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Somatic Embryogenesis and Plant Regeneration from Immature Embryos of Tropical Maize (*Zea mays* L.) Inbred Lines

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Abstract: Somatic embryogenesis and plant regeneration was achieved from immature embryos of six maize (*Zea mays* L.) inbred lines (CML216, CML78, CML331, TL18, TL27 and MU25). Callus was initiated on N6 medium supplemented with different concentrations of 2,4-D, 3% sucrose, 10 mg L^{-1} silver nitrate, 100 mg L^{-1} casein hydrolysate and 2.875 mg L^{-1} proline. Embryogenic callus was formed within two weeks of culture in callus maintenance medium. The concentration of 2,4-D, genotype and age of embryos had a significant effect ($p<0.5$) on the percentage of primary and embryogenic callus formed. The induction of primary callus ranged between 0 and 97% and embryogenic callus ranged between 0 and 70%. Somatic embryos were matured on N6 medium supplemented with 6% sucrose and 1 mg L^{-1} NAA. Embryogenic calli formed plantlets when it was transferred into the regeneration medium containing MS medium supplemented with 3% sucrose. The number of shoots formed ranged from 0 to 9.5 per plate. Callus initiation and plant regeneration were genotype dependent. CML216 had the highest number of shoots formed per culture. Regenerated plants were transferred into half MS medium supplemented with IBA for the development of healthy roots. Regenerated plants were successfully transferred into the greenhouse and into the field and they grew to maturity and set seeds in R_0 and R_1 generations.

Key words: Immature embryos, somatic embryogenesis, plant regeneration, *Zea mays*

INTRODUCTION

Maize (*Zea mays* L.) (2n = 20) is the most important staple human food crop in Kenya in semi-arid areas, medium and high potential regions (Ngugi, 2002). Over 90% of the country's rural population feed on maize. The annual consumption is approximately 125 kg per capita in Kenya (International Maize and Wheat Improvement Center, 2000). Maize is also an important animal feed and provides about 70% of rural employment. Maize ranks third after rice and wheat globally. It occupies a larger area than any other crop; hence it holds a key position in Kenya's economy.

Despite great effort made to increase maize production by use of conventional breeding in Kenya the demand has occasionally outstripped the supply due to various constraints such as drought, pests, diseases, weeds (*Striga*) and low soil fertility (Conway and Toenniessen, 2003; Kiiya *et al*., 2002; Mwangi and Ely, 2001; Bosquiz-Pérez *et al*., 1998). Conventional breeding has not been successful in increasing maize production. This has led to significant dependence on importation of large quantities of maize grains yet the country has the potential of sustainable production of the crop. Hence there is need to produce maize genotypes which are adapted to biotic and abiotic constraints. Transfer of transgenes by conventional breeding methods is laborious, time and space consuming and the recovery of progeny with transgenic and important agronomic traits is difficult due to incompatible heterotic groups and combining ability. Tissue culture and genetic transformation are the other techniques which can be used to complement conventional breeding to achieve increased and sustainable maize varieties in a relatively shorter time and will be of value on agricutural food industry. By use of genetic transformation, it is now possible to transfer desirable genes by the use of *Agrobacterium tumefaciens* to produce transgenic plants which are resistant to the production constraints.

An efficient *in vitro* regeneration system of the whole plant is often the limiting step in the application of genetic transformation technique for crop improvement of most of the maize genotypes. An efficient plant regenerative system through somatic embryogenesis is important for gene manipulation and improvement of maize by use of *A. tumefaciens*. This is because somatic embryos can be induced, repetitively and can also germinate into plantlets. Tissue culture technique has been used for successful *in vitro* regeneration of maize in

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some temperate and tropical maize genotypes from a range of tissues, such as pollen (He *et al*., 2006), pollinated ovaries (Tang *et al*., 2006), split seed (Al-Abed *et al*., 2006), mature embryo (Huang and Wei, 2004), immature embryo (Oduor *et al*., 2006; El-Itriby *et al*., 2003; Santos-Serejo and Aguiar-Perecin, 2000; Carvolho *et al*., 1997; Bohorova *et al*., 1995), shoot tips (O`Connor-Sánchez *et al*., 2002), shoot apical meristems (Zhang *et al*., 2002) through somatic embryogenesis and organogenesis. Although there are various reports on successful regeneration of maize, these are often limited to particular genotypes. Maize genotypes have a large variation in their competence to regenerate plantlets. Hence most of the lines remain inaccessible to improvement using genetic transformation techniques. Most of the genotypes fail to produce embryogenic callus from competent tissues or they fail to regenerate since they are recalcitrant *in vitro* response. The differences are associated with susceptibility to genetic programming and reprogramming of the competent cells to internal and external factors. Although regenerable maize embryogenic callus has been induced from various meristematic tissues, immature embryos have been shown to be most efficient in their regeneration competence as they contain a large number of actively dividing cells. In Kenya, regeneration has been reported in only four maize genotypes out of many which are available (Oduor *et al*., 2006). The response of maize tissues in culture is genotype dependent. Therefore there is need to assess a wide range of genotypes to identify those with high regenerative response which can be used during genetic transformation. Somatic embryogenesis and plant regeneration of tropical maize inbred lines available in Kenya has not yet been reported in the literature, hence up to date little is known about their response in tissue culture.

The build up of knowledge on the regenerative response of maize could not only be important for improvement of tissue culture response of elite genotypes but will also accelerate their improvement via genetic transformation technology. The objective of the present study was to assess callus initiation and plant regenerative response of six tropical inbred lines from immature embryos.

MATERIALS AND METHODS

Seeds of inbred lines (TL18, TL27 and MU25) were obtained from Kenya Agricultural Research Institute and three inbred lines (CML216, CML78 and CML331) from International Maize and Wheat Improvement Centre. The study was carried out between March 2006 and October 2007. Seeds were planted in the Plant and Microbial Sciences Research farm, Kenyatta University. Ears were covered with clear polythene bags until the emergence of the silk to avoid foreign pollen contamination. Self- or sib-pollination was done and ears were covered with the pollination bags to avoid cross pollination.

Ears were harvested 16 and 20 days after pollination (DAP). The ears were dehusked and surface sterilized for 5 min in 70% (v/v) ethanol and then for 20 min in 2.5% (v/v) sodium hypochlorite, wetting with two drops of Tween 20. This was followed by five times rinse in sterile distilled water. Immature embryos (1-2.5 mm) were aseptically isolated by cutting the tips of the kernels with a scapel without touching the embryo according to procedures used by Jiménez and Bangerth (2001). The embryos were placed with embryo axis in contact with Callus Initiation Medium (CIM) containing N6 medium (Chu *et al*., 1975) supplemented with 100 mg L^{-1} casein hydrolysate, 2.875 mg L^{-1} L-proline, 10 mg L^{-1} silver nitrate, 3% (w/v) sucrose and 0-20 mg L^{-1} 2,4-D (2,4-Dichlorophenoxyacetic acid) and 0.3% (w/v) gerlite. The media was autoclaved at 121°C for 20 minutes at a pressure of 1.06 kg cm^{-2} after adjusting the pH to 5.8 with 1N NaOH. Twenty-five embryos were placed in each 90×15 mm petri dish and replicated four times for each genotype. Cultures were incubated in the dark at 27±1°C. Percentage of immature embryos forming primary callus was recorded two weeks after culture. The developing callus was sub-cultured after every 14 days into the callus maintenance medium (CMM) as for callus initiation but without silver nitrate. Percentage of embryogenic callus formed was recorded four weeks after subculture.

Embryogenic callus was transferred into embryo maturation medium (EMM) containing N6 medium supplemented, 6% (w/v) sucrose, 1 mg L^{-1} NAA (Naphthalene acetic acid) and 0.3% (w/v) gerlite for embryo maturation. Cultures were incubated in the dark at a temperature of 27±1°C. Embryogenic callus was determined by examining the presence of somatic embryos under the Leica zoom 2000 microscope or hp scanjet 8200. After two weeks of culture on EMM medium, twelve pieces of embryogenic calli with somatic embryos about 100 mg were transferred into the Regeneration Medium (RM) containing MS medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose and solidified with 0.3% (w/v) gerlite (RM) for shoot induction. There were ten replicates for each genotype. The cultures were incubated at 27±1°C under 16 h/8 h (light/dark). The numbers of shoots formed per culture were recorded after 3 weeks of culture.

Plantlets were transferred onto a solidified half-strength MS medium supplemented with 3% sucrose and 0-1 mg L^{-1} IBA (Indole-3-butyric acid) for enhancement of root formation. The cultures were incubated at a temperature of 27±1°C under 16 h/8 h (light/dark). The number of roots formed was recorded after two weeks of culture.

Well-rooted plantlets from culture bottles were rinsed with water to remove the media and then transferred into pots containing peat moss. Plantlets were covered with a clear polythene bags which were punctured with several holes to allow gradual acclimatization to ambient humidity for 3-5 days. Plants (R_0) were then transferred into pots containing garden soil mixed with manure and sand (2:2:1) and later some of the plants were transplanted into the research farm and grown to maturity. Seeds of R_0 plants were grown to produce R_1 plants. The chemicals used in this report were purchased from Duchefa (Haarlem, The Netherlands).

The experiments were organized according to completely randomized design. The experiment was repeated three times. Analysis of variance (ANOVA) with SPSS for windows version 11.5 and MINITAB software version 23.22. were used to test the significant effect of the genotypes, 2,4-D concentrations and age of the embryos on callus initiation and plant regeneration. Means were separated using Tukey`s Honest Significant Difference (HSD) at 5% level.

RESULTS AND DISCUSSION

Callus initiation: Immature embryo were plated on N6 medium with their embryo axis in contact with the callus initiation medium (CIM) (Fig. 1a). This orientation helps to retard the germination of embryos and induce proliferation of scutellar cells (Green and Phillips, 1975). Callus initiation from immature embryos was observed after the fourth day of culture on CIM medium from the scutellum (Fig. 1b). This was due to the presence of meristematic cells in the scutellum. Al-Abed *et al.* (2006) reported the presence of the meristematic cells in the scutellum of maize embryos from which callus is induced. The percentage of the primary callus formed ranged from 0 to 97% and 0 to 83.5% for 16 and 20 DAP immature embryos, respectively (Table 1) while embryogenic callus ranged from 0 to 70% and 0 to 58% for 16 and 20 DAP respectively (Table 2) depending on the concentration of 2,4-D. Embryogenic callus was formed after the first subculture on callus maintenance medium. Globular somatic embryos were formed on the surface of the

Table 1: Effect of different concentrations of 2,4-D on the percentage of primary callus initiation response from immature embryos 16 and 20 DAP after 14 days of culture in callus initiation medium

	Genotype (%)*					
Conc. 2,4-D (mg L^{-1})	CML216	CML78	CML331	TL18	TL27	MU25
16 days after pollination (DAP)						
0	0.0a**	0.0a	0.0a	0.0a	0.0a	0.0a
0.1	22.0±3.1bc	19.0±1.9bc	32.0±2.0b	21.0±1.6b	15.3±1.3b	21.0±1.2b
0.3	23.8±2.8bc	21.8±2.6c	43.8±2.5b	33.0±0.9c	19.0±1.8b	22.5±1.6b
0.5	29.3±2.0c	32.5±1.9bc	56.3±2.0c	38.8±2.0cd	26.5±1.6bc	23.0±1.7b
0.7	32.3±2.5c	33.5±2.2bc	64.5±2.1d	46.3±2.0d	32.8±1.7c	26.5±2.0b
0.9	37.3±2.5d	38.8±1.7c	68.0±2.4d	47.3±3.1d	33.3±2.3c	28.5±3.4c
1	45.0±5.3d	40.0±1.6c	86.0±1.2e	54.8±2.8e	47.0±4.5d	30.0±4.1c
1.5	77.0±4.4e	45.0±3.5c	84.0±2.8e	68.0±4.0ef	66.0±2.6e	41.0±1.6d
2	82.0±4.8ef	70.0±3.8d	96.0±1.6g	93.0±3.0g	89.0±3.4f	58.0±0.9ef
2.5	86.0±2.6fg	77.0±1.9de	96.0±1.7g	92.0±2.8g	91.0±3.8f	62.8±2.9f
5	89.0±1.0fg	77.0±3.0de	94.0±1.2fg	92.0±4.3g	89.0±3.0f	58.0±2.3ef
10	97.0±1.9g	84.2±1.6e	90.0±2.6f	54.0±2.3e	95.0±1.0f	57.0±2.1ef
15	84.0±2.8ef	79.0±1.5de	96.0±1.6g	44.3±5.3d	86.0±4.8f	56.5±0.9ef
20	48.0±4.3d	71.0±3.4d	94.0±3.5fg	42.5±3.2d	62.0±2.6e	52.0±1.5e
Genotype mean***	54.6c	49.2b	71.5e	55.4c	53.7c	38.3a
20 days after pollination (DAP)						
0	0.0a**	0.0a	0.0a	0.0a	0.0a	0.0a
0.1	17.5±1.6b	15.5±1.2b	29.0±2.0b	17.8±0.9b	12.3±1.2b	16.3±1.4b
0.3	18.8±1.8bc	19.8±1.0b	39.8±2.5b	31.5±1.0b	17.8±1.2bc	21.0±1.1bc
0.5	24.3±1.5c	28.0±1.7c	52.3±2.0c	37.5±1.4bc	25.0±1.1bc	22.0±1.1b
0.7	30.5±2.2d	31.8±1.7cd	49.8±1.8c	39.5±0.9c	31.8±1.3d	24.5±1.7bc
0.9	33.8±1.7d	35.8±1.1d	57.8±1.2d	43.0±1.1c	32.3±1.8	27.0±2.3c
1	41.3±1.5e	36.3±1.7d	69.0±2.5e	43.0±3.0c	46.0±4.4d	29.0±2.1c
1.5	61.0±1.7f	37.8±1.4e	70.5±1.6ef	62.0±2.0e	50.0±1.1ef	38.0±3.8e
2	66.0±1.9fg	55.3±1.8d	78.5±1.0gh	81.0±4.1f	68.8±0.9g	56.0±3.5h
2.5	69.0±2.5g	66.0±1.8g	76.0±2.2f	81.0±4.4f	74.5±1.6gh	61.0±3.8fgh
5	67.0±2.3fg	66.0±0.9g	83.5±3.0h	82.0±5.3f	76.5±1.9hi	54.0±3.0f
10	67.0±1.3fg	70.0±2.3g	79.0±2.5gh	49.8±1.0d	81.5±1.3i	56.0±4.4gh
15	64.0±2.4fg	69.0±3.1g	79.8±2.6gh	49.0±1.6d	76.3±3.9hi	54.5±4.1f
20	32.0±1.4d	60.0±1.4f	78.3±1.5gh	42.3±1.5c	52.8±2.1f	42.0±2.6d
Genotype mean***	42.3a	42.2a	60.2c	47.1b	46.2b	35.8a

* Mean (percentage of the primary callus induction was evaluated by counting the number of immature embryos that formed callus after 14 days out of the total number of immature embryos cultured in CIM x 100). ** Values followed by different letter(s) within the columns are significantly different from each other according to Tukey`s HSD at 5% level, ***Values followed by the same letter(s) within the row are not significantly different from each other according to Tukey`s HSD at 5% level

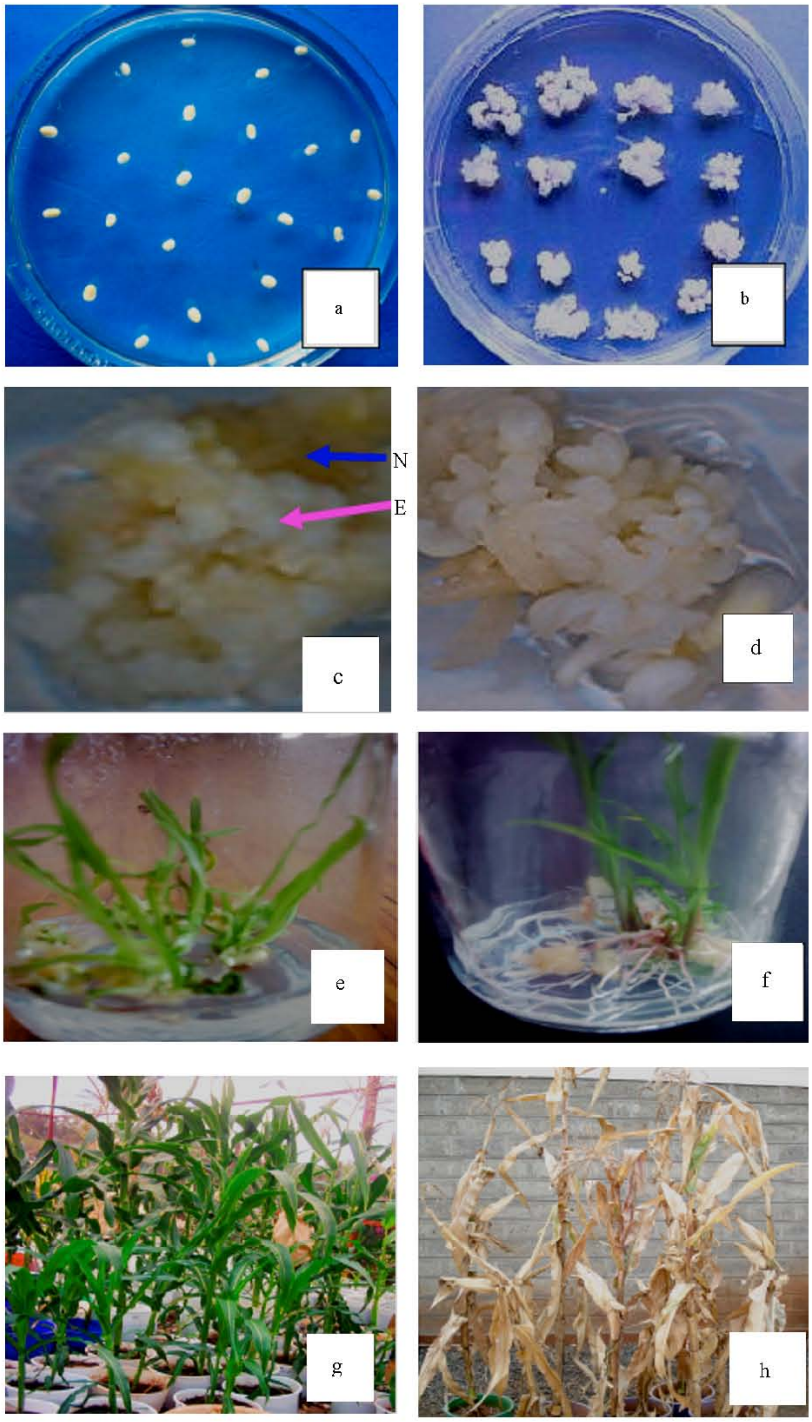

Fig. 1: Somatic embryogenesis and plant regeneration from immature embryos of maize immature embryos on callus initiation medium after one day of culture. (b) Callus initiation after 14 days of culture. (c) Embryogenic callus (E) with somatic embryo at globular stage on the surface of the proliferating scutellum and non-embryogenic callus (N). (d) Embryo maturation in N6 medium supplemented with 6% sucrose and 1 mg L^{-1} NAA showing heart-shaped somatic embryos. (e) Shoot induction from embryogenic callus on MS medium devoid of growth regulators. (f) *In vitro* rooting of maize regenerants. (g). *In vitro* regenerants (Ro) in greenhouse after 4 months in pots (h) Mature R_1 plants

Table 2: Effect of different concentrations of 2,4-D on the percentage of embryogenic callus induction response from immature embryos 16 and 20 DAP after 4 weeks of culture in callus maintenance medium

	Genotype (%)*					
Conc. 2,4-D (mg L^{-1})	CML216	CML78	CML331	TL18	TL27	MU25
16 days after pollination (DAP)						
0	0.0a**	0.0a	0a	0.0a	0.0a	0.0a
0.1	15.0±1.1b	0.0±0a	0±0	0.0±0a	0.0±0a	0.0±0a
0.3	18.0±1.2b	0.0±0a	0±0	13.0±1.7b	0.0±0a	0.0±0a
0.5	22.0±1.5bc	0.0±0a	0±0	18.3±1.7bcd	12.0±1.1b	0.0±0a
0.7	24.0±1.0bc	0.0±0a	0±0	20.0±1.8d	16.0±1.7cde	8.0±1.3b
0.9	27.0±1.1cd	8.0±1.4b	0±0	24.0±1.8de	18.0±1.5de	13.0±1.5c
1	42.67±3.3e	12.0±1.6bc	0±0	27.0±1.6e	18.0±1.8de	16.0±1.5c
1.5	70.0±4.9h	17.0±1.9d	0±0	40.0±2.2f	20.0±2.0e	18.0±1.6cd
2	69.0±4.3h	20.0±1.8d	0±0	30.0±3.3e	20.0±1.8e	21.0±1.8d
2.5	58.0±3.6fg	19.0±1.6d	0±0	18.0±1.8cd	20.0±1.6e	21.0±1.7d
5	50.0±3.2f	17.0±1.5d	0±0	15.0±1.5c	15.0±1.5cd	14.3±1.6c
10	40.0±3.6e	12.0±1.1c	0±0	13.0±1.7bc	12.0±1.3c	17.0±1.7cd
15	32.0±2.3de	10.0±1.2bc	0±0	11.0±1.6b	9.0±1.7bc	14.0±1.3c
20	25.0±2.9bc	7.0±1.2b	0±0	9.0±1.6b	6.0±1.0b	11.0±1.6bc
Genotype mean***	35.4e	8.7b	0a	17.0d	11.9c	11.0c
20 days after pollination (DAP)						
0	0.0a**	0.0a	0a	0.0a	0.0a	0.0a
0.1	11.0±1.1a	0.0±0a	0±0	0.0±0a	0.0±0a	0.0±0a
0.3	13.0±1.0bc	0.0±0a	0±0	12.0±1.1d	0.0±0a	0.0±0a
0.5	18.0±1.5cd	0.0±0a	0±0	16.0±1.1de	10.0±1.3c	0.0±0a
0.7	20.0±1.6d	0.0±0a	0±0	18.0±1.6e	13.0±0.7cd	6.0±1.1b
0.9	25.0±1.7de	5.0±0.9b	0±0	21.0±1.8ef	15.0±1.5d	8.0±1.3bc
1	30.0±1.7e	7.0±1.1b	0±0	23.0±1.3f	17.0±1.5d	12.0±1.4cd
1.5	58.0±3.5h	13.0±1.5ef	0±0	32.0±1.6g	19.0±1.8e	15.0±1.7de
2	57.0±3.4gh	17.0±1.5fg	0±0	19.0±1.7e	20.0±1.5e	18.0±1.9ef
2.5	50.0±3.6fg	16.0±1.8f	0±0	14.0±1.4d	16.0±1.1d	20.0±1.8f
5	46.0±3.4f	10.0±0.9de	0±0	13.0±1.1d	11.0±1.6c	13.0±1.1d
10	35.0±3.0e	9.0±1.5cd	0±0	11.0±0.7cd	11.0±0.9c	11.0±0.7cd
15	20.0±2.1d	6.0±1.2bc	0±0	8.0±0.9bc	5.0±0.7b	8.0±1.1bc
20	18.0±2.3cd	3.0±0.7ab	0±0	7.0±0.7b	5.0±0.9b	7.0±1.1bc
Genotype mean***	28.6f	6.1b	0a	13.9e	10.1d	8.4c

*Mean (percentage of the embryogenic callus formed was determined by counting number of immature embryos that formed embryogenic callus out of the total number of immature embryos cultured x 100, ** Values followed by different letter(s) within the columns are significantly different from each other according to Tukey`s HSD at 5% level, *** Values followed by different letter(s) within the row are significantly different from each other according to Tukey`s HSD at 5% level

embryogenic callus (Fig. 1c). Two types of embryogenic callus were formed, type I and II callus. Type 1 callus was compact, white to cream, while type II was friable and light yellow. The amount of type II callus formed was low in the present study. The formation of type I and II callus has been reported in maize (Jiménez and Bangerth, 2001; Tomes and Smith, 1985). Variation of the genotypes to form type I and II callus in maize has been reported to be due to additive gene effects while heterosis positively increases culture response (Tomes and Smith, 1985). Non-embryogenic callus was also formed which was soft, watery and yellow in colour (Fig. 1c) which ceased to grow and turned brown in subsequent subculture. The formation of embryogenic and non-embryogenic callus has been reported in other maize genotypes (Shohael *et al*., 2003; Jiménez and Bangerth, 2001) and sorghum (Gupta *et al*., 2006) from immature embryo. Successful establishment of competent cells which can regenerate into a whole plants in many plant species depends on the capability of identifying the appropriate cell types. In our study swelling of the scutellar tissue and germination of immature embryos cultured on a medium devoid of 2,4-D without callus initiation was observed. Studies have shown that 2,4-D is an important factor in the initiation and proliferation of primary and embryogenic callus from immature embryos of maize (Carvalho *et al*., 1997; Bohorova *et al*., 1995), mature embryos of maize (Huang and Wei, 2004) and from mature and immature embryos of wheat (Bi *et al*., 2007; Yu *et al*., 2003). Concentration of 2,4-D had a significant effect on the percentage of the primary and embryogenic callus initiated from immature embryos 16 and 20 DAP ($p<0.05$). Embryogenic callus was not formed in CML331 at all concentrations of 2,4-D. The optimum 2,4-D concentration for the initiation of embryogenic callus varied among the maize genotypes 1.5 mg L^{-1} (CML216 and TL18), 2 mg L^{-1} (TL27 and CML78) and 2.5 mg L^{-1} (MU25) from immature embryos 16 DAP (Table 2). The optimum concentration for the initiation of embryogenic callus also varied among genotypes from embryos 20 DAP. High concentration of 2,4-D reduced the percentage of embryogenic callus formed suggesting that 2,4-D had an inhibitory effect.

This could be due to the blockage of cell division and inactivation of cells which have the embryogenic potential. Embryogenic callus was not formed in a concentration of 2,4-D which was below 0.3 mg L^{-1} (TL18), 0.5 mg L^{-1} (TL27), 0.7 mg L^{-1} (MU25) and 0.9 mg L^{-1} (CML78). This shows that the concentration of 2,4-D for the initiation of embryogenic callus was genotype dependent

Age of the embryo (DAP) was a critical factor in determining the capacity of callus initiation from immature embryo. The percentage of primary and embryogenic callus formed from immature embryos 20 DAP was significantly lower than that formed from 16 DAP ($p<0.05$) (Table 1 and 2). This is probably due to reduction in the meristematic activity of the cells with ageing suggesting that the physiological and developmental state of the embryos is important in determining callus initiation response. Embryos which were less than 1 mm in length did not respond in culture. According to Bohorova *et al.* (1995), immature embryos of maize less than 0.5 mm in length did not respond in culture while Lu *et al.* (1983) reported similar results for embryo less than 1 mm.

The genotypes differed significantly ($p<0.05$) in their response to callus initiation from immature embryos 16 DAP and 20 DAP (Table 1 and 2). This is contrary to Oduor *et al.* (2006) in which significant differences were not detected on the Kenyan maize genotypes tested using immature embryos 17, 18, 19 and 20 DAP, showing that callus initiation is genotype dependent. CML216 had the highest percentage of embryogenic callus formed which was significantly different from other inbred lines (Table 2). The effect of genotype on the formation of callus has also been reported in other maize genotypes (Ratif *et al*., 2006; Bohorova *et al*., 1995). Significant differences were detected in the genotype x 2,4-D concentration interaction and in the genotype x DAP of embryos interaction in the formation of primary and embryogenic callus ($p<0.05$).

Maturation of somatic embryos and plant regeneration: Somatic embryos were matured when embryogenic callus was transferred to N6 medium with high concentration of sucrose (6%) and 1 mg L^{-1} NAA (Fig. 1d). Somatic embryos at the heart-shaped stage were observed. High concentration of sucrose has been reported to promote the maturation of somatic embryos in soyabeans (Körbes and Droste, 2005) and maize (Ratif *et al*., 2006; Che *et al*., 2006; El-Itriby *et al*., 2003; Bronsema *et al*., 1997). This is due to the osmotic stress caused by high concentration of sugar. According to Che *et al.* (2006), a large number of genes encoding for protein proliferating cell nuclear antigen and histone are down-regulated while those encoding for nucleases, glucosidases and proteases are up-regulated during somatic embryo maturation resulting in the reduction of cell division and growth. When embryogenic callus with somatic embryos were transferred into the Regeneration Medium (RM), they turned green and shoots were observed within two weeks (Fig. 1e). This could be due to the up-regulation of stress-related and transporter encoding genes followed by those encoding for photosynthetic and other chloroplast components (Che *et al*., 2006). Some of the somatic embryos did not regenerate to form plantlets. This could be due to down-regulation of genes controlling plant regeneration reported by Che *et al.* (2006) and Hodges *et al.* (1986) in which it was concluded that regeneration from immature embryo-derived callus was controlled by a few nuclear genes or a few gene clusters. Armstrong *et al.* (1992) proposed that there is a major gene or genes on the long arm of chromosome 9 of A188 genotype which is responsible for the promotion of embryogenic callus formation and plant regeneration in B73 and Mo17. According to Ikeda *et al.* (2006), embryogenesis related genes are involved during somatic embryogenesis in some plants which could be the reason for the genotypic differences in the response of the various genotypes observed in this study. Failure of some of the somatic embryos to regenerate plantlets has also been reported in other maize genotypes (Huang and Wei, 2004; Carvalho *et al*., 1997; Bohorova *et al*., 1995). In this study non-embryogenic callus did not regenerate plantlets, similar to reports in other maize genotypes (Jiménez and Bangerth, 2001; Bronsema *et al*., 1997).

The number of shoots formed by CML216 was significantly ($p<0.05$) higher compared to those formed by other genotypes (Table 3). This shows that regeneration was genotype dependent. The effect of genotype on plant regeneration has also been reported in other maize genotypes (Huang and Wei, 2004; El-Itriby *et al*., 2003; Bohorova *et al*., 1995; Tomes and Smith, 1985), wheat and tricale (Dornelles *et al*., 1997), sorghum (Gupta *et al*., 2006) and rice (Ge *et al*., 2006). This is contrary to results by Lu *et al.* (1983), in which all maize cultivars tested showed

Table 3: Shoot induction response of six maize genotypes cultured on MS medium devoid of growth regulators

Genotype	Mean No. shoots per culture*
CML216	9.5±0.3e**
CML78	2.5±0.3b
CML331	0.0a
TL18	5.3±0.2d
TL27	3.4±0.6c
MU25	3.5±0.3c

*Mean. Each value represents a mean of ten replicates each with twelve pieces of calli cultured on MS medium devoid of growth regulators. **Values having different letters within the column are significantly different from each other according to Tukey`s HSD at 5% level

Table 4: Effect of IBA concentrations on the number of roots formed in five tropical maize inbred lines

	Genotype (%)*					
Conc. of IBA (mg L^{-1})	CML216	CML78	TL18	TL27	MU25	Conc. mean
0	1.5±0.3a**	1.3±0.3a	1.5±0.3a	0.8±0.3a	1.0±0.0a	1.2a
0.2	2.8±0.5ab	1.5±0.3ab	2.3±0.3ab	1.0±0.0ab	1.3±0.3ab	1.7ab
0.4	3.0±0.0b	2.3±0.2ab	2.5±0.3ab	1.3±0.3ab	1.5±0.3ab	2.1b
0.6	3.3±0.5b	3.0±0.4bc	3.0±0.4bc	1.5±0.3bc	2.0±0.4bc	2.5bc
0.8	3.5±0.5b	3.3±0.8c	3.8±0.3c	2.0±0.0c	2.8±0.3c	3.2c
1	1.8±0.3a	1.3±0.2a	1.2±0.2a	1.0±0.0a	1.5±0.3a	1.3ab
Genotype mean***	2.7c	2.1bc	2.4bc	1.3ab	1.7b	

*Mean number of roots per plant, **Values having the same letter(s) within columns are not significantly different from each other according to Tukey`s HSD at 5% level, ***Values having the same letter(s) within the row are not significantly different from each other according to Tukey`s HSD at 5% level

more or less similar response in callus initiation and plant regeneration. Differences in the genotypic responses to regenerate observed in this study is likely to be due to the genetic diversity caused by *helitron* transposable elements which are responsible for capturing and moving genes around the maize genome which contribute to differences in gene expression (Buckler *et al*., 2006; Guo *et al*., 2004). In gramineous species such as wheat regeneration from embryonic callus has been reported to be successful on a medium devoid of 2, 4-D (Satyavathi *et al*., 2004) which is consistency with the present study.

Rooting response: Enhancement of root formation was achieved after one week in hormone free half strength MS medium while 4 days in medium supplemented with Indole Butyric acid (IBA) (Fig. 1f). This shows that IBA had a promotive effect on rooting. Increase in IBA concentration up to 0.8 mg L^{-1} caused an increase in the number of roots formed in all genotypes, while at a concentration of 1 mg L^{-1} IBA there was a reduction in the number of roots formed (Table 4). This probably may be due to the inhibitory effect of IBA when it is used in high concentration. The best concentration for root formation was 0.8 mg L^{-1}. There was a lot of branching of the roots when shoots were cultured on MS medium supplemented with 0.4, 0.6 and 0.8 mg L^{-1} IBA. Concentration of IBA had a significant effect ($p<0.05$) on the number of roots formed in H627, DLC1 and H513. Significant differences ($p<0.05$) were not detected on the effect of IBA concentration on the number roots formed in H625. CML216 genotype had the highest mean number of roots which was significantly different from those formed by TL27 and MU25 ($p<0.05$), however there was no significant different detected with those formed by TL18 and CML78 ($p>0.05$). This results show clearly that root formation was genotype dependent. There was no significant difference ($p>0.05$) in the interaction between the genotype and IBA concentrations on the number of roots formed.

Aclimatization and growth of *in vitro* regenerated plants in the greenhouse and in the field: *In vitro* regenerated plants (Ro) with well-developed roots were transferred into the pots containing peat moss for hardening and later into the soil in the pots in the greenhouse. Ninety four percent of TL18 regenerants (Ro), 98% of CML216, 60% of TL27, CML78 and MU25 of the *in vitro* regenerants survived in the greenhouse. *In vitro* regenerated R_o plantlets were grown in the greenhouse (Fig. 1g). Ninety percent of the regenerants (Ro) survived in the field. The seeds from Ro plants were planted, they grew to maturity and set seeds in R_1 plants (Fig. h). Hence regeneration system for obtaining fertile plants was established via somatic embryogenesis in the present study. Somatic embryogenesis has been recognized as the most reliable and efficient method for the production of *in vitro* fertile plantlets.

In conclusion, this study established a reproducible regeneration system of maize genotypes through somatic embryogenesis from immature embryos. Somatic embryogenesis is an important step in any successful plant transformation scheme, because from the callus each transformed cell has the potential to produce a plant. The study established that the genotype, 2,4-D concentration and age of explant had a significant effect on callus initiation and plant regeneration. This suggests that the physiological stage of the embryos is important in determining their response in culture. In our study the highest embryogenic callus and plant regeneration response was produced from inbred line CML216, hence this genotype can be used in genetic transformation studies. To the best of our knowledge this is the first report on successful somatic embryogenesis and plant regeneration of Kenyan tropical maize inbred lines from immature embryos.

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REFERENCES

Al-Abed, D., S. Rudrabhatla, R. Talla and S. Goldman, 2006. Split-seed: A new tool for maize researchers. Planta, 223 (6): 1355-1360.

Armstrong, C.L., J. Romero-Severson and T.K. Hodges, 1992. Improved tissue culture response of an elite maize inbred through back cross breeding and identification of chromosomal regions important for regeneration by RFLP analysis. Theor. Applied Genet., 84 (6): 755-762.

Bi, R.M., M. Kou, L.G. Chen, S.R. Mao and H.G. Wang, 2007. Plant regeneration through callus initiation from mature embryo of *Triticum*. Plant Breed., 126 (1): 9-12.

Bohorova, N.E., B. Luna, R.M. Brito, L.D. Huerta and D.A. Hoisington, 1995. Regeneration potential of tropical, subtropical, midaltitude and highland maize inbeds. Maydica, 40: 275-281.

Bosquiz-Pérez, N.A., S.O. Olojede and I.W. Buddenhaggen, 1998. Effect of maize streak virus disease on the growth and yield of maize as influenced by varietal resistance levels and plant stage as at time of challenge. Euphytica, 101 (3): 307-317.

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 (1): 57-65.

Buckler, E.S., B.S. Gaut and M.D. McMullen, 2006. Molecular and functional diversity of maize. Curr. Opin. Plant Biol., 9 (2): 172-176.

Carvalho, C.H.S., N. Bohorova, P.N. Bordallo, L.L. Abreu, F.H. Valicente, W. Bressan and E. Paiva, 1997. Type II callus production and plant regeneration in tropical maize genotypes. Plant Cell Rep., 17 (1): 73-76.

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): 1-14.

Chu, C.C., C.C. Wang, C.S. Sun, C. Hsu, K.C. Yin, C.Y. Chu and F.Y. Bi, 1975. Establishment of an efficient medium for anther culture of rice through comparative experiments on nitrogen source. Sci. Sin., 18: 659-668.

Conway, G. and G. Toenniessen, 2003. Science for African food security. Science, 299 (5610): 1187-1188.

Dornelles, A.L.C., F.I.F Carvalho, F.C. Federizzi, C.L. Handel, F. Bered, M.E.B. Sordi and F. Schnelden, 1997. Callus initiation and plant regeneration by Brazilian tricale and wheat genotypes. Braz. J. Genet., 20 (1): http://www. scielo. br/scielo.php? script=enandnrm= iso>. doi: 10.1590/S0100-84551997000100008. (Accessed on 15th October 2007).

El-Itriby, H.A., S.K. Assem, E.H.A. Hussein, F.M. Abdel-Galil and M.A. Madkour, 2003. Regeneration and transformation of Egyptian maize inbred lines via immature embryo culture and a biolistic particle delivery system. *In vitro* Cell. Dev. Biol. Plant, 39 (5): 524-531.

Ge, X., Z. Chu, Y. Lin and S. Wang, 2006. A tissue culture system for the different germplasms of *Indica* rice. Plant Cell Rep., 25 (5): 392-402.

Green, C.E. and R.L. Phillips, 1975. Plant regeneration from tissue cultures of maize. Crop Sci., 15 (3): 417-420.

Guo, M., M.A. Rupe, C. Zinselmeier, J. Habben, B.A. Bowen and O.S. Smith, 2004. Allelic variation of gene expression in maize hybrids. Plant Cell., 16 (7): 1707-1716.

Gupta, S., V.K. Khanna, R. Singh and G.K. Garg, 2006. Strategies for overcoming genotypic limitations of *in vitro* regeneration and determination of genetic components of variability of plant regeneration traits in sorghum. Plant Cell Tissue Organ. Cult., 86 (3): 379-388.

He, G., J. Zhang, K. Li, Z. Xiong, M. Chen, J. Chang, Y. Wang, G. Yang and B. Barnaba´s, 2006. An improved system to establish highly embryogenic haploid cell and protoplast cultures from pollen calluses of maize (*Zea mays* L.). Plant Cell Tissue Organ Cult., 86 (1): 15-25.

Hodges, T.K., K.K. Kamo, W.C. Imbrie, M. R. Becwar, 1986. Genotype specificity of somatic embryogenesis and regeneration in maize. Biotechnology, 4 (3): 219-223.

Huang, X.Q. and Z.M. Wei, 2004. High frequency plant regeneration through callus initiation from mature embryos of maize (*Zea mays*). Plant Cell Rep., 22 (11): 793-800.

Ikeda, M., M. Umehara and H. Kamanda, 2006. Embryogenesis-related genes. Its expression and roles during somatic and zygotic embryogenesis in carrot and *Arabidopsis*. Plant Biotechnol., 23 (2): 153-161.

International Maize and Wheat Improvement Center, 2000. Invest in CIMMYT-invest in the future. Mexico, D.F.: CIMMYT.

Jiménez, V.M. and F. Bangerth, 2001. Hormonal status of maize initial explants and of the embryogenic and non-embryogenic callus cultures derived from them as related to morphogenesis *in vitro*. Plant Sci., 160 (2): 247-257

Kiiya, W.W., R.M.A. Onyango, T.K. Mwangi and J.M.A. Ngeny, 2002. Participatory verification of maize varieties for lower, highland and upper midland transitional zones of North Rift Kenya. In: Proceedings of the 2nd Scientific Conference of the Soil Management and Legume Research Network Projects, June 2000, Mombasa, Kenya, Kenya Agricultural Research Institute.

Körbes, A.P. and A. Droste, 2005. Carbon sources and polyethylene glycol on soybean somatic embryo conversion. Pesq. Agropec. Bras., 40 (3): 211-216.

Lu, C., V. Vasil and I.K. Vasil, 1983. Improved efficiency of somatic embryogenesis and plant regeneration in tissue cultures of maize (*Zea mays* L.). Theor. Applied Genet., 66(4): 285-289.

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

Mwangi, P.N. and A. Ely, 2001. Assessing risks and benefits: Bt maize in Kenya. Biotechnol. Dev. Monitor, 48: 6-7.

Ngugi, K., 2002. Maize projects implemented by NDFRC-Katumani. Biotechnology Trust Africa. Annual Report 1998-2000, Nairobi, Kenya, pp: 60.

O'Connor-Sánchez, A., J.L. Cabrera-Ponce, M. Valdez-Melara, P. Téllez-Rodríguez, J.L. Pons-Hernández and L. Herrera-Estrella, 2002. Transgenic maize plants of tropical and subtropical genotypes obtained from calluses containing organogenic and embryogenic-like structures derived from shoot tips. Plant Cell Rep., 21 (4): 302-312.

Oduor, R.O., E.N.M. Njagi and J.S. Machuka, 2006. *In vitro* regeneration of dryland Kenyan maize genotypes through somatic embryogenesis. Int. J. Bot., 2 (2): 146-151.

Ratif, M., T. Fatma, T. Husnain, K. Bashir, M.A. Khan and S. Riazuddin, 2006. Regeneration and transformation of elite inbred line of maize (*Zea mays* L.), with a gene from *Bacillus thuringiensis*. South African J. Bot., 72 (3): 460-466.

Santos-Serejo, J.A. and M.L.R. Aguiar-Perecin, 2000. Genotypes with high somatic embryogenesis and plant regeneration capacity obtained from tissue culture. Sci. Agric., 57 (4): 717-722.

Satyavathi, V.V., P.P. Jauhar, E.M. Elias and M.B. Rao, 2004. Effects of growth regulators on *in vitro* plant regeneration in durum wheat. Crop Sci., 44 (5): 1839-1846.

Shohael, A.M., M.A.L. Akanda, S. Parvez and S. Mahfuja, 2003. Somatic embryogenesis and plant regeneration from immature embryo derived callus of inbred maize (*Zea mays* L.). Biotechnology, 2 (2): 154-161.

Tang, F., Y. Tao, T. Zhao and G. Wang, 2006. *In vitro* production of haploid and doubled haploid plants from pollinated ovaries of maize (*Zea mays* L.). Plant Cell Tissue Organ. Cult., 84: 233-237.

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 (5): 505-509.

Yu, G.R., J. Yin, T.C. Guo and J.S. Niu, 2003. Selection of the optimum genotype for immature embryo culture of wheat. J. Triticeae Crops, 23 (2): 14-18.

Zhang, S., R. Williams-Carrier and P.G. Lemaux, 2002. Transformation of recalcitrant maize elite inbreds using *in vitro* shoot meristematic culture from germinated seedlings. Plant Cell Rep., 21 (3): 263-270.