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Plant Regeneration of Ethiopian Tropical Maize (*Zea mays* L.) Genotypes

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Abstract: Five Ethiopian tropical maize genotypes were evaluated for their regeneration potential through somatic embryogenesis to establish regeneration system and select regenerable genotypes as prerequisites in applying genetic transformation for improved drought stress tolerance. Immature zygotic embryos were incubated for callus induction on Murashige and Skoog basal salts supplemented with 1.0, 1.5, 2.0 and 2.5 mg L⁻¹ 2, 4-dichlorophenoxyacetic acid. The genotypes differed significantly ($p < 0.01$) in callus induction frequency, formation of embryogenic callus, number of shoots per embryogenic callus regenerating plants, percent of embryogenic callus forming shoots and regeneration efficiency. Among the genotypes investigated, [CML387/CML176]-B-B-2-3-2-B [QPM] and Melkassa-6Q had higher capacity in embryogenic callus formation, regeneration efficiency and higher proportion of embryogenic calli forming shoots. Melkassa-6Q and Melkassa-2 produced significantly ($p < 0.05$) higher number of shoots per regenerated embryogenic callus. Higher frequencies of primary and embryogenic callus and regeneration efficiency were obtained from the use of 1 mg L⁻¹ 2,4-dichlorophenoxyacetic acid indicating this as optimal level for regenerating these genotypes. Except [CML312/CML206]-B-3-2-1-1-1 fertile plants were regenerated from all genotypes and set seeds which were viable to germinate and produce phenotypically normal plants. Melkassa-6Q, [CML387/CML176]-B-B-2-3-2-B [QPM] and Melkassa-2 were selected for use in genetic transformation.

Key words: Drought stress tolerance, Ethiopian tropical maize genotypes, genetic transformation, immature zygotic embryos, *in vitro* culture, regeneration potential, somatic embryos

INTRODUCTION

Maize (*Zea mays* L.) was introduced to Ethiopia in the late 1600 to early 1700s (Huffnagel, 1961). Since then it has gained tremendous importance as a staple food and to date it is an important component of the diet supporting lives of millions of the Ethiopian population.

Although maize is grown all over the country, the bulk of production (over 80%) comes from high potential areas (mid-altitude sub-humid zones) which cover about 60% of the total land area allotted to the crop (Nigussie *et al.*, 1996). This is attributed to significant productivity gains achieved through high yielding improved varieties developed through conventional breeding. Using high yielding hybrid varieties and improved crop management practices, farmers were able to produce average yield of 6 t ha⁻¹ (Tolessa *et al.*, 1997). However, similar yield gains could not be achieved in 40% of the country's maize growing environment where drought affects maize production significantly. Though conventional breeding led to identification of improved varieties having mechanisms of drought escape and/or tolerance, any year to year variation in rainfall is still seen

as a year to year variation in yield, with low rainfall years yielding less than high rainfall years. The major reason for such yield variation is lack of improved varieties having sufficient level of tolerance to drought stress. Narrow genetic base of the germplasm and lack of suitable selection criteria for tolerance to drought stress are the major reasons for lack of improved drought stress tolerant maize varieties in Ethiopia (Nigussie *et al.*, 2002). As a result the drought affected environment contributes less than 20% to the total national maize production (Nigussie *et al.*, 2002). With the current global climate change, more pronounced yield losses are expected from drought as temperature rises and rainfall distribution becomes more erratic (Battisti and Naylor, 2009). Hence there is a need to diversify the genetic basis of the locally adapted germplasm by introgressing genes responsible for improving tolerance to drought stress.

This can be achieved through genetic engineering which is relatively faster and more reliable means for varietal improvement than conventional breeding as it allows transfer of potential genes from within as well as outside the primary gene pool (Bhatnagar-Mathur *et al.*, 2008). Application of genetic engineering in improving

many maize genotypes is, however, limited by lack of suitable regeneration systems that result in normal and fertile plants from the *in vitro* grown transformed cells. Embryogenic callus induction and plant regeneration are generally genotype-dependent in maize (Ombori *et al.*, 2008). Hence, most of the genotypes remain inaccessible to improvement using the standard genetic transformation techniques since they fail either to produce embryogenic callus or to regenerate efficiently from the induced embryogenic calli (Che *et al.*, 2006). The differences are ascribed to susceptibility of the competent cells to genetic programming and reprogramming in response to internal and external factors (Ombori *et al.*, 2008). Regeneration capacity is also influenced by the composition of the culture media (Armstrong and Green, 1985). This calls for the need to study the regeneration potential of specific germplasm and optimization of media composition and culture environment prior to embarking on genetic transformation.

This study was designed to identify the best regenerable maize genotypes for subsequent *in vitro* genetic improvement for drought stress tolerance. We are reporting for the first time on successful plant regeneration of Ethiopian tropical maize genotypes through somatic embryogenesis.

MATERIALS AND METHODS

Plant materials and explant preparation: The experimental materials consisted of five Ethiopian tropical maize genotypes, including three open pollinated varieties (OPVs) (Melkassa-2, Mekassa-4 and Melkassa-6Q) and two inbred lines, [CML312/CML206]-B-3-2-1-1 and [CML387/CML176]-B-B-2-3-2-B[QPM]. Details of the pedigree and distinguishing agronomic characters of these genotypes are given in Table 1.

The genotypes were grown in the glass house at the Biosafety level II Plant Transformation Laboratory (PTL) of Kenyatta University from January to June 2010 to supply immature zygotic embryos for *in vitro* culture. To produce genetically uniform explants, the uppermost ear of each plant was covered with a transparent plastic bag of 8×4 inches before silk emergence, until it was either self or sib pollinated. After pollination, the ears were covered with

pollination bags to avoid any contamination from pollination of late coming silks with foreign pollen.

Immature zygotic embryos were collected 16 days after pollination, when they were observed to reach the size of 1.2-1.7 mm along their axis. 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 2-3 drops of Tween-20 for 20 min. The ears were then rinsed 2-3 times with sterile distilled water under the laminar flow cabinet. The immature zygotic embryos were excised aseptically by cutting the top part of the kernels with a sharp scalpel blade.

Callus initiation and maintenance: Callus Induction Medium (CIM) was prepared from MS (Murashige and Skoog, 1962) basal salts supplemented with 1.0, 1.5, 2.0 and 2.5 mg L⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D), L-Proline (2.875 g L⁻¹), casein hydrolysate (100 mg L⁻¹), silver nitrate (10 mg L⁻¹), together with 3% (w/v) sucrose as a source of carbon and 0.8% (w/v) agar as a solidifying agent. All culture media were autoclaved at 121°C for 20 min at a pressure of 1.06 kg cm⁻¹ after adjusting the pH to 5.8 using 1 N NaOH and/or 1 N HCl.

Twenty five immature zygotic embryos were cultured on the CIM for callus induction ensuring direct contact of the embryo axis with the medium in a 90×15 mm petri dish (supplied by PY-REX, East Africa Ltd) (Fig. 1a). 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 was composed of similar components with that of CIM but devoid of silver nitrate. The type of calli produced was noted at each step and frequency of embryogenic calli development was recorded after four weeks of culture on the CMM.

Embryo maturation and plant regeneration: Embryogenic calli with at least one somatic embryo were transferred onto Embryo Maturation Medium (EMM) which was composed of MS basal salts supplemented with 6% (w/v) sucrose, 1 mg L⁻¹ Naphthalene Acetic Acid (NAA) and

Table 1: Pedigree and important distinguishing agronomic characters of five Ethiopian tropical maize genotypes

| Genotypes | Pedigree | Days to silking (No.) | Plant height (cm) | Ear height (cm) | Yield potential (t ha ⁻¹) |
|---|-----------------|-----------------------|-------------------|-----------------|---------------------------------------|
| Melkassa-2 ^a | ZM-521 | 68 | 170-190 | 80-90 | 4.5-5.5 |
| Melkassa-4 ^a | ECA-EE-36 | 55 | 140-165 | 60-75 | 3.5-4.5 |
| Melkassa-6Q ^a | Pool 15 C; QPM | 60 | 170 | 72 | 4.5-5.5 |
| [CML312/CML206]-B-3-2-1-1 ^b | CML312 X CML206 | 55 | 150 | 60-70 | Na |
| [CML387/CML176]-B-B-2-3-2-B[QPM] ^b | CML387 X CML176 | 60 | 145 | 65-70 | Na |

QPM: Quality protein maize, ^aOPVs, ^binbred lines, Na: Data not available

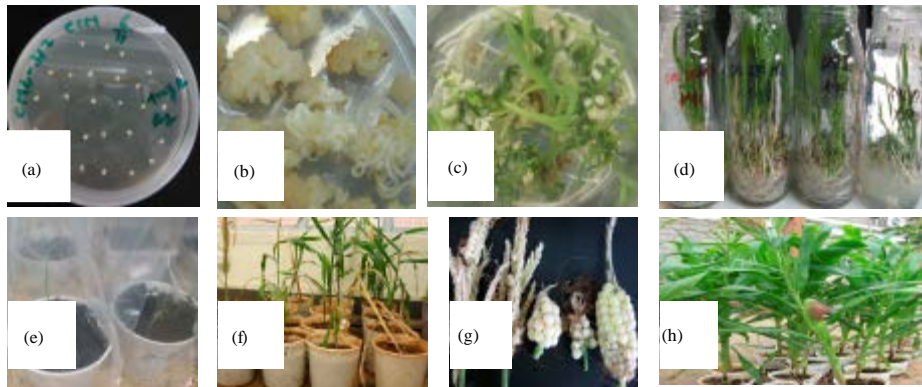


Fig. 1(a-h): Tissue culture procedure applied in *in vitro* regeneration of Ethiopian tropical maize genotypes: (a) Immature zygotic embryos plated on CIM with embryo axis in contact with the media, (b) Friable embryogenic calli with matured somatic embryos after two weeks on EMM, (c) Somatic embryos forming shoots and roots on regeneration media, (d) Plantlets on regeneration media immediately before transfer to peat moss, (e) Plantlets being acclimatized in peat moss, (f) Matured regenerants with seeds harvested, (g) R_0 seeds harvested from primary regenerants and (h) R_1 plants grown from R_0 seeds

0.8% (w/v) agar. These cultures were also retained in the dark at a temperature of $27 \pm 1^\circ\text{C}$. After two weeks of culture on the EMM, the embryogenic calli with somatic embryos were selected and transferred onto a hormone free shoot Regeneration Medium (RM). This medium was composed of MS basal salts with 3% (w/v) sucrose and solidified with 0.8% (w/v) agar. The cultures were incubated at a temperature of $27 \pm 1^\circ\text{C}$, 16h/8h (light/dark) photoperiod and light intensity of $71.65 \text{ J.m}^{-2}\text{s}^{-1}$ supplied from two cool-white fluorescent tubes of 18 watts each. The number of shoots formed per culture was recorded starting from the second week of culture on the RM. In this study, all plantlets produced roots on the RM and hence their transfer to a rooting medium was not necessary.

Acclimatization 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 transparent polythene bag punctured on the second day at several spots to allow gradual acclimatization to the ambient environment. After 7-10 days, the plantlets were transferred onto 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. The regenerants were either self or cross pollinated with the parental genotypes. The seeds

obtained from the R_0 plants were planted in soil and produced fertile normal plants.

Statistical analysis: 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 each replication containing 25 immature zygotic embryos. Analysis of variance (ANOVA) was carried out using GenStat discovery edition 3 (VSN international software for biosciences, www.vsn.co.uk/software/genestat) to test the statistical significance of differences among the genotypes and 2,4-D levels. Means which were significantly different were separated using the Least Significance Difference (LSD) test at 5% probability level.

RESULTS AND DISCUSSION

Callus initiation and maintenance: Calli were induced from the scutellum of immature zygotic embryos in four of the genotypes evaluated in the current study. This was signalled by swelling of the middle of the dome shaped scutellum four to five days after plating on the CIM. Afterwards, callus initiation was observed within the following 2-3 days with swelling of the scutellum accompanied by proliferation of small mass of undifferentiated cells. This is primarily associated with the presence of large number of competent cells in the scutellum for somatic embryogenesis (Ombori *et al.*, 2008).

Among the five genotypes tested, only [CML312/CML206]-B-3-2-1-1-1 failed to produce callus. The immature zygotic embryos of this line did show signal to form callus by swelling of their scutellum. However, this was not followed by proliferation of undifferentiated mass of cells, which is unlike the case in the other genotypes. In this particular case, all the immature zygotic embryos from this genotype were found to germinate at all levels of 2,4-D producing roots and shoots rather than initiating callus leading to somatic embryos.

Callus induction capacity, formation of embryogenic callus, number of shoots per embryogenic callus regenerating plants, percent embryogenic callus forming shoots and shoot regeneration efficiency from immature zygotic embryos varied significantly ($p < 0.01$) depending on genotype, indicating the prevalence of genetic differences among the genotypes for somatic embryogenesis. This was in agreement with Ombori *et al.* (2008). No significant interaction effect was observed from the combined use of different levels of 2,4-D and genotype, indicating that the genotypes had similar responses to the effect of 2,4-D levels used in the study. The inbred line [CML387/CML176]-B-B-2-3-2-B [QPM] and the OPV Melkassa-2 produced significantly ($p < 0.05$) higher mean callus induction frequency of 100% each indicating that all their immature zygotic embryos plated on the CIM produced callus (Table 2). On the other hand, the two OPVs, Melkassa-6Q and Melkassa-4, showed fairly high mean callus induction frequency of 93.33 and 87.67%, respectively but statistically significantly ($p < 0.05$) lower compared to the former two genotypes. With these OPVs, 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 undifferentiated mass of cells.

Except for [CML312/CML206]-B-3-2-1-1-1, which did not produce callus, the four Ethiopian maize genotypes tested in this experiment showed optimum performance irrespective of the levels of 2,4-D evaluated. Accordingly, the average callus induction frequency for the four concentrations of 2,4-D ranged from 74.1 to 77.8%. It can, therefore, be inferred from this finding that the lowest concentration of 2,4-D used in this study (1 mg L^{-1}) could be considered optimum for callus induction from immature zygotic embryos of those genotypes.

The type of calli induced by the different genotypes became apparent after two weeks of culture on the CMM. Calli differed in terms of morphology, size, rate of proliferation and regeneration capacity with proportions varying depending on genotypes. Among these were soft watery, cotton whitish, hard and round shaped calli which were non embryogenic and did not have regeneration capacity. Such calli were more common with Melkassa-4 mainly at 2,4-D levels exceeding 1 mg L^{-1} . These calli were yellowish in colour before they turned brownish. Subsequently, they became necrotic with further sub culturing on the CMM and finally died. Similar phenomena were reported in tropical maize genotypes (Binott *et al.*, 2008; Ombori *et al.*, 2008).

The embryogenic calli obtained in the present study were compact and whitish, while others were light yellow and friable type, which have been designated as Type I and Type II calli, respectively (Armstrong and Green, 1985). The number of Type II calli was more than the number of Type I calli (Fig. 2). Almost all calli formed in Melkassa-6Q and majority of the calli formed in Mekassa-2 and [CML387/CML176]-B-B-2-3-2-B [QPM] were of the Type II. Higher number of Type II calli reported here is in contrary to Ombori *et al.* (2008) who reported less number of Type II calli from the tropical maize inbred lines they evaluated. This could be ascribed

Table 2: Primary ^a and embryogenic^b callus frequencies of five Ethiopian tropical maize genotypes evaluated in response to four levels of 2,4-D

| Genotype | 2,4-D levels (mg L^{-1}) | | | | Genotype mean** |
|-----------------------------------|-------------------------------------|------------|------------|------------|-----------------|
| | 1.0 | 1.5 | 2.0 | 2.5 | |
| Melkassa-2 | ^a 100.0±0.0 | 100.00±0.0 | 100.00±0.0 | 100.00±0.0 | 100.0a |
| | ^b 69.3±13.2 | 78.70±24.7 | 62.70±6.8 | 60.00±5.6 | 67.7b |
| Melkassa-4 | ^a 86.67±7.5 | 80.00±8.6 | 93.30±4.9 | 90.67±6.8 | 87.67c |
| | ^b 36.4±16.9 | 35.00±1.3 | 13.10±7.2 | 11.70±5.5 | 24.0c |
| Melkassa-6Q | ^a 93.33±4.9 | 90.67±3.8 | 96.00±5.7 | 93.33±6.8 | 93.33b |
| | ^b 90.7±6.8 | 86.70±9.1 | 85.30±9.9 | 74.70±6.8 | 84.3a |
| [CML312/CML206]-B-3-2-1-1-1 | ^a 0.0±0.0 | 0.00±0.0 | 0.00±0.0 | 0.00±0.0 | 0.0d |
| | ^b 0.0±0.0 | 0.00±0.0 | 0.00±0.0 | 0.00±0.0 | 0.0d |
| [CML387/CML176]-B-B-2-3-2-B [QPM] | ^a 100.0±0.0 | 100.00±0.0 | 100.00±0.0 | 100.00±0.0 | 100.0a |
| | ^b 89.3±8.2 | 72.00±9.8 | 85.30±1.9 | 93.30±3.8 | 85.0a |
| 2,4-D level mean** | ^a 76.00a | 74.13a | 77.87a | 76.80a | |
| | ^b 57.1a | 54.50a | 49.30a | 47.90a | |

^aPrimary callus 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. ^bFrequencies 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 × 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

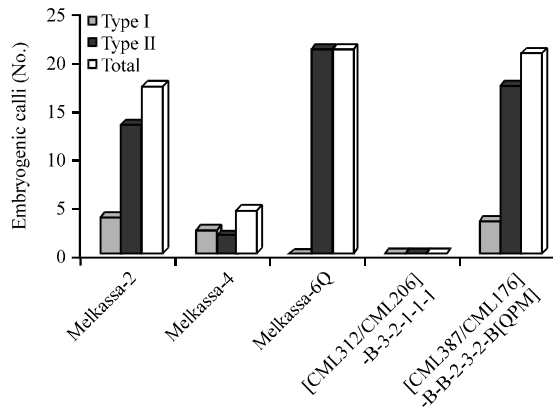


Fig. 2: Types of embryogenic calls formed by five Ethiopian maize genotypes

to the genotypic differences among the maize genotypes to form different types of embryogenic calli. Such variations were also reported to be associated with additive gene effects (Tomes and Smith, 1985). In line with this, Tomes (1985) also observed considerable response variations among maize genotypes with regard to the callus type produced and their subsequent ability of regeneration. There is strong evidence that the formation of Type II callus is under genetic control, as the frequency of initiation of Type II callus in the inbred line B73 was increased dramatically by introgression of chromosome segments from A188 through classical backcross breeding (Armstrong *et al.*, 1992). A region on the long arm of chromosome 9 has been proposed to contain major gene/genes that may regulate embryogenic competence.

Mean percentage of embryogenic calli combined across the four levels of 2,4-D ranged from 0.0 to 85.0 (Table 2). The two quality protein maize genotypes, [CML387/CML176]-B-B-2-3-2-B [QPM] and Melkassa-6Q, recorded significantly ($p < 0.05$) higher mean embryogenic callus frequency of 85.0 and 84.3%, respectively. On the other hand, Melkassa-4 produced significantly ($p < 0.05$) low mean embryogenic callus frequency (24.0%). Significant differences among maize genotypes in terms of embryogenic calli formation from immature zygotic embryos were also reported by other researchers (Bohorova *et al.*, 1995; Ombori *et al.*, 2008). The choice of better responding genotypes for embryogenic calli formation is, therefore, one of the key elements in improving *in vitro* plant regeneration from immature zygotic embryos in maize.

Even though there was no significant effect observed due to the levels of 2,4-D used in this study, MS medium supplemented with 1 mg L⁻¹ of 2,4-D closely followed by

1.5 mg L⁻¹ produced the highest frequency of embryogenic callus (57.1 and 54.5%, respectively). Thus, 1 mg L⁻¹ 2,4-D was found to be optimal for inducing embryogenic calli from immature zygotic embryos of the Ethiopian tropical maize genotypes tested in this study. This was, however, in contrary to Ombori *et al.* (2008), who reported the optimal levels of 2,4-D for the induction of embryogenic calli in other tropical maize genotypes, i.e., DLC1, H513, H627 and H625, to be in the order of 10, 2, 1.5 and 2.5 mg L⁻¹, respectively. These discrepancies could be due to the genetic differences among the maize genotypes tested in the two cases, with regard to their responses to the concentrations of 2,4-D for the formation of embryogenic callus. In the current study, though it was not statistically 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 (Oduor *et al.*, 2006; Ombori *et al.*, 2008). This could be ascribed to the blockage of cell division and inactivation of those cells that have embryogenic potential at higher concentrations beyond the optimum (Ombori *et al.*, 2008).

Somatic embryo maturation and plant regeneration:

Somatic embryos at the globular stage of development were visible on the embryogenic calli after two weeks of culture on the EMM (Fig. 1b). Somatic embryos turned green within one week after transfer to RM in the light (Fig. 1c). Somatic embryos obtained from the two quality protein maize genotypes, [CML387/CML176]-B-B-2-3-2-B [QPM] and Melkassa-6Q, were faster than the others' to turn green and germinate. Shoots were formed within 10 days after transfer to the RM. In general, Type II calli were found to be faster than Type I calli in shoot formation. High concentration of sucrose (6%) used in the EMM was reported to enhance maturation of somatic embryos in maize (Bronsema *et al.*, 1997). According to Che *et al.* (2006), there is a progressive decline in the expression of genes involved in cell proliferation and growth during somatic embryo maturation. These include such genes encoding histones and ribosomal proteins. On the other hand, expression rises for a group of genes encoding hydrolytic enzymes such as nucleases, glucosidases and proteases suggesting a breakdown and retooling of cell components during somatic embryo development, in response to the osmotic stress caused by high concentration of sucrose in the EMM. Some stress response genes were also reported to be up-regulated at the onset of germination as a normal developmental event or in response to the transfer of tissues onto a new culture medium. Germination and shoot greening are

accompanied by the activation in the expression of myriad of genes encoding photosynthetic and chloroplast components (Che *et al.*, 2006). Time differences in activation of these genes might have therefore, caused variation in the onset of germination and greening of somatic embryos derived from the different maize genotypes from Ethiopia.

Comparing the genotypes in terms of regeneration capacity, Melkassa-6Q was found to be the best with significantly ($p < 0.05$) higher regeneration efficiency of 234.0% (Table 3). On the other hand, [CML387/CML176]-B-B-2-3-2-B [QPM], Melkassa-2 and Melkassa-4 were statistically at par with each other with corresponding regeneration efficiency of 138.0, 98.0 and 82.0%. However, no plants were regenerated from the fifth inbred line, [CML312/CML206]-B-3-2-1-1-1. This indicates that the Ethiopian tropical maize genotypes tested have different genetic potential for plant regeneration. Similar variations in plant regeneration due to genotypic differences were also reported in maize by several workers (Bohorova *et al.*, 1995; Ombori *et al.*, 2008). The regeneration efficiency of 82.0% recorded with Melkassa-4 was not expected considering the relatively poor response of this genotype to tissue culture. In this case, only few embryogenic calli formed higher number of shoots, which in turn escalated the regeneration efficiency in this genotype. Owing to this fact, it may not be justifiable to consider this particular genotype for genetic transformation.

In this study, not all somatic embryos produced plantlets, hence showing poor correlation between the capacity to form somatic embryos and plant regeneration. Even in the most regenerable genotypes we reported here, only about 60% of the embryogenic calli were able to form shoots (Fig. 3), indicating that considerable proportions (40%) of embryogenic calli failed to regenerate. Failure of somatic embryos to regenerate to plantlets is also reported in other maize genotypes (Bohorova *et al.*, 1995; Ombori *et al.*, 2008). Their failures to germinate and form plantlets were ascribed to either down regulation of the gene that controls plant regeneration (Che *et al.*, 2006) or due to abnormal morphology (Korbes and Droste, 2005).

Melkassa-2 and Melkassa-6Q, revealed significantly higher number of shoots per embryogenic calli regenerated to plants (3.9 each) (Table 4) indicating that they produced about four shoots on the average per a clump of embryogenic callus that gave plants. These were followed by the quality protein maize inbred line [CML387/CML176]-B-B-2-3-2-B [QPM] that recorded a value of 2.35, indicating that it produced more than two shoots per a clump of embryogenic callus that produced shoots. Hence, these three genotypes, Melkassa-6Q, [CML387/CML176]-B-B-2-3-2-B [QPM] and Melkassa-2, could be considered for genetic transformation since they have a better response to subsequent regeneration. Better response of the former two quality protein maize genotypes to tissue culture indicates the opportunity Ethiopia has to develop drought tolerant transgenic maize genotypes with high level of the two essential amino acids, lysine and tryptophan.

Acclimatization and growth of regenerants: Plantlets were transferred to pots containing peat moss for hardening starting from three weeks of culture on RM (Fig. 1d). Fifty four, 24, 19 and 4 plantlets from Melkassa-6Q, Melkassa-2, [CML387/CML176]-B-B-2-3-2-B [QPM] and Melkassa-4, respectively, were transferred to the peat moss for acclimatization (Fig. 1e). Covered with punctured

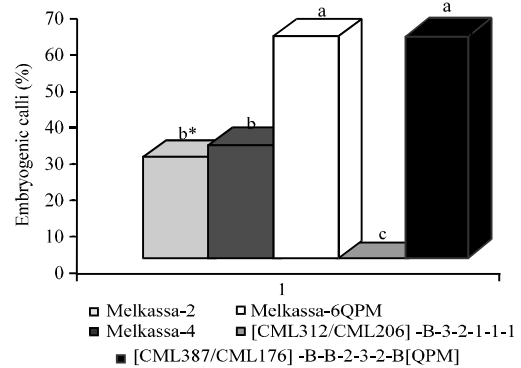


Fig. 3: Mean percent of embryogenic calli forming shoots in Ethiopian maize genotypes. *bars labeled with the same letter represent means not significantly different at $p < 0.05$ probability

Table 3: Regeneration efficiencies^a of five Ethiopian tropical maize genotypes evaluated at four levels of 2,4-D

| Genotype | 2,4-D levels (mg L ⁻¹) | | | | Genotype mean** |
|-----------------------------------|------------------------------------|------------|------------|-------------|-----------------|
| | 1.0 | 1.5 | 2.0 | 2.5 | |
| Melkassa-2 | 214.0±42.7 | 40.0±9.8 | 41.0±29.4 | 98.0±100.8 | 98.0b |
| Melkassa-4 | 204.0±234.8 | 0.0±0.0 | 0.0±0.0 | 122.0±149.9 | 82.0b |
| Melkassa-6Q | 244.0±121.2 | 168.0±38.5 | 220.0±68.4 | 304.0±126.9 | 234.0a |
| [CML312/CML206]-B-3-2-1-1-1 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0c |
| [CML387/CML176]-B-B-2-3-2-B [QPM] | 122.0±27.1 | 140.0±22.2 | 148.0±7.6 | 141.0±17.7 | 138.0b |
| 2,4-D level mean** | 157.0a | 70.0a | 82.0a | 133.0a | |

^aRegeneration 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

Table 4: Number of shoots^a formed per embryogenic calli regenerating plants in five Ethiopian tropical maize genotypes evaluated at four levels of 2,4-D

| Genotype | 2,4-D levels (mg L ⁻¹) | | | | Genotype mean** |
|----------------------------------|------------------------------------|-------|-------|-------|-----------------|
| | 1.0 | 1.5 | 2.0 | 2.5 | |
| Melkassa-2 | 3.14 | 3.33 | 4.67 | 4.72 | 3.97a |
| Melkassa-4 | 3.11 | 0.00 | 0.00 | 2.00 | 1.28c |
| Melkassa-6Q | 3.76 | 3.26 | 4.64 | 3.97 | 3.91a |
| [CML312/CML206]-B-3-2-1-1-1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0c |
| [CML387/CML176]-B-B-2-3-2-B[QPM] | 1.97 | 2.77 | 2.32 | 2.34 | 2.35b |
| 2,4-D levels mean** | 2.60a | 2.07a | 2.52a | 2.81a | |

^aNumber of shoots formed per embryogenic calli regenerating plants were determined as the ratio of number of shoots formed to the number of embryogenic calli forming shoots

polythene bag for 3 days, 90% of the plantlets survived the hardening stage and were further transferred onto pots containing garden soil mixed with manure and sand (2:2:1) in the greenhouse and grew to maturity (Fig. 1f). Most of the regenerants obtained from this study were normal and fertile enough to set seed (Fig.1g), although some minor abnormal phenotypes were observed at vegetative and flowering stages. The common abnormalities observed in the regenerants were dwarf phenotype with multiple ears on top, dwarf phenotype with multiple ears, i.e., one nodal and another on the tassel, dwarf phenotype with multiple branches per stem, each branch having an ear and hence seeds on the tassel. Such somaclonal variations were more pronounced in regenerants from Melkassa-2. Twenty five percent of the regenerants from this genotype had abnormal phenotypes, mainly of the dwarf type with seeds on the tassel. Larkin and Scowcroft (1981) described such somaclonal variations as epigenetic or genetic in their origin. 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 changes (Kaepler *et al.*, 2000). Hence, they cannot be passed on from one generation to another. Seeds obtained from the R₀ regenerants, including somaclonally affected plants, were re-grown in soil and produced normal R₁ plants (Fig. 1h) confirming that the phenotypic abnormalities are not heritable (Anami *et al.*, 2010).

CONCLUSION

In this study we have laid down a reproducible protocol for plant regeneration of Ethiopian tropical maize genotypes through somatic embryogenesis using embryogenic calli derived from immature zygotic embryos. Using this protocol, it is now possible to complement the country's maize genetic improvement program for drought stress tolerance through genetic engineering. Our study revealed that three genotypes, Melkassa-6Q, [CML387/CML176]-B-B-2-3-2-B [QPM] and Melkassa-2 responded well to regeneration, as indicated by higher

frequency of embryogenic calli production and regeneration efficiency. Hence, they can be used for genetic transformation. Besides, using the genes conferring tolerance to drought stress, these genotypes could also be genetically engineered for improved resistance to biotic stresses like insect pests and other abiotic constraints that are of high priority in limiting maize productivity in Ethiopia. In this regard, it is strongly advised to culture immature zygotic embryos of these genotypes at the size of 1.2-1.7 mm which could be achieved 16 days after pollination. MS salts supplemented with 1 mg L⁻¹ 2,4-D is recommended for successful regeneration of transformed cells of these genotypes.

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REFERENCES

- Anami, S.E., A.J. Mgutu, C. Taracha, G. Coussens and M. Karimi *et al.*, 2010. Somatic embryogenesis and plant regeneration of tropical maize genotypes. *Plant Cell Tissue Organ. Culture*, 102: 285-295.
- Armstrong, C., S.J. Romero and T.K. Hodges, 1992. Improved tissue culture response of an elite maize inbred through backcross breeding and identification of chromosomal regions important for regeneration by RFLP analysis. *Theor. Applied Genet.*, 84: 755-762.
- Armstrong, C.L. and C.E. Green, 1985. Establishment and maintenance of friable embryogenic maize callus and the involvement of L-proline. *Planta*, 164: 207-214.

- Battisti, D.S. and R.L. Naylor, 2009. Historical warnings of future food insecurity with unprecedented seasonal heat. *Science*, 323: 240-244.
- Bhatnagar-Mathur, P., V. Vadez and K.K. Sharma, 2008. Transgenic approaches for abiotic stress tolerance in plants: Retrospect and prospects. *Plant Cell Rep.*, 27: 411-424.
- Binott, J.J., J.M. Songa, J. Ininda, E.M. Njagi and J. Machuka, 2008. Plant regeneration from immature zygotic embryos of Kenyan maize inbred lines and their respective single cross hybrids through somatic embryogenesis. *Afr. J. Biotechnol.*, 7: 981-987.
- Bohorova, N.E., B. Luna, R.M. Briton L.D. Huerta and D.A. Hoistington, 1995. Regeneration potential of tropical and subtropical, mid altitude and highland maize inbreds. *Mayd.* 40: 275-281.
- Bronsema, F.B.F., W.J.F. Van Oostveen and A.A.M. Van Lammeren, 1997. Comparative analysis of callus formation and regeneration on cultured immature maize embryos of the inbred lines A188 and A632. *Plant Cell Tissue Organ Cult.*, 50: 57-65.
- Che, P., T.M. Love, B.R. Frame, K. Wang, A. L. Carriquiry and S. H. Howell, 2006. Gene expression patterns during somatic embryo development and germination in maize Hi II callus cultures. *Plant Mol. Biol.*, 62: 1-14.
- Huffnagel, H.P., 1961. Agriculture in Ethiopia. Food and Agriculture Organization of the United Nation Organization, Rome, Pages: 465.
- Kaeppler, S.M., H.F. Kaeppler and Y. Rhee, 2000. Epigenetic aspects of somaclonal variation in plants. *Plant Mol. Biol.*, 43: 179-188.
- Korbes, A.P. and A. Droste, 2005. Carbon sources and polyethylene glycol on soybean somatic embryo conversion. *Pesq. Agropec. Bras.*, 40: 211-216.
- Larkin, P.J. and W.R. Scowcroft, 1981. Somaclonal variation: A novel source of variability from cell cultures for plant improvement. *Theor. Applied Genet.*, 60: 197-214.
- Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.*, 15: 473-497.
- Nigussie, M., G. Bogale, G. Soboksa and B. Tolesa, 1996. Improving adaptation and yield dependability of maize in Ethiopia. Proceedings of the 1st Technology Generation, Transfer and Gap Analysis Workshop, December 25-27, 1995, Ministry of Agriculture, Nazret, Ethiopia, pp: 84-89.
- Nigussie, M., H. Mohammed, G. Seboksa, G. Bogale, Y. Beyene, S. Hailemichael and A. Hadis, 2002. Maize improvement for drought stressed areas of Ethiopia. Proceedings of the 2nd National Maize Workshop of Ethiopia, November 12-16, 2001, Addis Ababa, Ethiopia, pp: 15-26.
- Oduor, R.O., E.N.M. Njagi, S. Ndungu and J.S. Machuka, 2006. *In vitro* regeneration of dryland kenyan maize genotypes through somatic embryogenesis. *Int. J. Bot.*, 2: 146-151.
- Ombori, O., N.M. Gitonga and J. Machuka, 2008. Somatic embryogenesis and plant regeneration from immature embryos of tropical maize (*Zea mays* L.) inbred lines. *Biotechnology*, 7: 224-232.
- Omwoyo, O., N.M. Gitonga and J. Machuka, 2008. Plant regeneration via somatic embryogenesis of tropical maize (*Zea mays* L.) commercial hybrid lines. *J. Trop. Microbiol. Biotechnol.*, 4: 24-31.
- Tolessa, B., K. Mulatu, L. Wolde, M. Worku and L. Tulu, 1997. Reflections on the successful achievements of hybrid maize breeding program in Ethiopia. Proceedings of the 5th Eastern and Southern Africa Regional Maize Conference, June 3-7, 1997, CIMMYT, Addis Ababa, Ethiopia, pp: 67-71.
- Tomes, D.T. and O.S. Smith, 1985. The effect of parental genotype on initiation of embryogenic callus from elite maize (*Zea mays* L.) germplasm. *Theor. Applied Genet.*, 70: 505-509.
- Tomes, D.T., 1985. Opportunities and Limitations of the Genotypic Influences on the Establishment and Plant Regeneration from Callus and Cell Cultures of Crop Species. In: *Biotechnology in Plant Science*, Zaitlin, M., P. Day and A. Hollaender (Eds.). Academic Press, New York, USA., pp: 3-14.