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Full Length Research Paper

Regeneration of tropical maize lines (*Zea mays* l.) from mature zygotic embryo through callus initiation

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The use of immature zygotic embryos as an explant for maize regeneration has been hampered by the strictly limited suitable duration of immature embryos for culture. In contrast, mature zygotic embryos harvested from dry seeds are ubiquitous. However, generally mature embryos and especially tropical maize genotypes have been considered as the most recalcitrant for tissue culture work. Consequently tropical maize regeneration from mature embryos has not been reported so far. Here, we report successful regeneration of one inbred and one open pollinated tropical maize line from mature zygotic embryos using split seed technique. The maximum average callus induction recorded using LS basal salts and B5 vitamins supplemented with 3 mg l^{-1} 2,4-D alone was 90% and 52.5% when same level of 2,4-D was combined with Kinetin. A maximum of 75.6% Type II and 62.3% Type I callus was produced after maintaining calli on media composed of LS basal salts and B5 vitamins supplemented with 2 mg l^{-1} of 2,4-D. The frequency of regenerable calli induced was 21.14% for CIMMYT maize line 216 and 16.51% for Katumani. The number of shoots regenerated per callus induced on single split seed ranged from 1-5.

Key words: Maize tissue culture, scutellum, plumule, organogenic callus, embryogenic callus.

INTRODUCTION

Maize is among the major food crops of Africa and, therefore, its improvement has been a major priority to witness food self sufficient Africa. However, according to FAO reports (FAO, 2007) only six countries produced twice the amount they consumed, while eight countries imported 5 - 35% and eleven others 57 - 100% of their maize consumption in the year 2005. Hence, it is time to embrace fast, effective and reliable techniques, like biotechnology, in maize improvement program to ensure sufficient production (Machuka, 2001; Pingali and Pandy, 2001). One such technique is genetic transformation. The success of any transformation work depends on culture duration (to avoid too many deleterious effects from somaclonal variation) and efficiency of the regeneration system utilized.

So far almost all maize tissue culture and transformation involves the use of immature zygotic embryos as an explant source for regeneration (Armstrong and Green, 1985; Carvalho et al., 1997; Danson et al., 2006; Duncan et al., 1985; El-Itriby et al., 2003; Pareddy and Petolino, 1990; Shohael et al., 2003). However, immature embryos are seasonally available and have strictly limited suitable duration of culture, 14-19 DAP (Odour et al., 2006). This imposes tedious routine tissue culture activities within the specified time frame and continuous planting for continuous supply of the immature embryos. In contrast, mature embryos are readily available throughout the year in large quantities. Furthermore, despite few reports about the recalcitrance of tropical maize lines and mature embryos for tissue culture work (Bohorova et al., 1995; Hodges et al., 1986), successful regeneration of temperate maize lines and other cereal food crops from mature embryos has been reported by different authors (Akula et al., 1999; Green and Phillips,

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Abbreviations: 2,4-D, 2,4-dichloroaceticacid; BAP, benzyl aminopurine; DAP, days after pollination; KT, kinetin; KAT, Katumani and CML 216, CIMMYT maize line 216.

1974; Ozgen et al., 1998; Rueb et al., 1994; Wang, 1987; Ward and Jordan, 2001). Green et al. (1974) first reported that mature embryos of maize could be used to induce callus but no plantlets were regenerated. Wang (1987) successfully regenerated plants from mature embryos of two maize inbreeds, B73 and Mo17, but the regeneration was genotype dependent and the frequency was only 4 to 5%. Huang and Wei (2004) reported regeneration of temperate maize lines from mature embryos at a frequency ranging from 19.85 to 32.4%. Most recently Al-Abed et al. (2006) reported more efficient regeneration system for two hybrid and two inbred temperate maize lines using split mature seeds as an explant. Here, we report regeneration of one tropical maize inbred line and one open pollinated variety from mature embryos for the first time using split seed technique.

MATERIALS AND METHOD

Plant materials

One tropical open pollinated variety, namely Katumani and one tropical inbred line, namely CML 216 were used in this study. KAT dry seeds obtained from Kenya Seed Co. Ltd (Kitale, Kenya) and CML 216 seeds from Kenya Agricultural Research Institute (Nairobi, Kenya) were bulked in the Plant Transformation Laboratory screen house of Kenyatta University. Pollination was controlled by covering sprouting ears with transparent silk bags measuring 8”X4”.

Media

Media A: LS salts, B5 vitamins, 900 mg l^{-1} proline, 250 mg l^{-1} casein hydrolysate and 10 mg l^{-1} of filter sterilized $AgNO_3$ 30 g l^{-1} of sucrose and 8 g l^{-1} of agar.
Media B: MS salts and vitamins, 900 mg l^{-1} proline, 250 mg l^{-1} casein hydrolysate and 10 mg l^{-1} of filter sterilized $AgNO_3$ 30 g l^{-1} of sucrose and 8 g l^{-1} of agar.

Seed sterilization

Glumes surrounding the pedicle of a dry seed were removed with maximum precaution not to damage or expose embryos to the bleaching agent. Seeds were then washed with local liquid detergent and rinsed three to four times under running water. The washed seeds were immersed in 70% ethanol solution for 3 min and rinsed with distilled water three to four times. Seeds were then soaked in 85% commercial bleach Jik (Reclkit and Colman, Kenya) or 3% sodium hypochlorite solution twice for 15 min, each time using fresh solution followed by rinsing seeds three to four times in sterile distilled water (dH_2O) to completely remove remnants of Jik. Then seeds were subjected to one of the following soaking schemes; A) soaking in sterile dH_2O overnight, B) soaking in 28% commercial bleach overnight, C) soaking in 28% commercial bleach for 6 h followed by soaking in sterile dH_2O for 18 h, D) soaking in 28% commercial bleach for 2 - 3 h followed by soaking in sterile dH_2O for 21 - 22 h and E) soaking in sterile dH_2O overnight followed sterilization for 20 minutes in 28% commercial bleach. Seeds with pedicle surrounded by glumes were also sterilized and soaked in sterile dH_2O overnight. Media, water and culture bottles used in the experiment were steam sterilized at 121ºC at a pressure of 15 pounds per square inch (psi) for 21 min. pH of the media was adjusted to 5.8 using HCl/NaOH prior to sterilization.

Soaking and germination

Seeds were soaked in soaking media consisting liquid LS media supplemented with 3 mg l^{-1} of 2,4-D for 48 h. Semi solid MS media supplemented with 2 mg l^{-1} of 2,4-D was used for seed germination.

Callus induction

After 3 - 5 days germinated seeds were split longitudinally to expose shoot meristem, scutellum and coleorhiza simultaneously, and cultured on callus induction media with the split side facing the media. The callus induction media was media A supplemented with 0 - 6 mg l^{-1} of 2,4-D or 3 mg l^{-1} of 2,4-D combined with KT in the range of 0 - 2 mg l^{-1}. Ten split seeds were cultured per plate and incubated in the dark at 26 ± 2ºC. Two days later, growing radicle and plumule was removed to encourage callus initiation and returned back to growth room for 2 - 3 weeks of incubation. Highly proliferating calli induced using 2,4-D alone was transferred to Media A supplemented with 2 mg l^{-1} of 2,4-D alone while calli induced using 2,4-D combined with KT were transferred to media A supplemented with 2 mg l^{-1} of 2,4-D combined with 0.5 mg l^{-1} of KT. The callus was subcultured every two weeks during the 6 - 8 weeks incubation period in the dark at 26 ± 2ºC.

Regeneration

Organogenic calli were transferred to shooting media composed of MS media supplemented with 4 mg l^{-1} BAP and 2 mg l^{-1} KT and incubated at 26 ± 2ºC under 16/8 photoperiod. Embryogenic calli were, however, transferred to Media A supplemented with additional 30 g l^{-1} of sucrose for embryo maturation and incubated at 26 ± 2ºC in the dark. After maturation, embryos were incubated on MS media devoid of hormones at 26 ± 2ºC under 16/8 photoperiod.

Acclimatization

When plantlets developed two to three leaves, they were transplanted to plastic pot filled with moist pit moss (Kekkilä Oyj, Tussula, Finland). Plants were sprayed with water and covered with polyethylene paper bags to raise the relative humidity and watered regularly in the glass house. After two weeks, acclimatized plants were transferred to buckets filled with loam soil mixed with sand and Phytomix (Kenya Seed Co. Ltd, Kitale, Kenya) in 2:1 ratio and taken back to the glass house.

Data analysis

Ten explants (split seeds) were cultured per petridish and each experiment was replicated five times in completely randomize design (CRD). Analysis of variance (ANOVA) was carried out for callus induction and Type I and II callus production using GenStat software (http://discovery.genstat.co.uk.) at 95% confidence interval. Standard error was calculated using Microsoft Office Excel 2003 (Microsoft Corporation, USA).

RESULTS AND DISCUSSIONS

When mature dry seeds obtained from plants grown under field condition were sterilized following a technique outlined by Sairam et al. (2003), 95.4% germination and 81.58% contamination was recorded. However, when glumes surrounding the pedicle were removed prior to

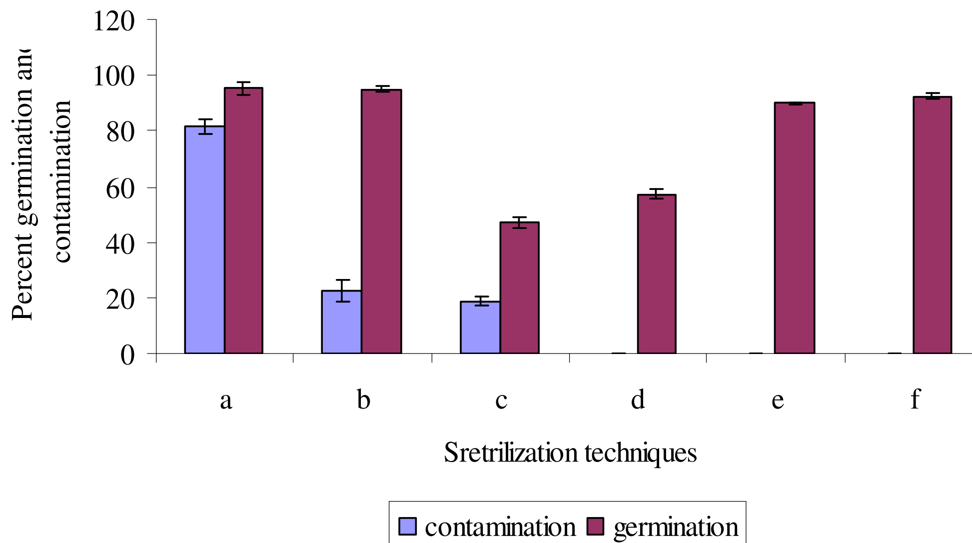

Figure 1. Sterilization of mature seeds: a) Soaking sterilized seeds in sterile dH_2O overnight without removing the glumes. b) Soaking sterilized seeds in sterile dH_2O overnight after removing the glumes. c) Sterilization of seeds in 2%NaHClO for 20 min after initial sterilization and overnight soaking in sterile dH_2O. d) Soaking sterilized seeds in 1%NaHClO overnight. e) Soaking sterilized seeds in 1%NaHClO for 6 h. f) Soaking sterilized seeds in 1%NaHClO for 2 - 3 h.

washing followed by sterilization using the same technique, contamination was brought down to 22.6%. The most probable reason for this could be the improved access to bleach upon removal of glumes. Soaking sterilized seeds in 1%NaOCl or 28% commercial bleach overnight completely controlled contamination but reduced germination to 57.3%. The most probable reason for the reduction of germination could be the prolonged exposure of embryos to bleach. In contrast, when sterilized seeds were soaked in 1%NaOCl for 2 - 3 h contamination was completely controlled and 92.6% germination was achieved. On the other hand, re-sterilization of seeds using 2% NaOCl or 57% commercial bleach for 20 min prior to soaking overnight in sterile dH_2O reduced contamination to 18.75% and germination to 46.9% (Figure 1).

The surface of the split seed started swelling after 2 - 3 days on callus induction media and visible calli appeared after 4 days for CML 216 and KAT, and 6 days for A188. White and soft callus was initiated on the scutellum and at the base of the plumule where it was attached to the scutellum. Callus induced on the surface of the scutellum was dominated by Type II (embryogenic) calli whereas callus induced at the base of the plumule was mainly Type I (organogenic) calli. On the other hand, watery and non-morphogenic callus was initiated on the surface of the split seed where the radicle was attached to the scutellum and it eventually formed roots. The highest average callus induction recorded for the inbred line CML 216 was 90% when media A supplemented with 3 mg l^{-1} of 2,4-D was used. The highest average callus induction recorded for KAT and A188 was 80 and 34.3% respectively when the same media was supplemented with 4.5 mg l^{-1} of 2,4-D. The lowest average callus induction recorded was 60% for CML 216 and KAT and 23.7% for A188 when same media was supplemented with 1.5 mg l^{-1} of 2,4-D (Figure 2A). The ANOVA test showed significant callus induction difference ($p \leq 0.021$) among the different levels of 2,4-D tested. However, the average callus induction difference among the lines tested was non-significant ($p \leq 0.926$).

When 3 mg l^{-1} of 2,4-D was combined with 0.5 mg l^{-1} KT the highest average callus induction recorded for CML 216 was 52.5%. KAT yielded the same 52.5% callus induction when 3 mg l^{-1} of 2, 4-D was combined with 1.0 mg l^{-1} of KT. However, A188 gave a maximum of 34.3% average callus induction when 3 mg l^{-1} of 2,4-D was combined with 1.5 mg l^{-1} of KT. Generally, as the amount of KT level increased, the amount of callus production per split seed declined and the amount of splits showing necrotic tissue increased. Among the three lines, A188 was most affected by tissue necrosis. The lowest callus induction frequency attained was 13.3% when 2.0 mg l^{-1} KT was combined with 3 mg l^{-1} of 2,4-D (Figure 2B). The ANOVA test for average callus induction showed a highly significant difference ($p \leq 0.001$) among the different levels of KT combined with 3 mg l^{-1} of 2,4-D. However, the difference among the three varieties tested was found to be insignificant ($p \leq 0.565$).

The scutellum of maize inbred line CML 216 detaches itself from the endosperm after about a week and produced white to pale yellow and translucent embryogenic calli. When this calli were subcultured on callus maintenance media, after 3 - 4 weeks, two distinct types of calli

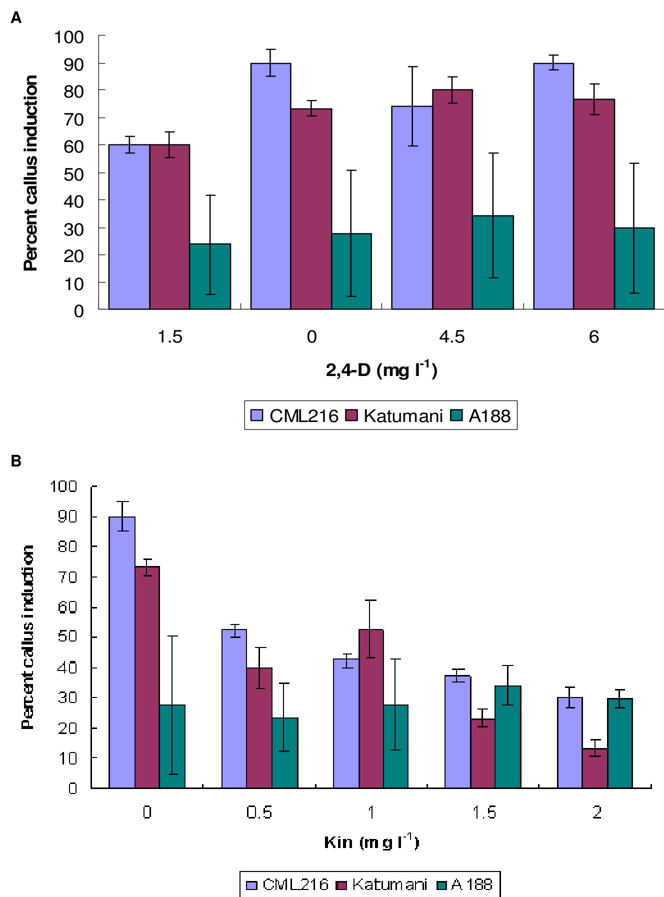

Figure 2. Effect of 2,4-D and its combination with kinetin on callus induction from mature seeds.
Error bars = Mean ± SE.

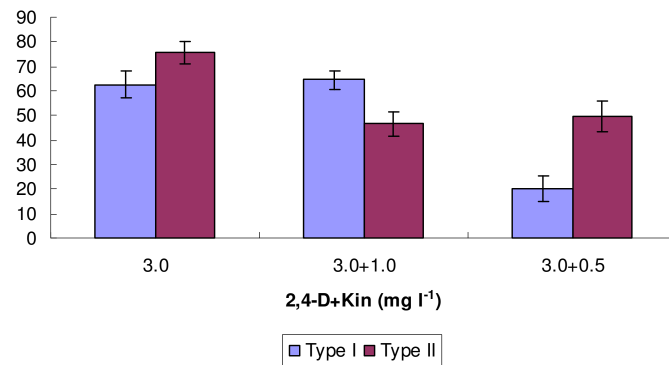

Figure 3. Type I and II callus induction from mature maize zygotic embryos: a) 3 mg l^{-1} 2,4-D. b) 3 mgl^{-1} 2,4-D and 1.0 mg l^{-1}3 mgl^{-1}. c) 2,4-D and 0.5 mg l^{-1}. Error bars = Mean ± SE.

were produced. Type II calli was induced on the surface of scutellum with three distinct transient stages of embryogenesis (Figure 4 F, G and H). Creamy yellowish Type I callus was, however, mainly induced at the base of the plumule where it was attached to the scutellum (Figure 4). The highest average Type II callus induction obtained was 75.6% when callus induced using Media A supplemented with 3 mg l^{-1} of 2,4-D was transferred to Media A supplemented with 2 mg l^{-1} of 2,4-D, whereas, the lowest average Type II callus induction was 20.1%. The highest average Type I callus induction obtained was 62.3% when calli induced on Media A supplemented with 3 mg l^{-1} of 2,4-D combined with 1.0 mg l^{-1} of KT was transferred to Media A supplemented with 2 mg l^{-1} of 2,4-D and 1.0 mg l^{-1} of KT. Whereas, the lowest average Type I callus induction was 46.7% (Figure 3). Highly significant difference ($p < 0.004$) was obtained among the three kinds of media selected for Type I callus induction. However, there was no significant difference ($p < 0.212$) among the three kinds of media selected for Type II callus induction. Generally calli induced and subcultured on media containing KT produced more organogenic calli than embryogenic calli as compared to media supplemented with 2,4-D alone. Higher frequency of necrosis and low average callus induction were also observed on media supplemented with KT. Overall, most split seeds produced both types of calli simultaneously. KAT, CML216 and A188 did not show significant differences ($p<0.212$) using either 2,4-D alone or in combination with KT for Type II callus production. Increasing the level of 2,4-D beyond 3 mg l^{-1} did not affect the frequency of callus induction or increased the amount of calli produced.

All plants regenerated in this study were obtained from organogenic calli. After incubating the organogenic calli on shooting media at 26 ± 2ºC under 16/8 photoperiod for a week multiple shoots started to appear. However, due to slow *in vitro* shoot development 4 - 6 weeks were required to transfer the shoots to rooting media. A total of 99 CML216 and 79 KAT organogenic calli were transferred to shooting media and 21 CML 216 and 13 KAT calli regenerated plantlets (Figure 4). Regenerated shoots were transferred to hormone free rooting media where roots started to appear within a week (Figure 4). The regeneration frequency was 21.2 and 16.5% for CML216 and KAT respectively. Number of multiple shoots developed per callus ranged 1 - 5 for both CML216 and KAT (Table 1).

Auxins, especially 2,4-D in the range of 1 - 3 mg l^{-1} are essential for the formation of embryogenic callus from cereal embryos (Bi et al.*,* 2007; Bhaskaran and Smith, 1990; Danson et al., 2006; El-Itriby et al., 2003; Oduor et al.*,* 2006). However, Al-Abed et al. (2006) and Huang and Wei (2004) used 3 and 4 mg l^{-1} of 2,4-D respectively to induce regenerable callus from mature embryos of maize. In agreement with this findings, 3 - 4 mg l^{-1} of 2,4-D was found to be the optimal concentration for regenerable callus induction from mature embryos of tropical maize lines. A combination of cytokinins at a lower concentration with auxins, especially 2,4-D has also been used by different authors to induce embryogenic calli from maize and other cereals crops (Al-Abed et al.*,* 2006; Chang et al., 2003; Huang and Wei, 2004). In consistent

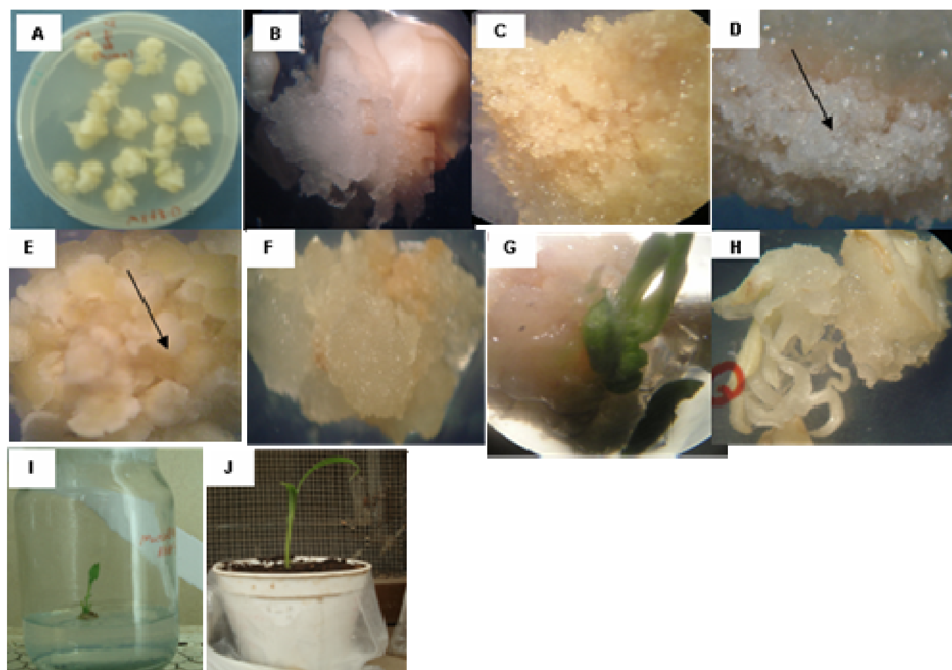

Figure 4. Plant regeneration from mature embryos of tropical maize lines. A&B) Callus induced on split seeds. C) Embryogenic callus induced. D) Globular embryos induced on maintenance media. E) Embryos on maturation media. F) Organogenic callus induced. G) shoots induced on from organogenic callus. H) multiple shoots induced on Katumani organogenic calli. I) Shoots on elongation media. J) acclimatization.

Table 1. Number of regenerated calli and number of shoots per callus.

Line	Number of split seeds cultured	Number of regenerated calli	Regeneration frequency (%)	Number of shoots per callus
CML 216	99	21	21.2	1-5
Katumani	79	13	16.5	1-5

with this findings, we report non significant difference for Type II callus induction by using either 2,4-D alone or in combination with lower levels of KT. However, the amount of Type II callus induced differed owing to the significantly higher amount of calli induced using 2,4-D alone.

In common with Al-Abed et al*.,* 2006 and Huang and Wei, 2004, we report induction of embryogenic calli on the surface of the scutellum. Similarly, Vasil et al. (1985) reported that zones of meristematic cells initiation were observed from the scutellum of maize immature embryos. However, all plants regenerated in this study were obtained from organogenic calli induced at the base of plumule where it is attached to the scutellum. This result was inconsistent with Delporte et al. (2001) who suggested that the origin of regenerable calli from mature embryos of wheat was epicotyl and mesocotyl regions of the embryo. Similarly, Danson et al*.* (2006) reported that with the exception of CYMMYT line Pool A3-6 and msv line CMB5 with embryogenic response of 12 and 8% respectively, none of the remaining tropical maize lines advanced beyond the 45 days subculture steps for the formation of the Type II callus. Initial tissue culture works on elite tropical maize lines CML72, CML216, CML323, and CML327 also produced only Type I callus (Bohorova et al*.,* 1995). The most probable reason for this could be the role of genetic background in the formation of the Type II callus (Armstrong and Green, 1985). Maturity level of embryos harvested from dry seeds could also play a major role for lack of regeneration through somatic embryogenesis. Similar response of low callus induction ability and regeneration was experienced for immature embryos harvested 21 DAP (Oduor et al., 2006).

The 16.5 and 21.2% regeneration frequency and 1 - 5 shoots per callus reported in this study is low as compared o previous reports. However, many other workers also reported lower regeneration frequency for tropical maize genotypes. Danson et al. (2006) reported signifi-

cant reduction in regeneration frequency of breeder preferred mid-altitude maize lines to that of highland adapted genotypes. Oduor et al. (2006) also reported a reduced regeneration frequency for tropical maize lines as compared to temperate lines from immature embryos. This low response is likely due to the recalcitrance nature of tropical maize genotypes to tissue culture conditions developed using temperate lines. However, considering the year round availability and abundance, the use of mature embryos as an alternative explant source can be useful in tropical maize tissue culture and transformation studies. Moreover, this is the first report regarding regeneration of tropical maize lines from mature embryos through a pathway that involves callus induction.

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