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Full Length Research Paper

Regenerability of elite tropical maize (*Zea mays* L.) inbred lines using immature zygotic embryo explants

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Five elite tropical maize inbred lines; CML395, CML443, CML442, MAS [MSR/312]-117-2-2-1-B-5-B) and CML216 as a control, were evaluated for their regenerability making use of calli derived from immature zygotic embryos. Murashige and Skoog basal salts supplemented with 1.0, 1.5, 2.0 and 2.5 mg/L 2,4-dichlorophenoxyacetic acid were used to induce callus. Callus induction frequency and formation of embryogenic callus varied significantly ($p<0.01$) depending on genotype and level of 2,4-dichlorophenoxyacetic acid. Shoot regeneration efficiency also differed significantly ($p<0.01$) depending on genotype. Significantly ($p<0.05$) higher callus induction and frequency of embryogenic callus were obtained at 1 mg/L 2,4-dichlorophenoxyacetic acid, indicating this as the optimal level for regenerating these inbred lines. CML395 and CML442 revealed significantly ($p<0.05$) higher callus induction and embryogenic callus frequency compared to CML443 and MAS [MSR/312]-117-2-2-1-B-5-B), while they were at par with the control inbred line CML216. Plants were regenerated from all the inbred lines except CML443 and were successfully acclimatized and grown to maturity. CML395 was the best regenerable line with significantly ($p<0.05$) higher regeneration efficiency of 109.3%. It was concluded that CML395, CML216 and CML442 can be used in *in vitro* genetic transformation.

Keywords: Elite tropical maize inbred lines, immature zygotic embryos, *in vitro* culture, plant regeneration, somatic embryos.

INTRODUCTION

Maize (*Zea mays* L.) is the primary grain crop grown for human consumption in Sub-Saharan Africa (SSA), comprising a significant part of the diet. In the East and Central African (ECA) countries, it is a staple food dominating the major proportion in the diets of the rural and urban population. Despite this fact, the actual maize yield in this part of the world is very low, rarely exceeding 1.3 t/ha, compared to the potential yield that is reported to be over 10 t/ha (Morris, 1998). Low productivity is ascribed to several biotic and abiotic stresses (Schechert et al., 1999; Ajanga and Hillock, 2000; Kanampiu et al., 2002). Among several abiotic stresses affecting maize productivity, drought takes the lead, accounting for a yield loss of about 70% and even causing total crop losses in extreme situations (FAOSTAT, 2002).

Breeding efforts have been targeting drought as a major constraint in maize production. To this end, the International Maize and Wheat Improvement Centre (CIMMYT) has been using conventional breeding through which it has developed several elite inbred lines that are well adapted to the tropical environment of the ECA region. These lines have played important role in hybrid maize production (Evenson and Gollin, 2003) due to high heterotic responses for grain yield and other agronomically desirable traits in their hybrid combinations. Elite inbred lines also have supreme significance in genetic engineering as direct recipients of transgenes of interest,

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thereby eliminating further steps of carrying the transgene to more elite genetic background through backcross breeding. Despite all their desirable qualities, maize inbred lines developed through conventional breeding are susceptible to drought stress at their critical growth stages, especially in the arid-and semi-arid tropics.

Considerable opportunities exist for developing better performing drought tolerant inbred lines in a relatively shorter time through biotechnology. Further steps could be adopted to dramatically improve and stabilize maize yield in drought stressed environment by the use of more advanced methods of genetic improvement through genetic engineering. For successful production of transgenic plants, foreign genes must be delivered to undifferentiated, dedifferentiated or dedifferentiating cells that are actively dividing or about to divide and that are capable of regenerating plants. For the production of transgenic lines in maize, the materials of choice are immature zygotic embryos (Ishida et al., 2007). These were reported as the best types of explants for subsequent efficient recovery of fertile plants in temperate (Green and Philips, 1975; Armstrong and Green, 1985) and tropical maize inbred lines (Binott et al., 2008; Ombori et al., 2008). This is primarily associated with the presence of large number of competent cells in their scutellum for somatic embryogenesis. Thus, the primary determinants of successful maize transformation are the responses of immature zygotic embryos to tissue culture - the type of cells that are grown from the immature zygotic embryos and their subsequent characteristics in growth and regeneration (Ishida et al., 2007). Formation of embryogenic callus is generally genotype-dependent in many plant species, including maize. Therefore, most elite inbred lines of maize remain inaccessible to improvement using the standard transformation techniques, because they either fail to produce embryogenic calli from their transformation competent tissues, or they fail to regenerate efficiently after the induction of embryogenic calli (Che et al., 2006). Regenerability is also influenced by the composition of the culture media used in the process (Armstrong and Green, 1985). This calls for the need to study the regenerability of specific germplasm prior to embarking on genetic transformation.

Most of the previous studies on maize regeneration used temperate germplasm (Green and Philips, 1975; Armstrong and Green, 1985), while due attention had not been given to tropical maize, particularly those adapted to the ECA region, until the pioneer work of Bohorova et al. (1995) that disclosed the regeneration potentials of tropical maize. It was only very recently that further studies were undertaken to evaluate the regenerability of more tropical maize genotypes (Danson et al., 2006; Oduor et al., 2006; Binott et al., 2008; Ombori et al., 2008; Rasha et al., 2008) using immature zygotic embryo explants. Considering its diverse genetic background, there is still a long way to go to exhaustively assess the regenerability of tropical maize. This study was carried out to evaluate the regeneration potential of five elite tropical maize inbred lines adapted to the ECA region, making use of calli derived from immature zygotic embryos. It was also intended to identify the best regenerable lines for subsequent use in *in vitro* genetic transformation aiming at improving tolerance to drought stress in this important food grain crop of the ECA region.

MATERIALS AND METHODS

Plant materials and explant preparation

The experimental materials consisted of five elite tropical maize inbred lines; CML395, CML442, CML443, MAS [MSR/312]-117-2-2-1-B-5-B) and CML216, which were obtained from CIMMYT, Nairobi, Kenya. The lines were selected on the basis of their adaptation to maize growing environments in the ECA region, resistance to diseases and uniformity in plant height, ear placement and other phenotypic traits. According to CIMMYT's classification based on their response to drought stress, CML442 and CML443 were grouped as drought tolerant, while CML395 and MAS [MSR/312]-117-2-2-1-B-5-B) were classified as susceptible to drought stress (Dan Makumbi, CIMMYT, Nairobi, Kenya, personal communication). In this experiment, CML216 was included as a control since its regeneration potential has already been confirmed (Ombori et al., 2008). The inbred lines were grown in the glasshouse at Plant Transformation Laboratory (PTL) of Kenyatta University from July 2009 to April 2010 to supply immature zygotic embryos for culture initiation *in vitro*. To produce genetically uniform explants, the uppermost ear of each plant was covered with a transparent plastic bag of 8 x 4 inches before silk emergence, until it was self pollinated. Self pollination was executed when the silks emerged to about 2 cm above the sheath and enough pollen was available. After pollination, the ears were covered with pollination bags to prevent contamination through cross pollination of the late coming silks with foreign pollen.

Immature zygotic embryos were continuously checked for their sizes under a microscope (Figure 1A to C), starting the 10th day post pollination, by removing kernels from different parts of the cob and excising the immature zygotic embryos using sterile scalpel blades. Maize cobs were then harvested when the immature zygotic embryos attained a size of 1.2 to 1.7 mm along their axis and used immediately or kept in the refrigerator at 4°C for 1 to 2 days within their husk for later use. The size of the immature zygotic embryos was selected considering the proposal by Ishida et al. (2007) who reported 1.2 mm to be suitable for subsequent *Agrobacterium* mediated genetic transformation. It was considered necessary to know how well these sizes of immature zygotic embryos from the inbred lines respond to tissue culture. In preparation for the experiment, the ears were de-husked and surface sterilized for 5 min in 70% (v/v) ethanol, prior to their treatment with a solution of 2.5% (v/v) sodium hypochlorite added with 2 drops of Tween 20 for 20 min, followed by 2 to 3 times rinse with sterile distilled water under the laminar flow cabinet. The immature zygotic embryos were then excised aseptically by cutting the top part of the kernels with a sharp scalpel blade.

Callus initiation and maintenance

Immature zygotic embryos having mean size of 1.2 to 1.7 mm (Figure 1A to C) were cultured on a callus initiation medium (CIM) (Figure 1D), ensuring direct contact of the embryo axis with the

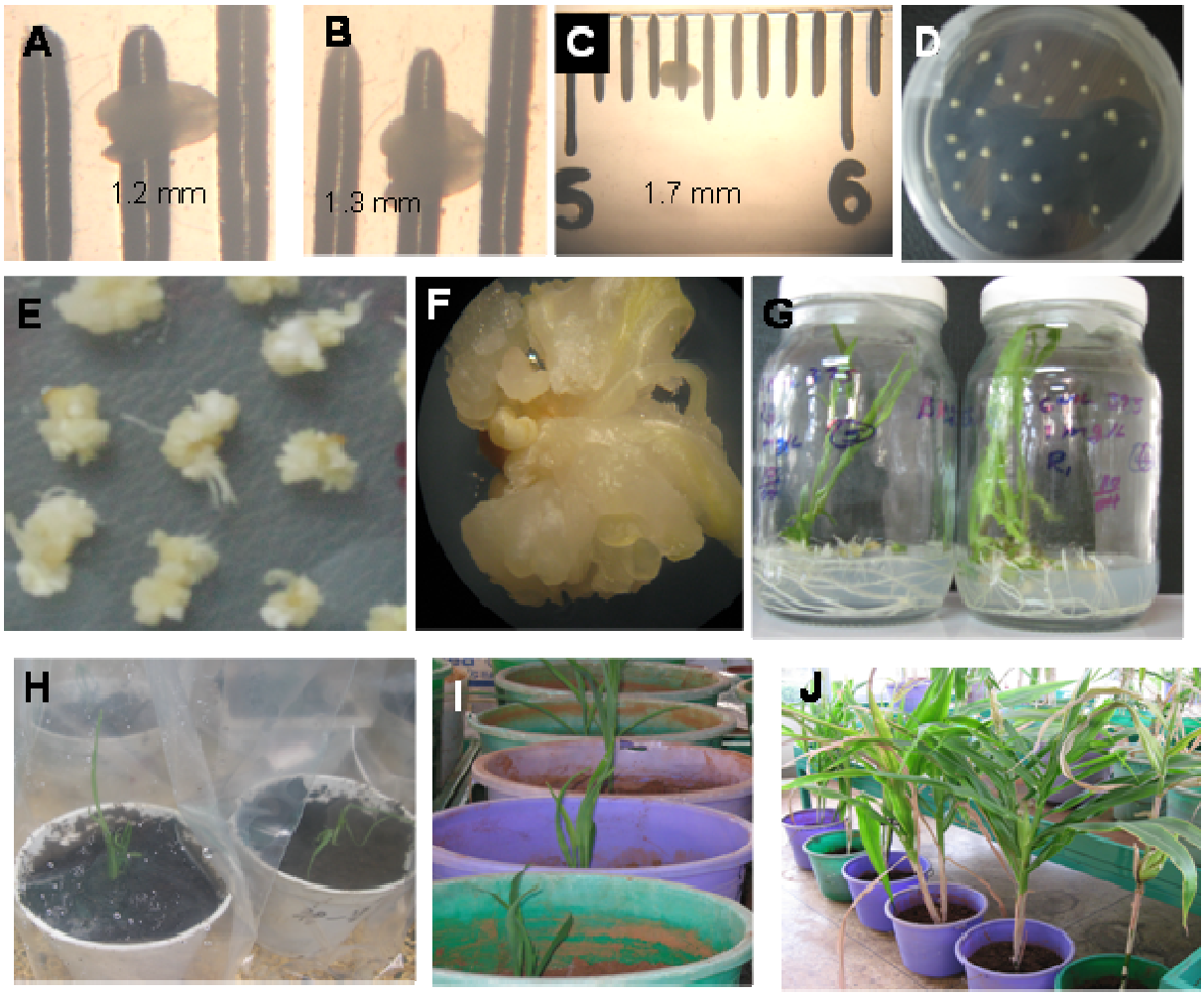

Figure 1. Tissue culture procedures applied in *in vitro* regeneration of five elite tropical maize inbred lines. (A to C) Size of immature zygotic embryos used for tissue culture. (D) Immature zygotic embryos after four days of culture on callus induction medium. (E) Friable type II embryogenic calli of CML442 on CMM. (F) Matured somatic embryos of CML442 turning green on CMM. (G) Plantlets on regeneration medium three weeks after culture. (H) Acclimatization of plantlets in the green house. (I) Plantlets grown in the potted soil in the green house. (J) Regenerants grown to maturity in the soil.

medium. This orientation is adopted to retard embryo germination and enhance proliferation of scutellar cells (Green and Phillips 1975). In this study, the CIM composed of MS (Murashige and Skoog, 1962) basal salts supplemented with 1.0, 1.5, 2.0 and 2.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), L-proline (2,875 mg/L), casein hydrolysate (100 mg/L), silver nitrate (10 mg/L), together with 3% (w/v) sucrose as a source of carbon and 0.8% (w/v) agar as a solidifying agent. The culture media were auto-claved at 121°C for 20 min at a pressure of 1.06 kg/cm after adjusting the pH to 5.8 using 1 N NaOH and/or 1 N HCl.

Twenty five immature zygotic embryos were cultured in a 90 × 15 mm Petri dish (supplied by PY-REX, East Africa Ltd) for each treatment (Figure 1D). The cultures were incubated in the dark at a temperature of 27 ± 1°C for two weeks. Callus induction frequency was recorded two weeks after culturing on CIM. The developing calli were sub cultured every 14 days onto a callus maintenance medium (CMM), which consisted of similar components with that of CIM but devoid of silver nitrate. The type of calli produced was noted at each step. Frequency of embryogenic calli was recorded after four weeks of culture on the CMM. The treatments were arranged in a 5 × 4 factorial experiment laid out in a completely randomized design (CRD). The experiment was repeated twice with three replications per treatment.

Embryo maturation and plant regeneration

Embryogenic calli were transferred onto embryo maturation medium (EMM) containing MS basal salts supplemented with 6% (w/v) sucrose, 1 mg/L naphthalene acetic acid (NAA) and 0.8% (w/v) agar for embryo maturation. Cultures were incubated in the dark at a temperature of 27 ± 1°C. After two weeks of culture on the EMM, embryogenic calli with somatic embryos were selected and transferred onto a hormone free regeneration medium (RM), containing MS basal salts supplemented with 3% (w/v) sucrose and solidified with 0.8% (w/v) agar for shoot induction. The cultures were incubated at a temperature of 27 ± 1°C, 16 h/8 h (light/dark) photoperiod and light intensity of 71.65 $J.m^{-2}s^{-1}$ supplied from two cool and white fluorescent tubes of 18 W each. The number of shoots formed per culture was recorded starting from the second

Table 1. Analysis of variance for callus induction and embryogenic callus frequency and regeneration efficiency evaluated in five elite tropical maize inbred lines and four levels of 2,4-D.

Sources of Variation	Df	Mean squares		
		CIF	ECF	RE
Genotypes(G)	4	1106.7**	16915.9**	22757.0**
2,4-D levels (GH)	3	256.98**	2582.85**	3241.0
G x GH interaction	12	112.53**	545.67**	1214.0
Error	40	19.20	33.69	2433.0
CV (%)		4.7	8.6	120.5

** Differences are statistically significant at P<0.01 probability level. **CIF**, Callus induction frequency; **ECF**, embryogenic callus frequency; **RE**, regeneration efficiency

week of culture on the RM. In this experiment, all plantlets produced roots on the RM and hence their transfer to rooting media was not necessary.

Hardening and growth of primary regenerants (R_0)

Primary regenerants (R_0) with well developed roots were removed from the RM and rinsed under tap water to remove the adhering media, prior to their transfer to plastic pots containing sterile peat moss. The plantlets were kept for 3 days covered with a clear polythene bag punctured on the 2nd day for gradual acclimatization to the ambient environment. After 7 to 10 days the plantlets were transferred onto the pots containing garden soil mixed with manure and sand at a ratio of 2:2:1, and kept in the greenhouse till they grew to maturity.

Data analysis

Data recorded on the number of primary callus, embryogenic callus and numbers of shoots were used to compute callus induction and embryogenic callus frequencies, and regeneration efficiencies, respectively. Analysis of variance (ANOVA) was carried out using the MINITAB statistical computer software (version 23.22) to test the statistical significance of differences among the genotypes and 2,4-D levels. Mean separation was carried out using least significance difference (LSD) test at 5% probability level.

RESULTS AND DISCUSSION

Callus initiation and maintenance

Four days after plating on CIM, the dome shaped scutellum of the immature zygotic embryos appeared to swell. Afterwards, callus initiation was observed within the following 2 to 3 days by swelling of the scutellum and concomitant proliferation of small mass of un-differentiated cells in all inbred lines. Callus initiation from the scutellum has been attributed to the presence of meristematic cells (Al-Abed et al., 2006).

Callus induction and formation of embryogenic callus from immature zygotic embryos of the tropical maize inbred lines varied significantly ($p<0.01$) depending on genotype and level of 2,4-D. Both parameters also differed significantly ($p<0.01$) due to the interaction effect of genotype × level of 2,4-D (Table 1). However, regeneration efficiency differed significantly ($p<0.01$) depending on genotype only. Inbred lines CML395, CML442 and the control CML216 produced mean callus induction frequency of 100 per cent each indicating that all immature zygotic embryos of these lines plated on CIM produced callus (Table 2). On the other hand, CML443 and MAS [MSR/312]-117-2-2-1-B-5-B) revealed significantly ($p<0.05$) lower mean callus induction frequency of 83.3 and 81.7 per cent, correspondingly. With these two lines, the scutella of some of the immature zygotic embryos failed to proliferate and induce callus. Instead they remained swollen and hard without showing any sign of further proliferation to un-differentiated mass of cells. Highly significant genotype × 2,4-D interaction effect for callus induction frequency indicated differential responses of the inbred lines to the levels of 2,4-D for optimum callus induction. Among the five tropical maize inbred lines tested in this study, three lines (CML395, CML442, and the control CML216) revealed optimum performance irrespective of the levels of 2,4-D (Table 2). On the other hand, CML443 and MAS [MSR/312]-117-2-2-1-B-5-B) performed better only on the medium supplemented with 1 mg/L 2,4-D. However, the callus inducing capacity of their immature zygotic embryos dropped significantly in cases where the culture media were fortified with more than 1 mg/L 2,4-D.

Considering the different hormone concentrations used, 1 mg/L 2,4-D gave significantly ($p<0.05$) higher mean callus induction frequency of 99.2% across all the inbred lines. Therefore, this level was confirmed to be optimum for callus induction from immature zygotic embryos of all the tropical maize inbred lines tested in the present study. Several authors have reported that 2,4-D is an essential growth hormone for the initiation of primary callus from immature zygotic embryos of maize (Bhorova et al., 1995; Carvalho et al., 1997; Ombori et al., 2008) and wheat (Yu et al., 2003; Bi et al., 2007). Normally, immature zygotic embryos of maize were known to germinate without callus initiation on callus induction media devoid of 2,4-D (Bronsema et al., 2001; Ombori et al., 2008). This was the rational for excluding zero level of 2,4-D from the current study.

Table 2. Callus induction frequencies[a] of five tropical maize inbred lines evaluated in response to four levels of 2,4-D.

Genotypes	2,4-D levels (mg/L)				Genotype mean**
	1.0	1.5	2.0	2.5	
CML395	100.0±0.0[a]***	100.0 ± 0.0[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]	100 ± 0.0[a]
CML442	100.0 ± 0.0[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]	100 ± 0.0[a]
CML443	96.0 ± 5.6[b]	81.3 ± 3.7[c]	76.0 ± 3.2[d]	80.0 ± 11.7[c]	83.3 ± 3.11[b]
MAS [MSR/312]-117-2-2-1-B-5-B)	100.0 ± 0.0[a]	73.3 ± 4.9[d]	80.0 ± 3.3[c]	73.3 ± 4.9[d]	81.7 ± 3.5[b]
CML216 (Control)	100.0 ± 0.0[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]	100 ± 0.0[a]	100 ± 0.0[a]
2,4-D level mean**	99.2 ± 0.8[a]	90.9 ± 3.13[b]	91.2 ± 2.95[b]	90.6 ± 3.46[b]	

[a]Callus induction frequencies were computed as the number of immature zygotic embryos forming primary callus divided by the number of immature zygotic embryos initially cultured per plate × 100, after two weeks of culture on the CIM. **Means followed by the same letter are not significantly different from each other according to LSD test at 5% probability level. *** Values followed by the same letter within columns and rows are not significantly different from each other according to LSD test at 5% probability level.

After two weeks of incubation on CIM, the calli were transferred to CMM on which embryogenic calli were visible after two weeks. Both type I and type II calli were formed, whereby type I calli were compact and white to creamy, while type II were friable and light yellow in colour. In the current study, three of the inbred lines, CML395, CML442 and the control CML216, mainly resulted in type II callus. However, not all inbred lines tested formed embryogenic calli at all levels of 2,4-D considered in this study. In other words, the formation of embryogenic calli was dependent on genotype and the level of 2,4-D. Non embryogenic calli, which were soft and watery and yellow in colour were commonly observed in CML443 and MAS [MSR/312]-117-2-2-1-B-5-B), mainly at 2,4-D levels higher than 1 mg/L. Inbred line CML443 gave embryogenic calli only when the MS basal salts were supplemented with 1 mg/L 2,4-D. On the other hand, whenever the concentration of 2,4-D was increased beyond this minimum level, the calli turned yellowish at first and then brownish at the later stage, then became necrotic with further sub culturing on CMM and finally died. Similar phenomena were observed in the inbred line MAS [MSR/312]-117-2-2-1-B-5-B) at all levels of 2,4-D. This is in agreement with the reports of Binott et al. (2008) who cultured immature zygotic embryos of Kenyan maize inbred lines and their respective single cross hybrids from which they obtained necrotic calli, which they named as type III that finally died upon sub culturing on CMM. With the other inbred lines, embryogenic calli proliferated and gave rise to globular somatic embryos.

Mean percent of embryogenic callus combined across the four levels of 2,4-D ranged from 13.2 to 97.4 (Table 3). The highest embryogenic callus frequency was recorded for CML442 which was significantly ($p<0.05$) different from the other inbred lines. However, the inbred line CML395 was second to CML442, giving rise to mean embryogenic callus frequency of 92.7% and was statistically on par with the control CML216. All these three inbred lines (CML395, CML442 and CML216) performed better at all levels of 2,4-D in terms of embryogenic callus frequency as they did in terms of callus induction frequency. On the contrary, CML443 and MAS [MSR/312]-117-2-2-1-B-5-B) performed relatively better only at the lowest level of 2,4-D used in this study (1 mg/L). Of all the inbred lines tested, CML443 performed least and gave the lowest mean frequency of embryogenic calli (13.2%), and produced no embryogenic callus at 1.5 to 2.5 mg/L 2,4-D. Per cent of embryogenic calli formed by MAS [MSR/312]-117-2-2-1-B-5-B) also decreased with increasing levels of 2, 4-D although the mean frequency (43.2%) was higher than that of CML443. This was corroborated by reports of other workers (Bohorova et al., 1995; Ombori et al., 2008) who obtained significant differences among different genotypes of maize that formed embryogenic callus from immature zygotic embryos. Studies have stressed on the involvement of embryogenesis related genes in the course of somatic embryogenesis of some plant species (Ikeda et al., 2006). The choice of regenerable genotype is, therefore, one of the key elements in improving *in vitro* regeneration of plants, including maize, from immature zygotic embryos.

Significantly higher frequency of embryogenic calli (86.8%) was obtained by using 1 mg/L 2,4-D in CIM. Thus, 1 mg/L 2,4-D was optimal for inducing embryogenic calli from immature zygotic embryos in the tropical maize inbred lines considered here. This was, however, in contrary to the findings of Omwoyo et al. (2008), where the optimal levels of 2,4-D for the induction of embryogenic calli in other tropical maize genotypes, DLC1, H513, H627 and H625, were in the order of 10, 2, 1.5 and 2.5 mg/L, respectively. The discrepancies could be due to genetic differences among maize genotypes in responding to the concentrations of 2,4-D for the formation of embryogenic callus. In the current study, significant reductions in embryogenic calli were observed with increasing levels of 2,4-D, indicating the inhibitory effect of this hormone at higher concentrations. Similar trends were also reported by several authors (Bronsema et al., 2001; Odour et al., 2006; Ombori et al., 2008). This could be due to blockage of cell division and inactivation

Table 3. Embryogenic callus frequencies[a] of five tropical maize inbred lines evaluated in response to four levels of 2,4-D.

Genotypes	2,4-D levels (mg/L)				Genotype mean**
	1.0	1.5	2.0	2.5	
CML395	100.0 ± 0.0[a]***	100.0 ± 0.0[a]	89.0 ± 12.36[c]	81.3 ± 4.98[d]	92.7 ± 3.11[b]
CML442	98.6 ± 1.88[ab]	98.6 ± 1.88[ab]	95.3 ± 4.1[b]	97.0 ± 2.16[ab]	97.4 ± 0.9[a]
CML443	52.9 ± 7.86[e]	0.0 ± 0.0[h]	0.0 ± 0.0[h]	0.0 ± 0.0[h]	13.2 ± 7.0[d]
MAS [MSR/312]-117-2-2-1-B-5-B)	83.0 ± 7.5[d]	26.7 ± 8.2[g]	34.7 ± 4.98[f]	28.67 ± 5.24[g]	43.2 ± 7.4[c]
CML216 (Control)	99.3 ± 0.94[ab]	100.0 ± 0.0[a]	84.6 ± 0.94[d]	82.0 ± 1.63[d]	91.5 ± 2.5[b]
2,4-D level mean**	86.8 ± 5.0[a]	65 ± 11.6[b]	60.7 ± 10.1[b]	57.8 ± 9.95[b]	

[a]Frequencies of embryogenic calli were determined by dividing the number of calli having at least one somatic embryo by the number of immature zygotic embryos initially cultured per plate x 100, after four weeks of culture on the CMM. **Means followed by the same letter are not significantly different from each other according to LSD test at 5% probability level. *** Values followed by the same letter (s) within columns and rows are not significantly different from each other according to LSD test at 5% probability level.

Table 4. Regeneration efficiencies[a] of five tropical maize inbred lines evaluated using embryogenic calli formed at four levels of 2,4-D.

Genotypes	2,4-D levels (mg/L)				Genotype mean**
	1.0	1.5	2.0	2.5	
CML395	120.0 ± 16.3	113.3 ± 103.7	73.8±49.2	130.1±39.4	109.3±19.55[a]
CML442	27.8 ± 39.3	6.7±9.4	41.1±15.6	27.8±25.9	25.8±8.48[bc]
CML443	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0[c]
MAS [MSR/312]-117-2-2-1-B-5-B)	52.6 ± 56.8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	13.2 ± 10.98[c]
CML216 (Control)	100.0 ± 90.9	20.0 ± 28.3	39.7 ± 40.4	65.3 ± 30.9	56.3 ± 18.62[b]
2,4-D levels mean**	60.1 ± 18.21[a]	28.0 ± 17.3[a]	30.9 ± 10.85[a]	44.7 ± 14.73[a]	

[a]Regeneration efficiencies were computed as the ratio of number of shoots regenerated to the number of embryogenic calli put on the regeneration medium and expressed as percentage. **Means followed by the same letter(s) are not significantly different from each other according to LSD test at 5% probability level.

of those cells that have embryogenic potential (Ombori et al., 2008).

Somatic embryo maturation and plant regeneration

Embryogenic calli obtained from all the genotypes and levels of 2,4-D tested were placed on EMM and kept in the dark for two weeks. Somatic embryos at the globular stage of development were visible on the embryogenic calli after two weeks of culture on EMM. Embryogenic calli of CML442 were, however, unique forming globular somatic embryos on the CMM quite ahead of the embryogenic calli of the other lines. Somatic embryos of this line were seen to premature by turning green in colour (Figure 1F). Upon subsequent transfer to EMM, they germinated within one week time. Hence, they were immediately taken to the RM where they further developed to plantlets. High concentration of sucrose (6%) used in EMM was reported to enhance maturation of somatic embryos in maize (Bronsema et al., 1997). During somatic embryo maturation there is a progressive decline in the expression of genes involved in cell proliferation and growth, such as genes encoding histones and ribosomal proteins. In response to the osmotic stress caused by high concentration of sucrose in EMM, expression rises for a group of genes encoding hydrolytic enzymes such as nucleases, glucosidases and proteases, thereby suggesting a breakdown and retooling of cell components during somatic embryo development (Che et al., 2006). Somatic embryos of CML395, CML216 and MAS [MSR/312]-117-2-2-1-B-5-B) turned green within one week after transfer to RM in the light. Some stress response genes were reported to be up-regulated at the onset of germination as a normal developmental event or in response to transfer of tissue to new culture medium. Germination and shoot greening are accompanied by the activation in expression of myriad of genes encoding photosynthetic and chloroplast components (Che et al., 2006). Time differences in activation of these genes might have caused variation in the onset of germination and greening of somatic embryos derived from these tropical maize inbred lines.

Comparing the inbred lines in terms of regenerability, CML395 was found to be the best with significantly ($p<0.05$) higher regeneration efficiency (109.3%), which was almost twice and four times the regeneration efficiency of CML216 and CML442, respectively (Table 4). This shows that inbred lines CML395, CML442 and CML216 may be used for genetic transformation since

they respond to regeneration. It was, however, noted that not all somatic embryos produced plantlets showing poor correlation between capacity to form somatic embryos and plant regeneration. Failure of somatic embryos to germinate and form plantlets was ascribed to either down regulation of the gene that controls plant regeneration (Che et al., 2006) or due to abnormal morphology (Körbes and Droste, 2005). Danson et al. (2006) screened tropical maize inbred lines, including CML395, CML442, CML444 and Pool A3-6, for production and regeneration of friable and embryogenic type II callus using the N6 basal salts. However, except Pool A3-6, all the remaining lines were reported not producing viable callus. It can, therefore, be confidently stated that MS basal salts are better than N6 basal salts for calli induction and subsequent plant regeneration of these inbred lines. This was in consistence with the findings of Armstrong and Green (1985) and Shohael et al. (2003). In addition, several other factors have also been associated with callus formation as well as plantlet development from somatic embryos of maize. The initiation and maintenance of maize tissue culture and successful regeneration of plants depend on the genotype used, choice of explants, and developmental stage of the source of the ex-plant, culture media and the environ-ment at each stage of the culture process (Phillips et al., 1988; Armstrong, 1994). The age of immature zygotic embryos and their placement on the culture medium are the other major factors affecting initiation and main-tenance of maize cells and tissues *in vitro* (Green and Phillips, 1975).

Somatic embryos of CML442 were the first to form shoot buds and give plantlets within one week time after their transfer to a hormone free RM. However, this line was not good in its plant regeneration potential as its capacity in forming friable embryogenic calli which formed many roots than shoots on the RM. This could be the principal reason why production of callus capable of plant regeneration has been the main objective of many regeneration experiments of tropical maize whereby the ability to regenerate somatic embryos derived from callus culture has remained genotype dependent. The embryogenic calli of CML443, which were produced only on media with 1 mg/L 2,4-D did not give rise to plantlets, even though they turned green after spending three weeks on the RM clearly showing recalcitrant nature of this line. MAS [MSR/312]-117-2-2-1-B-5-B) also gave plantlets only at the same level of 2,4-D (1 mg/L). Despite their competent callus induction frequency, both CML443 and MAS [MSR/312]-117-2-2-1-B-5-B) were found to produce non regenerating calli at almost all levels of 2,4-D considered in this study. Similar results were obtained in wheat by Zheng and Konzak (1999) who reported that continuous presence of 2,4-D concentration that satisfied callus induction inhibits further development and subsequent plant regeneration**.** The continuous presence of 2,4-D during the induction phase, beyond the critical point at which genes encoding products for plant regeneration are expressed is detrimental to the normal development of calli and may cause loss of their regeneration capacity.

Acclimatization and growth of regenerants

Plantlets with well developed roots were transferred to pots containing peat moss for hardening starting from two weeks of culture on RM. Fifty seven, 27, 14 and 7 plantlets from CML395, CML216, CML442 and MAS [MSR/312]-117-2-2-1-B-5-B), respectively, were trans-ferred to the peat moss for acclimatization (Figure 1H). Covered with punctured polythene bag for 3 days, 90% of the plantlets survived the hardening stage and were then transferred onto pots containing garden soil mixed with manure and sand (2:2:1) in the greenhouse and grew to maturity (Figure 1I and J). Most of the regenerants were normal and fertile, although some minor abnormal phenotypes were observed at flowering stage. The common abnormalities observed in the regenerants were silks and then seeds on the tassel, which usually are the results of tissue culture induced somaclonal variation. Such somaclonal variations have been described as epigenetic or genetic in origin (Larkin and Scowcroft, 1981). Epigenetic changes are stated as alterations in gene expression that are potentially reversible, and not due to sequence changes. They would, therefore, involve a mechanism of gene silencing or gene activation that are not due to chromosomal aberrations or sequence change (Kaeppler et al., 2000). Hence they cannot be passed on from one generation to another. On the other hand, genetic changes are heritable and arise as a result of changes in the chromosome structure and number.

In conclusion, our study revealed that CML395, CML216 and CML442 are regenerable among the five elite CIMMYT tropical maize inbred lines evaluated. The study further indicated that 1 mg/L 2,4-D is the optimal level for effective callus induction, embryogenic callus formation and plant regeneration. Therefore, these three lines can be recommended for further use in *in vitro* genetic transformation to improve their tolerance to drought stress. In this regard, it is strongly advised to culture immature zygotic embryos of CML395, CML216 and CML442 at size of 1.2-1.7 mm which is achieved 16 mean days post pollination. MS salts fortified with 1 mg/L 2,4-D are recommended for successful regeneration of transformed cells. Further studies need to be done on optimization of callus induction and regeneration of CML443 and MAS [MSR/312]-117-2-2-1-B-5-B) at vary-ing concentrations of 2,4-D that were not tested in this study to ascertain their regeneration potential.

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REFERENCES

Ajanga S, Hillocks RJ (2000). Maize cob rot in Kenya and its association with stalk borer damage. Crop Prot. 19: 297-300.

Al-Abed D, Rudrabhatla S, Talla R, Goldman S (2006). Split-seed: a new tool for maize researchers. Planta, 223: 1355-1360.

Armstrong CL, Green CE (1985). Establishment and maintenance of friable embryogenic maize callus and involvement of L-proline. Planta, 164: 207-214.

Armstrong CL (1994). Regeneration of plants from somatic cell culture; applications for *in vitro* genetic manipulation. In Freeling M and Walbot V (eds.). The maize hand book. New York, NY, USA, Springer-Verlage, pp. 663-671.

Bi RM, Kou M, Chen LG, Mao SR, Wang HG (2007). Plant regeneration through callus induction from matured embryo of *Triticum*. Plant Breed. 126(1): 9-12.

Binott JJ, Songa JM, Ininda J, Njagi EM, Machuka J (2008). Plant regeneration of Kenyan maize inbred lines and their respective single cross hybrids through somatic embryogenesis. Afr. J. Biotechnol. 7(8): 981-987.

Bohorova NE, Luna B, Brito RM, Huerta LD, Hoisington DA (1995). Regeneration potential of tropical, subtropical, midaltitude and highland maize inbreds. Maydica, 40: 275-281.

Bronsema FBF, van Oostveen WJF, van Lammeren AAM (1997). Comparative analysis of callus formation and regeneration on cultured immature maize embryos of the inbred lines A 188 and A632. Plant Cell Tissue Organ Culture, 50: 57-65.

Bronsema FBF, van Oostveen WJF, van Lammeren AAM **(**2001). Influence of 2,4-D, TIBA and 3, 5-D on the growth response of cultured maize embryos. Plant Cell Tissue Organ Cult. 65: 45-56.

Carvalho CHS, Bohorova NE, Bordallo PN, Abreu LL, Valicente FH, Bressan W, Paiva E (1997). Type II callus production and plant regeneration in tropical maize genotypes. Plant Cell Rep. 17: 73-76.

Che P, Love TM, Frame BR, Wang K, Crriquiry AL, Howel SH (2006). Gene expression patterns during somatic embryo development and germination in maize Hi II callus cultures. Plant Mol. Biol. 62: 1-14.

Danson JW, Lagat M, Mbogori M (2006). Screening tropical maize lines for the production and regeneration of friable and embryogenic type II callus. Afr. J. Biotechnol. 5(23): 2367-2370.

Evenson RE, Gollin D (2003). Assessing the impact of the Green Revolution, Science, 300, May. World Bank (2007), World Development Indicators CD-ROM.

FAO Statistics (2002). http::/www. FAO. Org

Green CE, Phillips RL (1975). Plant regeneration from tissue culture of maize. Crop Sci. 15: 417-421.

Ikeda M, Umehara M, Kamanda H (2006). Embryogenesis-related gene: Its expression and roles during somatic and zygotic embryogenesis in carrot and Arabidopsis. Plant Biotechnol. 23: 153-161.

Ishida Y, Yukoh Y, Komari T (2007). *Agrobacterium*-mediated transformation of maize. Nat. Prot. 2(7): 1614-1621.

Kanampiu F, Ransom J, Gressel J, Jewell D, Friesen D (2002). Appropriateness of Biotechnology to African Agriculture: Striga and maize as paradigms. Plant Cell Tiss. Organ Cult. 69: 105-110.

Kaeppler SM, Kaeppler FH, Rhee Y (2000). Epigenetic aspects of somaclonal variation in plants. Plant Mol. Biol. 43: 179-188.

Körbes AP, Droste A (2005). Carbon sources and polyethylene glycol on soybean somatic embryo conversion. Pesquisa Agropecuària Brasileira 40(3): 211-216.

Larkin PJ, Scowcroft WR (1981). Somaclonal variation: a novel source of variability from cell cultures for plant improvement. Theor. Appl. Genet. 60: 197-214.

Morris ML (1998). An overview of the world maize economy. In M. L. Morris(ed.). Maize seed industries in developing countries. Lynne Rienner Publishers, Inc and CIMMYT, Int. pp. 13-34.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant 15: 473-497.

Oduor RO, Njagi ENM, Ndung'u S, Machuka J (2006). In vitro regeneration of dry land Kenyan maize genotypes through somatic embryogenesis. Int. J. Bot. 2: 146-151.

Ombori O, Gitonga NM, Machuka J (2008). Somatic embryogenesis and plant regeneration from immature embryos of tropical maize (*Zea mays* L.) inbred lines. Biotechnology, 7(2): 224-232.

Omwoyo O, Gitonga NM, Machuka J (2008). Plant regeneration via somatic embryogenesis of tropical maize (*Zea mays* L.) commercial hybrid lines. J. Trop. Microbiol. Biotechnol. 4(1): 24-31.

Phillips RL, Somers DA, Hibbered KA (1988). Cell/tissue culture and *in vitro* manipulation. In: Sprague GF and Dudley JW (eds.) Corn and corn improvement, 3rd ed., pp. 345-387.

Rasha AOA, Ali AM, Matheka JM, Machuka J (2008). Regeneration of Sudanese maize inbred lines and open pollinated varieties. Afr. J. Biotechnol. 7(11): 1759-1764.

Schechert AW, Welz HG, Geiger HH (1999). QTL for resistance to *Stesphaeria turcica*, in tropical African maize. Crop Sci. 39: 514-523.

Shohael AM, Akanda MAL, Parvez S, Mahfuja S, Alam MF, Islam R Joarder N (2003). Somatic embryogenesis and plant regeneration from immature embryo derived callus of inbred maize (*Zea mays* L.). Biotechnology, 2: 154-161.

Yu GR, Yin J, Guo TC, Niu JS (2003). Selection of the optimum genotype for immature embryo culture of wheat. J. Triticeae Crops, 23(2): 14-18.

Zheng MY, Konzak CF (1999). Effect of 2,4-Dichlorophenoxyacetic acid on callus induction and plant regeneration in anther culture of wheat (*Triticum aestivum* L.). Plant Cell Rep. 19: 69-73.