature of 26±2°C. Sprouted shoots with well developed root system were directly hardened whereas those with no or few roots were transferred first to half strength MS medium (RM) to enhance rooting under similar conditions as those for shooting. Hardening was done in pots (100 ×100 × 100 mm depth) containing peat moss (KEKKILA Co. Ltd, Tuusula, Finland) and covered with white transparent polythene bags to maintain relative humidity of ca. 80% for 3 days. After 5 to 7 days, surviving regenerants were transplanted to plastic pots (150 mm radius with 330 mm depth) containing forest soils for development and maturity.

Experimental design and data management

Experiments for calli initiation and embryogenic calli formation were designed as a 4 × 4 factorial (4 varieties treated with 4 concentration levels of 2, 4-D) arranged in a completely randomized design (CRD) with each experiment conducted in triplicates. Callus induction frequency (CIF) was scored as the percentage of total number of immature embryos which formed calli in CMM per total number (35 embryos per plate) of initial immature zygotic embryos cultured. Embryogenic calli formation frequency (ECF) was deter-

Table 2. Developmental features and attributes of Tanzanian OPV maize varieties used for tissue culture.

Variety	Type	Year released	Recommended altitudes (M)	Days to anthesis*	Days to silking*	ASI (Days)*	Yield (t/ha)		Agronomic attributes
							Potential	Expected	
Kito	OPV	1983	0-1,500 Low to mid altitude	92	94	2	6.0	2.50-3.5	Small stature varieties, early maturing variety
Situka M-1	OPV	-	0-1,500 Low to mid altitude	99	105	6	-	-	Drought tolerant
Staha	OPV	1983	0-1,500 Low to mid altitude	92	98	6	6.5	4.0-4.5	Tolerance to maize streak virus disease
TMV-1	OPV	1987	0-1,500 Low to mid altitude	98	104	6	4.25	-	Has white, flinty grain, is streak resistant, and has intermediate maturity

ASI: Anthesis-silking interval *These developmental features were obtained from the current study, whereas other data were obtained from East African Seed (T) Ltd (EASEED Arusha, Tanzania).

mined as the percentage number of calli containing at least a single somatic embryo in EMM per number of the initial immature embryos cultured. Regeneration frequency (RF) was calculated as the percentage number of shoots proliferated per total number of EC transferred to SM. The data analysis was done using GenSat Discovery Edition 3 (VSN International software for biosciences (www.vsni.co.uk/software/genstat/). Means were separated using LSD at 5% level of significance.

RESULTS AND DISCUSSION

Source of ex-plants and determination of anthesis-silking interval

Determination of anthesis-silking interval (ASI) was carried out in all the four varieties (Kito, Situka M-1, Staha and TMV-1). Although, days to silk and anthesis varied among the varieties, the ASI was found to be six days for all the varieties except for Kito which was two days (Table 2). ASI is an important parameter that is useful in determining the right time for pollination and eventual collection of embryos at the right age and size. In the present study, immature zygotic embryos of 1 to 1.5 mm obtained at the age of 14 to 16 day after pollination were found to be the best sources of ex-plant. Other studies have reported the right embryo size and ages for various tropical maize varieties to be 1 to 1.3 mm obtained 15 days after pollination (Binott et al., 2005), 1 to 2 mm obtained 17 to 20 days after pollination (Oduor et al., 2006) and 1 to 2.5 mm obtained 16 to 20 days after pollination (Ombori et al., 2008).

Callus induction, maintenance and maturation

Maize embryos started to form primary calli within 4 to 5 days on CIM. These calli were observed to originate at the scutellum of the embryo (Figure 1A). Inclusion of $AgNO_3$ in CIM has been reported to influence type II embryogenic calli proliferation (for review see El-Itriby et al., 2003; McCain and Hodges, 1986; Vain et al., 1989). After 28 days of culture on CMM, a mass of dedifferentiated cells (Figure 1B) was visible. Two types of calli were observed which were classified as non embryogenic, *NE* (Type I) and embryogenic, *EC* (Type II) calli (Figure 1C). Type I calli were characterized as being white, compact, organized, watery and slow growing, whereas type II calli looked creamy yellow, brittle or friable and fast growing. Type II embryogenic calli are the most preferred as they often regenerate to plantlets upon exposure to SM (Oduor et al., 2006). Ex-plants on MS medium amended with 2, 4-D resulted into calli production whereas those on control plate (devoid of 2, 4-D) germinated instead of forming calli. These results further demonstrate the role of growth regulator (2, 4-D) in calli induction. While working with maize embryos of A188, Bronsema et al. (2001) observed a similar trend where EC formation was only achieved at 0.2 or higher up to 2 mg/l of 2, 4-D, but below 0.02 mg/l 2, 4-D embryos germinated and did not form calli at all. Growth regulators such as auxins are normally included in culture media and play an important role in somatic embryogenesis.

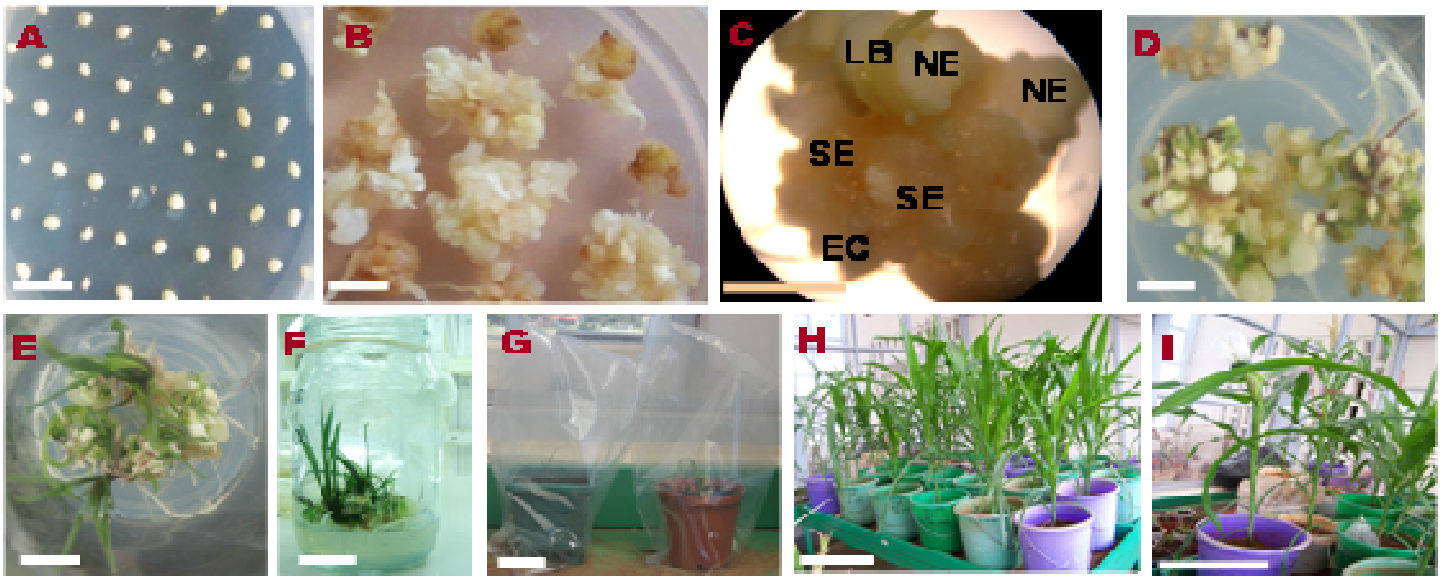

Figure 1. *In vitro* regeneration procedure of Tanzanian open pollinated maize varieties; A: Immature embryos bulging 1-3 days of culture on CIM, *bar* = 1 cm; **B:** Embryos forming calli 14 days on CMM, *bar* = 1 cm; **C:** Callus displaying both type I, *NE*, and type II, *EC*, somatic embryos *SE*, leaf bud, *LB* emerging from mature somatic embryo, *bar* = 1 mm; **D:** Somatic embryos greening 2-5 days on SM upon exposure to light, *bar* = 1 cm; **E:** Leaf buds emerging from mature somatic embryos on SM, *bar* = 1 cm; **F:** Two weeks growing maize shoots on SM, *bar* = 1 cm; **G:** hardening of plantlets in peat moss in the glass house, bar=1cm; **H:** Regenerated maize plants growing in forest soils in the glass house, *bar*=30cm; **I:** *In vitro* maize regenerants tasselling and silking at Kenyatta University PTL glass house, *bar* = 30 cm.

Frequently used auxins are 2, 4-D, picloram and dicamba. Although they perform similar functions, these auxins differ significantly in their effect-tiveness when used in various plant species and concen-trations (Satyavathi et al., 2004). In this study, only 2, 4-D was used as source of auxins to induce calli because it was cheaply available compared to other types of auxins.

Analysis of variance revealed that variation of 2, 4-D concentrations had no significant effect on CIF means within varieties. However, significant ($p<0.01$) differences with respect to CIF and ECF means were observed among the varieties, indicating that the varieties have genetic differences in inducing callus and embryogenic callus. Similarly ECF means were significantly influenced by the variation of 2, 4-D concentrations and the effect of interaction of 2, 4-D concentration by variety. This sug-gests that there was an independent and combined effect of 2, 4-D concentrations and varieties on embryogenic calli formation.

Based on analysis of variance means were separated by LSD to compare the means at 5% significance level. Among the varieties tested, Kito, Situka M-1 and Staha had significantly higher CIF means of 84.4, 92.7 and 88.7%, respectively, compared to TMV-1 (43.3%t) (Table 3). On the other hand, Kito demonstrated the highest ECF mean (69.5%) followed by Situka M-1 (49.77%), Staha (35.43%) and TMV-1 (15.67%). The highest ECF means for Kito, Situka M-1, Staha and TMV-1 (Table 3) were optimally obtained at 2.5, 1, 1 and 1.5 mg/l 2, 4-D concentrations, respectively. These results are not sur-prising as genotype dependences have been reported particularly in cereals (Przetakiewicz et al., 2003; Satyavathi et al., 2004; Oduor et al., 2006; Ombori et al., 2008). The variation in callus induction and embryogenic calli formation within varieties is often attributed to many factors including but not limited to media composition, 2, 4-D concentration and duration of exposure, type of ex-plant, embryo size, age of the ex-plant and softness or tenderness of the ex-plant (Green and Phillip,1975; Slesak et al., 2005).

In the current study, we observed formation of somatic embryos (SE) on calli while on CMM. However, SE pro-liferation and growth appeared retarded on EMM. Among the varieties tested, Kito was observed to abort SE on EMM. Such slow growth and retarded proliferation of EC during somatic embryo maturation are often linked to gene regulation. The ability of 2, 4-D in triggering the embryogenic pathway may be related to its capacity to induce stress genes, which have been shown to contri-bute to the cellular reprogramming of somatic cells toward embryogenesis (Kitamiya et al., 2000). In this study, slow growth of embyos was observed during maturation. Che et al. (2006) reported that reduced cell proliferation and slow growth during somatic embryo maturation is associated with down regulation of histone and ribosomal protein genes and up-regulation of genes such as those encoding hydrolytic enzymes (nucleases, glucosidases and proteases) and a few storage genes (an α-zein and

Table 3. Callus induction, embryogenic calli formation and regeneration of four Tanzanian OPV of maize as influenced by 2, 4-D concentration.

EXP	2,4-D(mg/l)	Varieties				2, 4-D MEAN*
		Kito	Situka M-1	Staha	TMV-1	
CIF (%)	1.0	73.3	**96.0**	89.3	40.0	**74.67±6.8^{a}**
	1.5	**90.7**	94.8	84.0	33.3	**75.7±7.78^{a}**
	2.0	87.6	89.3	**92.0**	**54.7**	**80.89±6.3^{a}**
	2.5	86.2	90.7	89.3	45.3	**77.89±6.6^{a}**
	VARIETY MEAN*	**84.4±4.4^{a}**	**92.7±2.8^{a}**	**88.7±3.0^{a}**	**43.3±3.6^{b}**	
ECF (%)	1.0	61.3	**85.9**	**52.0**	10.7	**52.48±8.5^{a}**
	1.5	64.0	64.9	36.0	**22.7**	**46.88±8.5^{a}**
	2.0	75.3	8.3	29.3	13.3	**31.58±8.6^{b}**
	2.5	**77.3**	40.0	24.4	16.0	**39.43±7.5^{a}**
	VARIETY MEAN*	**69.5±4.6^{a}**	**49.7±10.7^{b}**	**35.4±3.9^{c}**	**15.7±2.5^{d}**	
RF (%)	1.0	11.7	7.8	**63.7**	**40.0**	**30.78±11.9^{a}**
	1.5	1.8	**41.7**	23.1	35.7	**25.57±9.20^{a}**
	2.0	00.0	00.0	22.2	00.0	**5.56±5.56^{b}**
	2.5	00.0	00.0	21.4	00.0	**5.36±5.36^{b}**
	VARIETYMEAN*	**3.4±2.2^{b}**	**12.4±8.3^{a}**	**32.6±9.9^{a}**	**18.9±10.9^{a}**	

Value = Means ±SE; *Values followed by different letter indicates statistically significant difference by LSD at 5% level; **EXP:** experiment; **CIF (%):** Callus induction frequency; **ECF (%):** Embryogenic calli formation frequency; **RF (%):** Regeneration frequency.

caleosin). However, down regulated genes recovered drastically during shoot emergence.

Regeneration

Shoot proliferation was observed 3 to 4 days after transferring EC to hormone free SM. Upon exposure to light, EC were observed to green in 2 to 3 days (Figure 1D) followed by shoot buds within 3 to 4 days (Figure 1E). In this study, all shoots developed roots on SM (Figure 1F). Shoots with stable root systems were hardened on peat moss (Figure 1G). Hardened plantlets were transplanted to forest soils (Figure 1H) and grown to maturity. No abnormal phenotypes compared to the non tissue culture counterparts were observed (Figure 1I). It has been shown that long duration of culture *in vitro* can impose some morphological changes in regenerated plants. Such variations are often attributed to somaclonal variations which usually are epigenetic (Ombori et al., 2008). Nevertheless, these results showed that regeneration frequencies were not correlated to CIF and ECF means. Great variations were observed among the genotypes with regard to regeneration frequencies. Staha was comparatively the most regenerable variety with RF of 32.6% than even Kito which had the highest callus induction and embryogenic callus formation. On the other hand, TMV-1 that was less responsive to calli formation showed high regeneration ability compared to Situka M-1 that had good performance on CIM. This may probably be attributed to several shoots that emanated from few EC of TMV-1 varieties.

Embryogenic calli formation is one of the most important changes that may highly determine regeneration potential of a given variety. Generally, the ability to form EC was not always comparable to regeneration as almost all varieties gave low RF. This low RF could have been attributed by some factors notably somatic embryo abortion as was demonstrated with Kito.

Occasionally, EC were observed to green and produce massive roots without shooting. Such responses are often associated with the type and concentrations of auxins. Auxins can exert residual effect to regenerating tissues thereby causing *in vitro* epigenetic changes which affect regeneration. While studying the effect of two auxins, 2, 4-D and picloram, on scutellum culture response of durum wheat, He and Lazzeri (2001) noted that addition of auxin to media had no significant effect on embryogenesis. However, addition of these auxins clearly affected regeneration, with cultures induced on picloram containing media showing higher regeneration frequencies than those induced on 2, 4-D. Leyser (2001) reported that the auxin signal is received by plant cells and rapidly transduced to a wide range of responses in the growth and development of plant organs, including changes in the direction of growth, shoot and root branching and vascular differentiation.

In the current study, we observed a general trend for all the varieties studied, in which high shoot induction was recorded on calli that were initially cultured on MS media amended with low 2, 4-D concentration than on those previously cultured on higher 2, 4-D concentrations. Although, 2, 4-D is required initially for calli induction and somatic embryo formation, it is also important to reduce its concentration for further embryo development and sprouting (Bronsema et al., 2001). Furthermore, there was massive necrosis of EC on SM due to initial cell death of aborted somatic embryos thus contributing to low RF. This cell death could have probably been due to released phenol-like chemical compound on the medium which may be toxic to surviving cells/tissues. These findings suggest that when this happens it is important to subculture the surviving calli on a fresh SM media. Zhao et al. (2010) while working on sweet sorghum (*Sorghum bicolor* Moench) observed that the non embryogenic calli would turn brown and die during subsequent culture and regeneration. Also, the non embryogenic calli often exuded a reddish orange pigment which could inhibit callus growth and the addition of 10 mg/l vitamin C and polyvinylpyrrolidone (PVP) could decrease the pigment secretion.

Conclusion

Efficient *in vitro* procedures for calli initiation, maintenance and regeneration of four commercially important Tanzanian open pollinated varieties of maize were developed. Situka M-1, Staha and Kito were found to be the most responsive varieties. However, somatic embryos from Kito were aborted on maturation medium. Somatic embryo abortion in Kito may partly be avoided by bypassing the maturation stage. TMV-1 was the least responsive in terms of calli induction and embryogenic calli formation although it demonstrated a relatively higher regeneration frequency than Kito and Situka M-1. This work represents the first report of *in vitro* regeneration of Tanzanian maize varieties and thus can act as a basis for future *in vitro* improvement of Tanzanian maize varieties through genetic transformation, recovery of somaclonal variants, haploidisation, somatic hybridization and micropropagation.

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