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In vitro Regeneration of Dryland Kenyan Maize Genotypes Through Somatic Embryogenesis

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Abstract: In this study, we report successful regeneration of Kenyan white maize using immature embryos derived from three selected Kenyan dryland synthetic maize hybrids; Dryland Hybrid (DH) 02, Pwani Hybrid (PH) 01 and PH04 and an open pollinated variety-OPV (Katumani, KAT). The embryos were cultured in N6 medium supplemented with proline, casein hydrolysate, silver nitrate and 2% sucrose containing different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) and incubated in total darkness at 28±2°C to initiate callus. Mature somatic embryos were sub-cultured in shoot promotion medium consisting of hormone free Murashige and Skoog (MS) media supplemented with 2 mg L^{-1} glycine and 2% sucrose. Root formation was induced by sub-culturing the shoots on half strength MS medium supplemented with 2% sucrose and 0.1 mg L^{-1} of Naphthalene Acetic Acid (NAA). Regenerated plantlets were successfully acclimatized in soil and raised to maturity. Fertile progeny were also recovered.

Key words: *In vitro* regeneration, white maize, somatic embryogenesis

INTRODUCTION

Maize (*Zea mays* L.) is the number one food staple in Eastern and Southern Africa and an important crop globally for other uses, e.g., as a source of income, animal feeds, manure and for industrial uses. Kenya has an average per capita maize consumption of 103 kg $years^{-1}$ of which 91% is used for food (Pingali and Panday, 2001). Maize yield and production in Kenya is 1.7 tons ha^{-1} and 2.7 million tons, respectively and is in the decline due to both biotic and abiotic constraints faced by the crop. Constraints to maize production include pests (e.g., stalk borers, weevils, larger grain borers), diseases (e.g., white leaf blight, maize streak virus), weeds (e.g., *Striga* spp.), drought and low nitrogen concentration (Ajanga and Hillocks, 2000; Kanampiu *et al*., 2002; Schechert *et al*., 1999). Traditional breeding had limited success to overcome these constraints hence the need to use biotechnological approaches (genetic transformation, somaclonal variation) to overcome the constraints.

Efficient *in vitro* regeneration of normal and fertile plants from single cells, tissues and organs is a basic prerequisite for the production of genetically transformed plants. Immature embryos have been the most widely used explant in many cereals, including maize (Green and Phillips, 1975; Armstrong and Green, 1985; Shillito *et al*., 1989). Successful plant regeneration has also been reported from calli initiated from anthers (Ting *et al*., 1981), glumes (Suprasanna *et al*., 1986), immature inflorescences (Pareddy and Petolino, 1990), immature tassels (Songstad *et al*., 1992), leaf segments (Ray and Ghosh, 1990), seedling segments (Santos *et al*., 1984), shoot tips (O'Connor-Sanchez *et al*., 2002), shoot apical meristems (Zhang *et al*., 2002) and mature embryos (Huang and Wei, 2004). The production of compact embryogenic callus arises at low frequency and only for specific genotypes (Armstrong and Green, 1985). Regeneration ability is also influenced, to a great extent, by the media composition (Armstrong and Green, 1985; Songstad *et al*., 1992). To date, most regeneration studies have focused on temperate, yellow endosperm maize and only few have been reported for white maize (Bohorova *et al*., 1995; El-Itriby *et al*., 2003).

In Kenya, over 75% of the total landmass belongs to the arid and semi arid agroecology and is unsuitable for rain fed agriculture. For this reason, our laboratory has initiated a project to engineer drought stress tolerance in Kenyan maize through genetic engineering. As the initial step towards transformation, this study was undertaken to develop a reproducible regeneration protocol(s) for three popular Kenyan dryland maize synthetic hybrids and one OPV through somatic embryogenesis.

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MATERIALS AND METHODS

Seeds from three popular dryland synthetic maize hybrids [Dryland Hybrid 02 (DH02), Pwani hybrid 01 (PH01) and PH04] and one Open Pollinated Variety [(OPV), Katumani, KAT] were obtained from Kenya Seed Co. Ltd (Kitale, Kenya) and grown in the Botany farm of Kenyatta University between January 2004 and December 2004. Pollination was controlled by covering the sprouting ears with transparent silk bags measuring 8×4 inches. Ears were collected at different Days After Pollination (DAP) for subsequent tissue culture experiments according to Register *et al.* (1994). Ears 17-21 DAP with embryo sizes ranging between 1-3 mm in length were harvested and used immediately or held in a refrigerator for 1-2 days at 4°C while still in the husk. Dehusked ears were surface sterilized for 3 min in 70% alcohol followed by soaking in 2.5% sodium hypochlorite plus one drop of wetting agent (Tween 20) for 18 min, then washed 3 times with sterile distilled water under aseptic conditions. Following sterilization, procedures for callus induction and maintenance were modified from Frame *et al.* (2002). Excised embryos were cultured on the callus Initiation Medium (IM) with the flat (embryo axis) side down in contact with the medium. The IM was composed of N6 medium (N6 salts and vitamins) (Chu *et al.*, 1975) supplemented with 20 g L^{-1} sucrose, proline (2.9 g L^{-1}), 100 mg L^{-1} casein hydrolysate, 10 mg L^{-1} silver nitrate, 2 mg L^{-1} glycine and 2,4-dichlorophynoxyacetic acid (2,4-D) in the range of 0-12 mg L^{-1}. The pH of the medium was adjusted to 5.8 with either 1M NaOH or 1M HCl. Gelrite (3 g L^{-1}) was added as a solidifying agent before autoclaving the media at 121°C at a pressure of 15 pounds per square inch (psi) for 25 min. The calli were incubated at 26±2°C in the dark. Callus was transferred to fresh Maintenance Medium (MM) at 2-3 week intervals depending on growth rate. The MM was composed of N6 medium containing 2 mg L^{-1} 2,4-D but devoid of silver nitrate. However, other conditions and supplements were similar to those used in IM. Friable type II calli (Green and Phillips, 1975; Armstrong and Green, 1985) were transferred to somatic embryo maturation medium composed of N6 medium supplemented with sucrose (60 g L^{-1}), 1 mg L^{-1} α-napthaleneacetic acid (NAA), glycine (2 mg L^{-1}), gelrite (3 g L^{-1}). Cultures were incubated at 26±2°C in the dark for 10-20 days depending on the genotype.

For plant regeneration, calli excised at suitable sizes (5×5×5) mm were transferred to MS medium (Murashige and Skoog, 1962) (MS salts and vitamins) supplemented with sucrose (20 g L^{-1}), glycine (2 mg L^{-1}), gelrite (3 g L^{-1}) and Benzyl Amino Purine (BAP) ranging between 0 -4.0 mg L^{-1} and transferred to fresh medium after every 20 days. After shoots had developed from calli, shoot tips were excised and cultured on half strength MS medium supplemented with sucrose (20 mg L^{-1}), glycine (2 mg L^{-1}) and NAA ranging between 0-1.0 mg L^{-1} for 14 days. Regenerated plantlets were acclimatized by transplanting into pots containing sterilized vermiculite and sand in the ratio of 2: 1, respectively. The pots were covered with polythene paper for the first 3 days and later transplanted into pots containing loam soil mixed with sand and Phytomix™ (Kenya Seed Co. Ltd) after 4 days. Differences in responses to different parameters were tested using single factor analysis of variance (ANOVA). The analysis was done using SPSS software (version 11.5) with associated Least Significant Differences (LSD) function at 95% level of confidence. Experiments were designed in completely randomized design with each experiment repeated at least thrice.

RESULTS AND DISCUSSION

When immature embryos are used as explant sources, it is not only important that plants be healthy and robust, but that flowering time be fully characterized in order to obtain reproducible and competent embryos for tissue culture. In this regard, KAT was the first to flower and set seed, followed by DH02, PH01 and PH04 in that order (Table 1). This evaluation was also necessary for reasons of synchrony in tassel and ear formation to allow self- or cross-pollination.

Calli were initiated from immature maize embryos at different DAP (Table 2). Overall, there was no significant difference among the maize genotypes tested in their callus formation using embryos at 17, 18, 19 and 20 DAP except at 21 DAP (ANOVA, $p = 0.05$). KAT and PH01 consistently exhibited slightly higher responses than DH02 and PH04. Increase in callus weight was also faster in KAT and PH01 (Table 3). Embryos at 21 days post pollination germinated and showed reduced calli formation when cultured in the same medium. The best results were obtained from embryos within a size range of 1-2 mm long. At this stage, the scutellum had turned opaque and measured approximately twice the length of the embryonic axis. Visible callus appeared within 3-5 days of culture in callus initiation medium in all the maize genotypes. Compact, opaque, white to pale yellow embryogenic calli formed within 2 weeks (Fig. 1A and B). Tissue culture of maize immature embryos typically produces two different types of embryogenic callus. Type I callus is a compact, organized and slow-growing callus. Type II callus is soft, friable and fast-growing and is the

Table 1: Key developmental features and attributes of maize varieties used in tissue culture

Variety name	Year of release	Type	Altitude (meters)	Flowering period* (days after planting)	Maturity (months)	Special attributes
KAT	1987	OPV	900-1350	42-49	3-4	Early maturing/drought avoidance
DH02	1995	TC	900-1500	47-55	3-4	Early maturing/drought avoidance
PH01	1989	VC	1-1200	57-65	3- 4.5	Moderate drought tolerance
PH04	1995	VC	1-1200	67-74	4-5	Moderate drought tolerance. Big grain stable

*Flowering period was obtained from present studies, whereas other data was obtained from Kenya Seed Co. Ltd, Kitale, Kenya

Table 2: Effect of embryo age (days after pollination-DAP) on callus induction

Maize genotype	Percentage callus induction (Mean±SE) at 17, 18, 19, 20 and 21 DAP*				
	17	18	19	20	21
DH02	77±1.44	79±1.55	80±1.58	79±1.29	63±2.69
KAT	81±2.32	83±2.36	87±1.11	89±1.49	77±1.44
PH01	84±1.78	85±1.08	85±1.97	87±0.91	80±2.27
PH04	78±2.56	79±1.29	80±2.27	80±1.08	68±2.63

*No significant difference among the hybrids at 17, 18, 19 and 20 DAP except at 21 DAP (ANOVA, p = 0.05)

Table 3: Calli growth rate on N6 maintenance media supplemented with 2 mg L^{-1} 2-4, D*

Days in culture	Fresh weight of calli (g) in maize genotypes			
	DH02	KAT	PHO1	PH04
0	0.06±0.00	0.06±0.00	0.06±0.00	0.06±0.00
4	0.11±0.005b	0.16±0.020b	0.29±0.015a	0.15±0.020b
8	0.15±0.010b	0.20±0.250b	0.48±0.050a	0.25±0.010b
12	0.24±0.015c	0.40±0.050d	0.62±0.065a	0.26±0.010c
16	0.33±0.030c	0.54±0.080d	0.80±0.040a	0.28±0.010c
20	0.36±0.020c	0.85±0.080d	1.05±0.030a	0.28±0.005c
24	0.38±0.020c	1.08±0.110d	1.33±0.050a	0.29±0.005c
28	0.38±0.025c	1.22±0.075d	1.48±0.025a	0.28±0.005c
32	0.38±0.020c	1.26±0.065d	1.46±0.025a	0.27±0.005c

*Values with the same letter (s) are not significantly different (ANOVA, p = 0.05)

Table 4: Effect of 2, 4-D on embryogenic callus induction in four maize genotypes

Concentration of 2,4-D (mg L^{-1})	Embryogenic callus induction (%) in maize genotypes (mean ±SE)			
	DH02	KAT	PHO1	PH04
0	0±0	0±0	0±0	0±0
0.2	21±1.32	24±0.70	30±1.82	20±1.82
0.4	31±0.70	34±2.08	60±1.82	32±1.82
0.6	41±1.13	48±0.91	64±1.82	45±2.38
0.8	52±1.58	59±1.08	70±1.22	56±1.63
1.0	60±1.63	74±2.08	74±0.91	65±1.47
1.5	75±1.68	80±1.63	83±2.04	75±0.91
2.0	79±1.29	88±1.31	90±0.85	80±0.64
4.0	56±2.17	60±2.04	63±1.29	58±1.29
12	15±0.91	19±2.49	25±2.04	18±1.47

one that is targeted due its high regeneration capacity (Armstrong and Green, 1985; Lu and Vasil, 1983; Carvalho *et al*., 1997). The production of type II callus arises at low frequency and only for specific genotypes (Lu and Vasil, 1983; Carvalho *et al*., 1997; Hodges *et al*., 1986).

Visible callus appeared within 3-5 days of culture in IM in all the maize genotypes. Compact, opaque, white to pale yellow embryogenic calli formed after sub-culture

Table 5: Effect of BAP on shoot induction

BAP (mg L^{-1})	Average number of shoots (Mean±SE)			
	DH02	KAT	PH01	PH04
0.0	4±0.64	7±0.91	8±0.48	5±0.63
0.1	1±0.41	3±0.64	3±0.64	2±0.48
0.2	1±0.48	2±0.48	2±0.63	1±0.84
0.5	1±0.41	1±0.48	1±0.29	1±0.25
0.8	1±0.48	1±0.29	1±0.29	1±0.29
1.0	1±0.48	1±0.29	0±0	0±0
1.5	1±0.25	0±0	0±0	1±0.29
2.0	0±0	0±0	0±0	0±0
4.0	0±0	0±0	0±0	0±0

on to MM (Fig. 1B and C). A range of 2, 4-D concentrations was tested to establish the effective concentration for embryogenic callus initiation using 19 DAP embryos (Table 4). The best response for callus induction was achieved with 2 mg L^{-1} 2,4-D. At this optimum concentration, callus induction responses of KAT and PH01 were not significantly different from each other but significantly different from PH04 and DH02 (ANOVA, p = 0.05). Embryogenic callus induction from cereal embryos due to auxins and especially 2,4-D in the range of 1-3 mg L^{-1}, is well documented (Bhaskaram and Smith, 1990). The frequency of callus induction from younger immature embryos ranged from 59 to 83%, thus implicating the role of the age of the explant in callus morphogenesis (Table 4). Juvenile tissues are usually more responsive to tissue culture than mature ones (Huang and Wei, 2004; Benson, 2000).

Although a range of cytokinins are known to affect *in vitro* plant cultures (Bhaskaran and Smith, 1990), addition of BAP to regeneration medium had little effect on shoot induction in the present study (Table 5). The embryos started to form green spots rapidly after the 3rd day of culture in light with shoots emerging after the 12th day of culture (Fig. 1C). The best shoot induction response was on hormone free MS media (Fig. 1D). As in other reports (Armstrong and Green, 1985; Lu and Vasil, 1983; Bhaskaran and Smith, 1990), somatic embryos developed into plantlets on regeneration medium free of plant growth regulators (Armstrong and Green, 1985; Huang and Wei, 2004; Lu and Vasil, 1983). One possible explanation for this is that the somatic embryos capable of germination to give rise to new plantlets have already formed and their fate may be predetermined by the initiation media (Huang and Wei, 2004). Although

Fig. 1: Plant regeneration from immature embryos of maize. A: Freshly isolated immature embryos cultured on IM containing 2,4-D (2 mg L^{-1}), bar = 1.5 cm. B: Calli on MM, bar = 1.5 cm. C: Greening embryos hormone free MS medium in light. Bar = 1.5 cm. D: Multiple shoots on hormone free MS medium, bar = 2 cm. E: Root formation on half strength MS with 0.1 mg L^{-1} of NAA, bar = 2 cm. F: Acclimatization of regenerated plantlets in pots containing sterilized vermiculite and sand in the ratio of 2:1, respectively, bar = 5 cm. G: Normal fertile regenerated plantlets in pots, bar = 20 cm. H: Viable seeds obtained from a regenerant, bar = 2 cm

hormone free MS media could support root formation, we have also demonstrated that NAA (0.1 mg L^{-1}) enhances root induction when shoots are transferred to half strength MS media (data not shown). Bohorova *et al.* (1995), found that MSR medium containing 0.5 mg L^{-1} indole-3-acetic acid and 1 mg L^{-1} BAP was more efficient than N6 medium free of plant growth regulators for maize plant regeneration (rooting). The overall regeneration frequency of the genotypes used in this study was: 51.7 (PH01), 45 (KAT), 35 (PH04) and 24% (DH02). In this case, there seems to be a correlation between callus induction and regeneration capabilities, which may be genotype dependent. The acclimatization process was successful with 95% of regenerants surviving the process (Fig. 1F). A total of fifty regenerants were obtained with 85% growing normally without morphological abnormalities to maturity (Fig. 1G) and viable seeds obtained (Fig. 1H).

In conclusion, the regeneration protocol reported here meets the two requirements for a good suitable regeneration system; explants capable of regenerating into plants at high frequency through somatic embryogenesis or organogenesis and, minimum *in vitro* period from explant stage until whole plant development. This minimizes the amount of time in culture that reduces somaclonal variation responsible for abnormalities experienced in tissue culture. The regeneration protocol reported here is currently being used for *Agrobacterium*-mediated genetic transformation (Frame *et al*., 2002; Ishida *et al*., 1996) of the four Kenyan maize genotypes described here using immature embryo explants. Other transformation methods such as particle bombardment (Gordon-Kamm *et al*., 1990) may also be applied. We are presently widening the scope of these regeneration protocols to encompass elite Kenyan and regional adapted inbred lines commonly used in breeding programs.

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