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Full Length Research Paper

Regeneration of Kenyan Maize Inbred Lines and Their Single Cross Hybrids from Immature Embryos via Somatic Embryogenesis

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Field grown, self pollinated maize genotypes were planted in KARI (Kiboko and Kabete) research stations between January 2004 and May 2005. Immature maize embryos from twelve parental inbred lines and their respective single cross hybrids were evaluated for their ability form callus, somatic embryos and subsequent regeneration into plants. The embryos were excised from surface sterilized kernels harvested at different physiological stages, namely 10 - 24 days after pollination (DAP). They were used as explants to initiate callus on solid N6 basal media with varying level of 2,4-D (0 - 20 mg L^{-1}) and regenerated on hormone free MS media. Optimal induction of primary callus at 2 mg L^{-1} averaged 83% and 67 in hybrids and inbred lines respectively. Somatic embryo competence was demonstrated in 6 inbreeds and 4 hybrids. However, plant regeneration was only achieved in 4 inbreeds and 3 hybrids. 90% percent of regenerants were normal and fertile. The successful regeneration of some of the inbred lines and/or hybrids provides a basis for development of genetic transformation using *Agrobacterium tumefaciens* to improve priority traits such as enhanced insects/pest and drought tolerance.

Key words: Inbred lines and hybrids, immature embryos, *in vitro* plant regeneration, recalcitrancy.

INTRODUCTION

Maize (*Zea mays* L.*)* is the third most planted cereal crop after wheat and rice worldwide. It is staple food in Kenya grown by 95% of the rural population who cultivate10 hectares or less for subsistence as well as for sale (Ayaga, 2003). The production of this crop is on the decrease due to increased population, limited land, environmental and biotic stresses. Over the years, conventional breeding has been used as a tool to overcome these constraints. This has resulted in deve-lopment of modest increments in yields and agronomic characteristics such as disease resistance and drought adaptability to different agroecological zones, as well as output traits such as enhanced levels of macro and micro nutrients (Machuka, 2004). For recalcitrant traits for which improvement through classical breeding holds little promise, molecular breeding methods involving marker assisted selection and genetic transformation now provide viable alternatives in several crops, including maize (Bruce et al., 2002; Frame et al., 2002). However, the pre-requisite for crop genetic transformation is existence of a reliable plant regeneration system.

In cereals such as wheat, barley and maize, immature immature embryos have been the favourite explant for *in vitro* culture and plant regeneration (Armstrong and Green, 1985; Green and Phillips, 1975; Ray and Ghosh, 1990). Except for a few studies (Bohorova et al., 1995; El-Itriby et al., 2003), Most studies of regeneration have utilized genotypes adapted to temperate zones, with scarce or no attention and resources focused on assessing the regeneration potential of maize germplasm adapted to the eastern African region. The objective of the study reported here was to evaluate

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Abbreviations: TH, hybrid; TL, inbredline; DAP, days after pollination; 2,4-D dichlorophenoxyacetic acid.

the tissue culture responses of selected inbred lines popularly used in the Kenyan maize breeding program for the purposes of genetically manipulating them to express phenotypes of agronomic benefits such as disease, pest/insects resistance as well as drought tole-rance among others using *A. tumefaciens* and biolostic gene delivery systems. The effect of parental genotype on regeneration potential of derived hybrids was also investigated.

MATERIALS AND METHOD

Plant materials

Field grown, self pollinated maize genotypes were planted in KARI - Kiboko and Kabete research stations between January 2004 and May 2005. Twelve inbred lines namely; TL08, TL09, TL18, TL 19, TL 20, TL21, TL22, TL23, TL 24, TL25 TL26, and TL27 were used to generate six single cross hybrids ;TH21, TH23, TH24,TH 25, TH26 and TH27. Where TL and TH designates inbredline and hybrid respectively. The choice of maize lines was based on their good agronomic traits such as high yields, drought tolerance, and resistance to maize streak virus, *turcicum* leaf blight resistance and *Striga* weed. The inbred lines were identified as parents of major hybrids in commercial production or currently used in breeding programs in Kenya. They were planted after every three weeks under overhead irrigation at drip irrigation system at Kiboko and Kabete respectively. They all flowered at different times with early maturity being observed at Kiboko and late at Kabete. The two sites were established to enable the availability of embryos throughtout the experimental period.

Ears were harvested at different physiological stages, namely 10, 15, 18, 21 and 24 days after pollination (DAP) and utilized immediately for culture initiation or they were stored at +4^{o}C for up to 3 days for later use.

Callus induction and maintenance

The induction media medium contained N6 salts and vitamins according to Chu et al. (1975). Supplem ented with 25 mM proline, 0.2 g L^{-1} Casein hydrolysate, 1.0 g L^{-1} glycine and 0.3% gelrite (w/v). Various levels of 2, 4-D (0.0-, 20.0 mg L^{-1}) were added to determine the effect of this phytohormone on callus induction efficienty. The ears were s urfac ed sterilized acc ording to Frame et al. (2002). Twenty immature embryos were aseptically excised and plated solid callus induction media with scutellar side in contact with media and incubated for 2 - 3 weeks under conditions described by Bohovora et al. (1999). The experiment was replicated three times in completely randomized block design. The calluses induced were then sub cultured onto maintenance media which had the same composition as callus induction medium for a further 2 – 3 wks. Data on callus induced at different levels of 2,4-D in the media was recorded as well as the number of callus formed per genotype at different physiological ages in all the experiments. Types of calli formed by each genotype were noted at this level.

Somatic embryo formation

Calli were transferred to maturation medium which was essen-tially the same as the callus induction media except that it was devoid of 2,4-D and had 60 g L^{-1} of sucrose. The cultures were transferred onto fresh maturation medium biweekly. After 4 weeks incu-bation on maturation medium at 27°C in the darkness, the embryogenic competence for each genotype was evaluated. The callus that converted to somatic embryos was recorded in each genotype.

Plant regeneration and acclimatization

Embryogenic calli were transferred onto 4.43 g/L MS-based (Murashige and Skoog MS (1962) regeneration medium supple-mented with 30 g L^{-1} sucrose without any growth regulators as described by Frame et al. (2002). Regenerating cultures were incubated at 16 h of light and 8 h of darkness. After 2 - 3 weeks, the number of embryos converting to plantlets out of the total embryos plated on induction medium determined the percentage of plantlets regenerated. Regenerated shoots were transferred to rooting medium which was half strength 8 – 10 cm tall; plantlets with healthy roots were transferred into pots containing mixture of 3:1 vermi-culate and sterile soil, and grown in humid condition for 2 weeks to acclimatize. They were further transferred to bigger of 5 L pots in green house grown to maturity and seed was harvested. Gemina-tion viability of R_1 seed was tested by planting five seed of each genotype per pot and the number of seed germinated scored as percentage viability.

Statistical analysis

Statistical analysis was carried out using ANOVA (SPSS, 1990). Parameters analyzed included callus induction efficiency, somatic embryo formation and plants regeneration expressed as percen-tages and R_1 seed germination viability. Means were separated using Student Newman-Keuls Test (SNK).

RESULTS

Effect of embryo physiological age on callus induction

Callus was induced from embryos excised from ears at 10, 15, 18, 21 and 24 DAP. Embryo size contributed very significantly to differences in callus induction among inbred lines ($p < 0.0002$) and their hybrids ($p < 0.0005$). A large decrease in the frequency of callus induction was seen when immature embryos from all genotypes were harvested at 10 and 24 DAP. Among the hybrids callus induction was optimal at 15 DAP when the embryos were 1.0-1.3 mm long (Figure 2). Among inbred lines, the optimal stage was 18 - 21 DAP when embryos were 1.5 - 2.0mm long (Figure 3). At 10 DAP, calli initiation was slow, and the induced calli often turned soft, loose, browned and gradually necrosed and died. Older embryo harvested at 24 DAP germinated precociously. Callus induction from single cross hybrids was higher than the parental inbredline combination. Moreover, it was observed that the hybrids were able to induce callus 5 – 6 days and inbreds 14 – 21 days upon incubation in callus induction media.

Effects of 2, 4-D levels

Different levels of 2, 4-D were evaluated to study the effect of this growth regulator on callus induction. There was no callus induction in medium devoid of 2, 4-D. Instead, embryos readily germinated to form shoots and roots after 4 – 6 days on induction medium. However

Table 1. Percentage callus induction efficiency for both parental and hybrid lines. Means followed by the same letter are not significantly different according to SNK test at ($p < 0.05$). Bold type depicts hybrids.

Maize genotype	Callus induction (%)
TL08*(p)*	77.40 ± 3.70b
TL09*(p)*	77.80 ± 3.92b
TH21 *(h)*	**77.7 ± 5.2b**
TL18	82.40 ± 3.86a
TL19	69.87 ± 7.26b
TH23	**88.8 ± 3.3a**
TL20	67.00 ± 7.03 bc
TL21	54.73 ± 7.93d
TH24	**77.5 ± 5.0 b**
TL22	55.87 ± 5.17cd
TL23	66.60 ± 5.51bc
TH25	**90.7 ± 4.0 a**
TL23	66.60 ± 5.51bc
TL24	50.13 ± 5.27d
TH26	**77.7 ± 4.2b**
TL26	81.87 ± 4.52a
TL27	57.33 ± 5.77cd
TH27	**85.9 ± 5.2a**

Table 2. Effect of different levels of 2, 4-D (0. 0 - 20.0 mg/L) on % callus induction efficiency on 7 selected maize genotypes. Numbers with the same letter are not significantly different ($p < 0.05$).

2,4-D Conc. mg/L	Mean for % mean callus induction
0.0	0.000 f
0.1	16.67 d
0.5	25.00 c
1.0	39.00 b
2.0	61.00 a
5.0	42.00 b
10.0	11.00 e
20.0	2.67 f

there was optimal callus induction in the presence of 2 mg L^{-1} of 2, 4-D in induction medium (Table 2). The calli formed were nodular and white irrespective of genotype (Figure 1,b). The frequencies of callus induction on average were 67% in inbredlines and 83% among hybrids (Table 3). Calli induced at high levels of (5 mg L^{-1}) of 2, 4-D was watery and fragile and did not mature into somatic embryo nor regenerated and. Callus induction was found to be genotype dependent (Table 1).

Formation of embryogenic calli

As for callus induction, somatic embryo formation was

Table 3. Average of callus induction between inbred lines and hybrids.

Maize genotypes	Average % callus induction efficiency
TL(Inbreds)	67.3
TH(hybrids)	83.05%

Table 4. Comparison of % means somatic embryo formation between parental inbred lines and their respective single cross hybrids. Numbers with the same letter are not significantly different ($p < 0.05$).

Parental inbred line* and its ability to form embryogenic calli (mean±SD)	Hybrid line** and its ability to form embryogenic calli (mean±SD)
TL08-E(5.0± 1.8b)	
TL09-NE(0.0)	TH21-NE(0)
TL18-E(6.3± 3.1)	
TL19-E(4.8± 1.9b)	TH23-E(13.3± 4.1ab)
TL20-NE(0.0)	
TL21-E(1.0± 0.6b)	TH24-E(7.8± 2.1b)
TL22-NE(3.3±.1.7b)	
TL23-E(9.0± 6.7b)	TH25-E(27.8± 6.5a)
TL24-NE(0.0)	
TL23-E(8.0± 3.4b)	TH26-NE(0)
TL26-NE(0.0)	
TL27-E(4.7±2.4b)	TH27-E(7.8± 1.7b)

genotype dependent. Type I calli accounted for 17.6% of embryos formed whereas type II calli accounted for 52.9% of total calli examined. The remaining 29.4% was calli that we classified as type III (Figure 3). The latter were brown non-morphogenic calli that did not proliferate further despite subsequent subcultures and was therefore discarded in all the experiments (Figure 1, i). Somatic embryo formation was genotype dependent. One or two parental inbred lines had to be embryogenic for the derived hybrid to also form embryogenic capacity (Table 4). For example the inbred lines TL22 and TL23 were embryogenic and their hybrid TH25 which displayed even greater regeneration capacity than both its parents when compared singly. The inbred line TL26 was non-embryogenic, but when crossed with the embryogenic line TL27, gave rise to an embryogenic hybrid (TH27) (Table 4). 6 inbred lines and 4 hybrids callus efficiently converted to somatic embryos (Table 4).

Plant regeneration

The capacity to regenerate plantlets was not correlated with the propensity for callus induction or somatic embryo formation since not all embryogenic calli converted to plantlets. After embryogenic calli were transferred to regeneration media, their surfaces turned green within

Figure 1. Stages in immature embryo culture, somatic embryogenesis and plant regeneration in18 locally adapted Kenyan maize genotypes used in breeding programmes. **a.** 18 DAP derived embryos plated on day one. Callusing immature zygotic embryos 21 days on callus induction media. **c.** Compact type II embryogenic callus on maturation medium. **d**. Emerging plantlets. **e**. Rooted plantlets in baby jars. **f.** Acclimatization of a plantlet in green house. **g**. Maize seed derived from regenerated maize plant. **h**. Germination viability of regenerant seeds. **i**. Poorly developed and necrosed embryogenic callus derived from TL 09 an example of Type O callus. **j**. Tassel seed, an example of somaclonal variation *in vitro* manipulation.

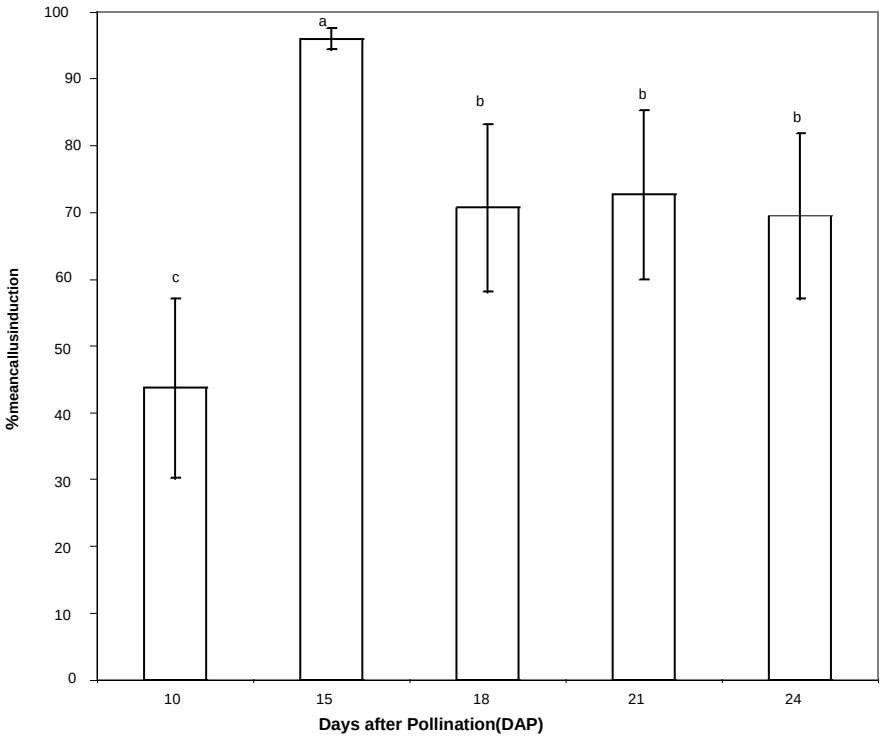

Figure 2. Effect of embryo physiological age on callus induction in six selected hybrids. Means followed by the same letter are not significantly different according to SNK test at ($p < 0.05$).

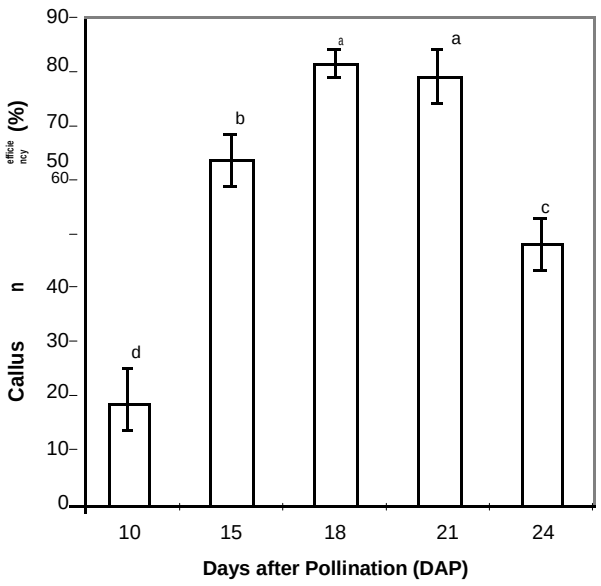

Figure 3. Effect of embryo physiological age on callus induction efficiency in eleven selected parental inbred lines. Means followed by the same letter are not significantly different according to SNK test at ($p < 0.05$).

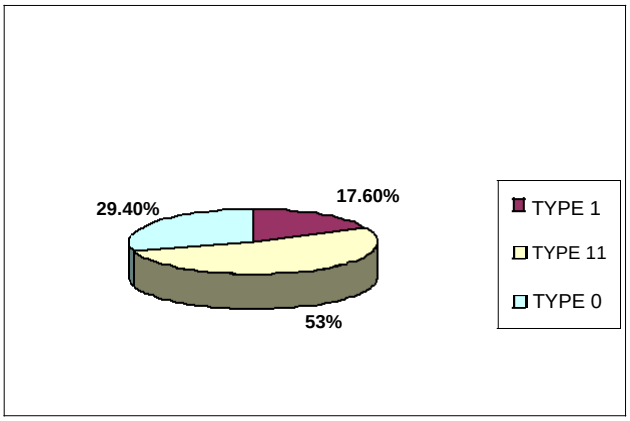

Figure 4. Types of calli generated *in vitro* by 18 maize genotypes evaluated. Type I accounted for 17.6%, Type II, 53% and 29.4% formed Type 0.

the one week and plantlet regeneration occurred within 21 days. Under the conditions described here, 90% of plantlets regenerated developed into morphologically normal and fertile, plants. However plant regeneration was only achieved in 4 inbred lines (TL18, TL19, TL23 and TL27) and 3 hybrids (TH23, TH25 and TH27). Recalcitrancy was observed in 61% of lines evaluated. Somaclonal variations were observed in a few such as tassel seed (Figure 1, j).

DISCUSSION

Molecular plant breeding is largely dependent on available *in vitro* regeneration protocols that are amenable for use in genetic transformation for improvement of the target traits. The success of regeneration procedures is affected predominantly by genotype, the type of explants material employed and media composition (Lindsay and Jones, 1989). Since the early tissue culture studies in maize were first reported by Green and Philips (1975), immature zygotic embryos have become the explant of choice in cereals (El-Itriby et al*.,* 2003; Ward and Jordan, 2001; Oduor et al., 2006). In the present study, it was established that the optimal physiological stage for callus induction among Kenyan maize genotypes was 15 - 21 DAP. Among temperate lines, the reported age of embryos is usually in the range of 8 - 13 DAP (Frame et al., 2002; Ishida et al., 1996; Lu and Vasil, 1983; Vain et al., 1989). Generally the auxin, 2, 4-D in the range of 1 – 3 mg L^{-1}, is essential for the establishment of embryogenic callus from cereal embryos (Bhaskaran and Smith, 1990). This is true for both tropical and subtropical maize genotypes. The results of this study showed that the presence 2 mg L^{-1} of 2,4-D in culture medium was critical for callus induction and embryogenic callus formation from immature embryos which concurred with the findings of Armstrong and Green, 1985; Bohorova et al., 1995; Carvalho et al., 1997). However, Oduor et al. (2006) was able was able efficiently regenerate 2 dry land and 2 highland Kenya maize varieties using 1.5 mg/L of the same plant regulator.

Genotype specificity of somatic embryogenesis and regeneration in maize has been reported previously (Hodges et al*.*, 1986; Tomes and Smith, 1985; Willman et al., 1989). The present studies has confirmed that callus induction, somatic embryo formation and plant regeneration was found to be genetically linked and hybrids showed better tissue culture response compared with their inbred lines counterparts. Recently, Jedidah et al. (2006) esta-blished that of 22 maize evaluated, majority initiated type
II calli and only 2 highland adapted lines regenerated into plantlets.

Conclusion

Since the hybrid embryos had greater regeneration capacities than their parental inbredline counterparts, they are likely to be the better choice for genetic transformation that utilizes the inbred lines reported here.

The regeneration of these genotypes sets the basis for genetic transformation of adapted Kenyan maize genotypes to compliment conventional breeding efforts. We are currently expanding the spectrum of inbred lines under tissue culture investigation to include many other elite lines and genotypes (including popular open pollinated varieties -OPV) used in breeding programs. We have also started to investigate the transformation capabilities of these adapted inbred lines and OPVs using *A. tumefaciens*.

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Collaborating Institution

Kenyatta University, Department of Biochemistry and Biotechnology.

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