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Research Article

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Somatic embryogenesis and optimization of regeneration system from immature embryos in maize inbred lines

Justus Anyieni Obara*, Richard Mulwa, Maurice Oyoo, Miriam Karwitha

Department of Crops, Horticulture and Soils, Egerton University, P.O Box 536-20115, Egerton, Kenya

ABSTRACT

Maize production and productivity is on a sharp decline due to abiotic and abiotic stresses, therefore, an efficient regeneration protocol is an important tool that can contribute to maize improvement and gene-function studies to improve food security for the ever-growing population. The objective of this study was to optimize a regeneration system for CML 444 inbred line with CML 442 maize inbred line used as a reference. Callus was generated by incubation of immature embryos in Murashige and Skoog (MS) medium with vitamins supplemented with 0 - 4 g L^{-1} of 2, 4-D hormones, 900 mg L^{-1} proline, 250 mg L^{-1} caseinin hydrolysate and 10 mg L^{-1} of filter sterilized $AgNO_3$, 30 g l^{-1} of sucrose and 3 g L^{-1} gelrite. Somatic embryo maturation was achieved by transferring 6-week old callus to MS medium with vitamins prepared as previously in callus induction with 60 g L^{-1} of sucrose and zero plant growth regulators (PGR). Shoot initiation was conducted in MS medium with vitamins supplemented with BAP, NAA at varied concentrations and a 0 mg L^{-1} control. Plants at a 3-leaf stage that had not rooted were transferred to MS media with vitamins with IBA at a concentration of 0 - 0.3 mg L^{-1}. The 2, 4-D rates were significantly ($p \leq 0.001$) different for callus onset and callus induction. The genotype × rate interaction effects showed that 0 and 2 g L^{-1} 2, 4-D had the lowest and highest mean, respectively in both lines during onset and induction of callus. The lines had a significant ($p \leq 0.001$) effects on shooting induction, however, their means were not significantly different. Similarly, the means for the hormones were not significantly different for shooting induction. The lines, IBA rate and their interaction were significantly ($p \leq 0.05$) different for rooting induction. The means for the lines were significantly different for rooting induction in different IBA rates. Conversely, the mean for the IBA rates was significantly different for rooting induction. This study found that plant growth regulators rates during the callus induction stage plays a key role during regeneration. This protocol was a success and could provide a fundamental platform for future transformation in this line.

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***Corresponding Author:**
Justus Anyieni Obara
E-mail: justusanyieni@gmail.com

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INTRODUCTION

Maize (*Zea mays* L.) is among the widely cultivated crops and arguably the most economically important cereal worldwide. It is majorly cultivated in tropical and sub-tropical regions. The crop is well suited to survival in its environment due to its C4 photosynthetic system that lays a platform for higher rates of photosynthetic activity in comparison to other cereal crops. This salient feature has made the crop become best adapted to the environment, hence, most adopted across the globe (Muppala *et al.*, 2020). The demand for maize was projected to have increased by 50% to over 800 million tons per year by 2020 and is predicted to surpass both rice and wheat consumption globally. The uses of maize vary as some use it for food, feed, fodder and industrial raw materials such as bio-ethanol (Santos *et al.*, 2017; Rufino *et al.*, 2018).

Despite the diverse uses of the crop, its production and productivity is on a sharp decline due to abiotic and abiotic stresses. Improving maize crop production is fundamental in overcoming the negative impacts of these stresses and sustaining and/or increasing agricultural production under such environmental constraints. High frequency *in vitro* plantlet regeneration is core to modern crop improvement approaches such as genetic transformation. Regeneration in monocots pose a major challenge as compared to dicots due to variation in tissue structure and composition. Factors such as genotype, age of the explant, *in vitro* media composition play

key roles in maize tissue culture (Muppala *et al.*, 2020). The fundamental approach of genetic engineering in improving maize is limited due to the lack of suitable regeneration and recalcitrance in maize *in vitro* propagation. Hence, most of the genotypes remain inaccessible to genetic upgrading due to poor/or non regenerability (Ombori *et al.*, 2008; Liliane & Charles, 2020).

The establishment of a maize regeneration system is the prerequisite for its utilization of maize improvement (Wang *et al.*, 2012). *In vitro* regeneration in maize is achieved via organogenesis or somatic embryogenesis. Somatic embryogenesis has the advantage of producing bipolar structures and can be germinated and regenerated in one step. The approach possesses the capacity for automation and rapid scale up, which has been found to be a limiting factor in organogenic systems. It further reduces breeding cycles in relation to organogenesis. Regeneration through somatic embryos has been proven to present higher genetic integrity as somatic embryos, like zygotic (seed) embryos, develop with a good connection between root and shoot, and the tendency of the growth response of a plant in orientation to stimulus is eliminated as often seen with propagation by cuttings (Chen *et al.*, 2012; Pervin *et al.*, 2019; Egertsdotter *et al.*, 2019).

Inquest for successful *in vitro* maize regeneration, different studies have used different maize explants; mature embryos (Ali *et al.*, 2014; Tiwari *et al.*, 2015; Mushke *et al.* 2016), coleoptile nodes Kumar *et al.* (2018), nodal regions Vladimir *et al.* (2006), leaf tissues Ahmadabadi *et al.* (2007), anthers Ismaili and Mohammadi (2016), tassel and ear meristems Pareddy and Petolino (1990), protoplast Morocz *et al.* (1990), and shoot meristems Sairam *et al.* (2003). Amongst these, regeneration from immature embryos has been termed to present a number of benefits such as being convenient during inoculation and easy to induce callus (Jiao *et al.*, 2019). One key reason for predominantly using immature embryos during *in vitro* cultivation is their high competency in tissue culture (Ahmadabadi *et al.*, 2007). In spite of preferring immature embryo derived callus regeneration in maize, the approach presents limitations such as; low output, limited suitable stage for culture 14-19 days post-pollination and high genetic dependency during *in vitro* culture (Abebe *et al.*, 2008).

Successful maize *in vitro* culture has been found to be reliant on factors such as genotype, type of explant, culture media and conditions according to Mostafa *et al.* (2020) rendering just a small number of maize genotypes regenerable. Thus, it would be important for the stratification of *in vitro* growth conditions for various genotypes for convenient breeding during doubled haploid, somaclonal variation, gene engineering and somatic hybridization (Manvannan *et al.* (2010); Olawuyi *et al.*, 2019). Hence, this study was designed to assay *in vitro* renewability in CML 444 maize inbred line from immature embryos for subsequent genetic improvement.

MATERIALS AND METHODS

Experimental Site and Study Materials

Maize inbred lines CML, 444 and 442 were used in this study. The plants were sourced from CIMMYT-Kiboko, Kenya. The two lines were chosen because they are susceptible to maize lethal necrosis viruses (Table 1) and are highly preferred during polycrossing because of their ability to confer high grain yielding traits. CML 444 and 442 have been used in developing hybrid varieties that combine high yield and resistance against potyviruses. The experiment was conducted at the Egerton University Field 3 and Kenya-China Joint Laboratory for Crop Molecular Biology between June 2020 and March 2021.

Growing of Immature Embryo Donor Maize

Growing of immature embryo donor maize (Figure 1) was done in the green house according to the procedure of Abebe *et al.* (2008) with modifications. Black polythene bags measuring 15×10×20 cm and filled with 18 kg of normal garden soil was used to germinate the plants. Four seeds were sown per bag. About 100 g of fertilizers, N-P-K in the ratio15-9-12, was incorporated into each bag 2 week after planting. Essential agronomic practices were carried out and daily watering done in the morning or evening. Seedlings were thinned in 2 seedlings per pot after 14 days. Planting was staggered at 14 day intervals to ensure continuous immature embryo supply. During tassling, ears and tassels were covered with brown bags in order to curb pollen spills and uncontrolled pollination. Three days after tasselling, tassels were bent and the bag tapped for enough pollen collection. About 2-3 days after silking, husks were stripped down approximately 2.5 cm from the top. Tassel bags were removed and folded to prevent pollen spills. The ear bags were removed to expose the silks. Using a sterile blade silks were cut. Selfing was performed using the collected pollen. Screening of ears for embryo size evaluation was carried out from 9 days after pollination. Harvesting was done on the 21st day after pollination.

Figure 1: 40 days old CML 444 maize donor plants in the greenhouse

Table 1: MLN susceptible checks evaluation under SCMV, MCMV and MLN in different inbred lines in a research conducted at KARLO-Kabete and CIMMYTKenya and Kwazulu Natal-South Africa

MCMV				SCMV				MLN	
Pedigree	AUDPC	Days to 50% Anthesis	Days to 50% Silking	Pedigree	AUDPC	Days to 50% Anthesis	Days to 50% Silking	Pedigree	AUDPC
MLN001	270^{a}	71.33^{abcdefgh}	73.67^{abcdefghijk}	Atlas	286^{a}	68^{bcdefghijkl}	73.67^{defghijklm}	Atlas	196^{a}
MLN006	270^{a}	59.67^{abc}	62^{abc}	MLN042	286^{a}	66^{abcdefghij}	69^{abcdefghij}	MLN001	471.3^{ab}
Atlas	270^{a}	70^{abcdefgh}	74.33^{abcdefghijk}	MLN041	288^{a}	69^{bcdefghijklm}	70.67^{bcdefghijklm}	MLN013	518^{abc}
MLN012	286^{a}	75^{bcdefgh}	77^{abcdefghijk}	MLN013	321^{ab}	77.33^{klm}	80.67^{klmn}	MLN006	535.5^{abcd}
MLN016	287^{a}	81^{abcdefgh}	83^{abcdefghijk}	MLN019	378^{abc}	71.33^{cdefghijklm}	70.33^{bcdefghijkl}	MLN019	551.2^{bcde}
MLN008	292^{a}	77.33^{bcdefgh}	78.67^{bcdefghijk}	PA405	385^{abc}	61.67^{abcd}	62.67^{abcd}	MLN052	555.9^{bcdef}
MLN007	298^{a}	76^{bcdefgh}	82.67^{cdefghijk}	MLN028	453^{abcd}	70.67^{cdefghijklm}	73^{defghijklm}	MLN016	565.8^{bcdef}
N211	454^{ab}	64.67^{abcdefg}	66.33^{abcdef}	MLN020	474^{abcde}	63^{abcdefg}	60^{ab}	MLN018	573.4^{bcdefg}
MLN009	459^{ab}	72.33^{abcdefgh}	75^{abcdefghijk}	MLN056	566^{abcdef}	66^{abcdefghij}	70.33^{bcdefghijkl}	MLN002	582.8^{bcdefgh}
MLN050	636^{bc}	58.33^{ab}	59.33^{ab}	CML444	1311^{hi}	73.67^{ghijklm}	74.33^{efghijklmn}	MLN056	601.1^{bcdefgh}
KS23-6	844^{cd}	77.33^{bcdefgh}	77.67^{abcdefghijk}	CML312	1323^{i}	70^{cdefghijklm}	70.33^{bcdefghijkl}	KS23-6	606.7^{bcdefgh}
CML312	1132^{fgh}	70.67^{abcdefgh}	73.33^{abcdefghijk}	CML442	1326^{i}	70.67^{cdefghijklm}	73.33^{defghijklm}	N211	663.8^{bcdefgh}
CML444	1166^{fgh}	84.33^{fgh}	86^{efghijk}	CML 539	1363^{i}	66^{abcdefghij}	68.67^{abcdefghij}	CML444	666.8^{bcdefgh}
CML 539	1165^{fgh}	69.33^{abcdefgh}	71.33^{abcdefghij}	OH28	1369^{i}	69.67^{cdefghijklm}	72.33^{defghijklm}	CML312	722.2^{bcdefgh}
CML442	1178^{fgh}	76.33^{bcdefgh}	75.67^{abcdefghijk}	MLN006	960^{bcdefghijk}	58.67^{ab}	63.76^{abcde}	CML 539	890.2^{efgh}
PA405	1182^{fgh}	67.33^{abcdefgh}	70.67^{abcdefghij}					OH28	913.5^{gh}
OH28	1259^{h}	73.33^{abcdefgh}	75^{abcdefghijk}					CML442	922.8^{h}
Mean	1019.94	73.099	76.12	Mean	1116.7	69.932	72.609	Mean	740.38
s.e.	69.15	5.993	6.159	s.e.	196.7	3.142	3.352	s.e.	79.94
cv%	6.8	8.2	8.1	cv%	17.6	4.5	4.6	cv%	10.8

*Mean followed by the same letters in the same column are nor significantly different from each other at P <0.001

Harvesting Immature Embryos and Sterilization

Immature embryos of 21-28 days post pollination were selected and harvested. Explant sterilization was carried out based on Abebe *et al.* (2008) and Masters *et al.* (2020) protocol with modifications. The cobs were dehusked followed by 50 % NaOCl and 0.01% Tween 20 solution soaking for 10 minutes. Rinsing was done three times in autoclaved distilled water. The materials were transferred into 70% ethanol for 3 min and rinsed in autoclaved distilled water 3 times. Ears were cut open at the top and immature embryos were picked out.

Media Preparation and Explant Inoculation

The experiment was laid out in a 4×2 factorial in a completely randomized design with three replications. Explant inoculation and callus induction was carried out using Bohorova *et al.* (1999) and Holderbaum *et al.* (2019) protocols with modifications. MS media with vitamins supplemented with 100 mg L^{-1} ^{case}in hydrolysate, 2 mg L^{-1} L-Proline, 10 mg L^{-1} silver nitrate, 30 g L^{-1} sucrose, various combinations of 2,4-D (0, 2, 3 and 4 g L^{-1}. pH was adjusted to 5.8 and 3 g L^{-1} Gelrite was added. The media was autoclaved at 121 °C for 15 min. 30 ml of MS media was dispensed into Petri plates (100 mm diameter). 10 embryos (Figure 2) were placed on each Petri plate with the embryo axis in contact with callus induction media. Plates were kept in the dark at 26 ± 2°C. 2 days post inoculation, growing radicle and plumule (precautious germination) were excised to encourage callus formation. Callus induction was carried out for 6 weeks while sub-culturing was done after every two weeks for the six weeks. Necrotic callus was being discarded in every sub-culture. *In vitro* growth parameters; callus onset and induction rate were recorded in the different 2, 4-D treatments.

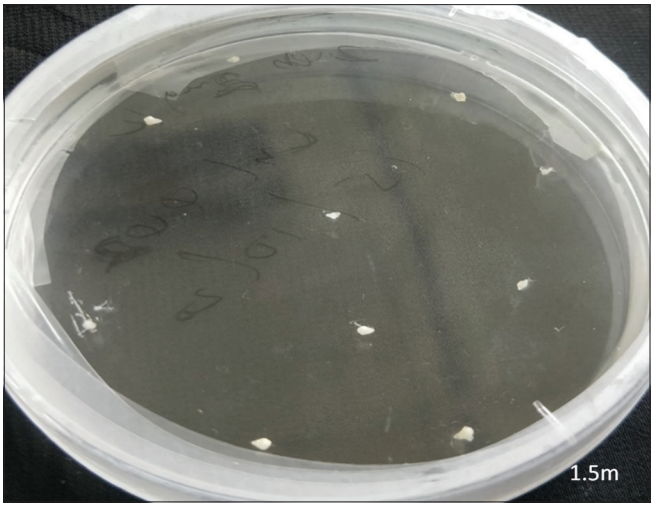

Figure 2: Inoculated maize immature embryos at day one of callus induction

Callus Maturation

On the sixth week after callus induction and upon differentiation of the induced callus on maintenance media maturation was carried out in MS media supplemented with vitamins, 100 mg L^{-1} ^{case}in hydrolysate, 2 mg L^{-1} L-proline, 10 mg L^{-1} silver nitrate, 60 g L^{-1} sucrose, devoid of growth hormones with. 30 ml of media was dispensed into Petri plates (100 mm diameter). Ten calli of about 10 mm were placed in every Petri plate. The maturation period took 4 weeks with a change of media every 2 weeks.

Plant Regeneration

Shooting media: The experiment was laid in a 3×2×2 factorial in a completely randomized design with three replications.

MS media with vitamins supplemented with 30 g L^{-1} sucrose with varying concentrations of plant growth regulators control (devoid of growth regulators), 0.5 mg L^{-1} BAP, 1 mg L^{-1} BAP/1.2 mg L^{-1} NAA and 1.5 mg L^{-1} BAP/1.4 mg L^{-1} NAA was prepared. pH was adjusted to 5.8 and 3 g L^{-1} gelrite was added. Autoclaving was done at 121°C for 15 min. 40 ml of the media was dispensed into each culture bottle (100 × 50 mm). To each bottle, 3 calli of approximately 10 mm were placed in shooting conditions and kept at 26 ± 2°C under 16 h light/8 h dark photoperiod. Lighting was provided by white fluorescent lamps emitting approximately 130 µmol/m2/s of photosynthetically active radiation (PAR) at the surface of the bottles. After three weeks, shoots that had not been rooted were transferred to rooting media. The experiment was 3×2 factorial in a completely randomized design with three replications. Root induction media was prepared following a similar protocol as shooting induction media. IBA hormone was used instead at concentrations; 0 mg L^{-1}, 0.2 mg L^{-1} and 0.3 mg L^{-1}. Shoots of 3 cm were excised close to the base of the callus with the aid of a scalpel. One culture bottle contained a single shoot. All bottles were incubated at 26 ± 2°C under 16 h light/8 h dark photoperiod. Lighting was provided by white fluorescent lamps emitting approximately 130 µmol/m2/s of photosynthetically active radiation (PAR) at the surface of the bottles.

Acclimatization and Greenhouse Transfer

Individual plants at 6 leaf stage were transplanted into a 3-inch pot containing autoclaved pre-wetted peat moss. The pots were covered with a plastic humidity dome and placed in a tray (30 cm x 50 cm) with 1-inch drain holes. Humidity was reduced by making 1 inch holes in the polythene bags to expose the plant's abiotic stressors to enhance hardening. Pots were placed on the laboratory bench with sufficient light illumination at 25 °C day/20 °C night for three days before being moved to the greenhouse. Domes were removed and plants were transferred to the greenhouse with an average temperature of 32 °C during the day and 20 °C at night. Watering was done moderately to avoid drying and waterlogging for proper root development. Plants were left in the small pots till they recovered from transplant stress for two weeks. After two weeks, acclimatized plants were transferred to larger pots of about 5L filled with loam soil mixed with sand in the greenhouse. Watering was maintained in the greenhouse.

Statistical Tools

Data was collected on callus onset, frequency of callus induction, shooting, rooting and the number of plantlets regenerated. Data was subjected to general linear model (GLM) for analysis of variance using SAS software version 8.2. The analysis for 2, 4-D, BAP and NAA, and IBA hormones were based on the following models;

$$Y_{ijkl} = R_i + L_i + T_k + LT_{ik} + \varepsilon_{ijkl} \quad \text{Equation 1}$$

Where, Y_{ijkl} =Observation of the experimental units, R_i=effects due to replicates, Y_{ijkl} =effects due to lines, Y_{ijkl} =effects due to treatments (2, 4-D hormones), LT_{ik} =interaction effects due to lines and treatments, ε_{ijkl} =residual.

$$Y_{ijkl} = R_i + L_i + H_k + LH_{ik} + \varepsilon_{ijkl} \quad \text{Equation 2}$$

Where, Y_{ijkl} =observation of the experimental units, R_i =effects due to replicates, L_i =effects due to lines, H_k =effects due to BAP and NAA hormones, LH_{ik} =interaction effects due to lines and hormones, ε_{ijkl} =residual.

$$Y_{ijkl} = R_i + L_i + I_k + LI_{ik} + \varepsilon_{ijkl} \quad \text{Equation 3}$$

Where, Y_{ijkl} =observation of the experimental units, R_i =effects due to replicates, L_i =effects due to lines, I_k =effects due to IBA hormone, LI_{ik} =interaction effects due to lines and IBA, ε_{ijkl} =residual.

Upon significant difference, mean separation of the treatments was conducted using Fisher's Least Significant Difference test at P≤0.05 level of significance.

RESULTS

Callus Onset and Induction

A day after inoculation, immature embryos had increased from 2 mm to 4 mm. Callus appeared on the 3rd day for CML 444 and 5th day for CML 442 (the scutellum opened up after 3 days and produced white to pale yellow and translucent embryogenic calli). Precautious germination was observed during callus induction as shown in (Figure 3 a, b). Analysis of variance showed that lines and lines×rates interaction had no significant effects (p≥0.001) effects on callus onset and induction however, 2, 4-D rates had significant (p≤0.001) effects on callus onset and induction (Table 2). Similarly, all the means for 2, 4-D rates had a significant (p≤0.05) different effect (Tables 3, 4). In the interaction between the lines and rates, 2 mg l^{-1}had the highest mean followed by 3, 4 and 0 g L^{-1} (Table 5). At 3 g L^{-1}, callus was smaller 3 mm and smallest at 0 g L^{-1}. At 2 g L^{-1} browning/ necrosis was patchy while necrosis was highly observed at 3 g L^{-1} across the two lines.

On sub-culturing these calli on maintenance media for 3 weeks two distinct types of calli were produced. In the three different concentrations, CML 444 had the induction highest frequency of Type I callus, 75.3% at 2 g L^{-1} 2, 4-D and the minimum frequency of 23 % at 4 g L^{-1}. In CML 442, a maximum of 75.6% embryogenic (Type I) callus induction was obtained at 2 g L^{-1} 2, 4-D with a minimum frequency of 25 % at 4 g L^{-1}. In both lines, Type II Callus were initiated at a lower frequency

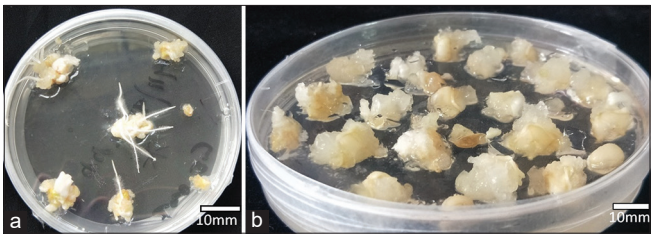

Figure 3: (a) Callus showing precautious germinations and (b) embryogenic callus during callus maintenance.

Table 2: Mean squares of the analysis of variance for callus onset and induction for maize lines CML 442 and CML 444 at different rates of 2, 4-D hormone in MS media

Source of variation	df	Callus onset	Callus induction
Replicates	2	182.292	45.167
Lines	1	88.167	51.042
Rates	3	11115.444***	11093.931***
Line×Rates	3	31.389	9.375
Error	14	33.577	485.667
R^2		0.986	0.986
CV (%)		11.215	11.590

*** Significant at P≤0.001, rates=2, 4-D, CV=coefficient of variation

Table 3: Means for callus onset and callus induction for the two genotypes (CML 442 and CML 444) in MS media

Maize lines	Callus onset	Callus induction
CML444	53.583[a]	51.417[a]
CML442	49.750[a]	48.500[a]
$LSD_{0.05}$	5.0738	5.1572

Means followed by same letter are not significantly different at P≤0.05

Table 4: Means for callus onset and callus induction at different rates of 2, 4-D for maize lines CML 444 and CML 442

2, 4-D rates	Callus onset	Callus induction
0	5.000[d]	2.500[d]
2	97.1667[a]	96.500[a]
3	77.833[b]	74.167[b]
4	26.667[c]	26.667[c]
$LSD_{0.05}$	7.1754	7.2954

Means followed by same letter are not significantly different at P≤0.05

averaging 10%. The onset of callus greening was observed at the fifth week of induction with 5% calli showing green erected somatic bodies.

Maturation

The somatic embryos observed from 5th week of callus induction were more frequent during maturation phase. Increase in number of greening callus was an additional salient feature (Figure 4).

Shooting was based on Pathi *et al.* (2013) protocol with modifications

Regeneration was achieved through utilizing BAP at concentration 0, 1.2, 1.4 mg L^{-1} and 0, 0.5, 1, 1.5 mg L^{-1} respectively for NAA. A total of 250 CML 444 and 180 CML 442 calli were transferred for shooting. An increase in calli greening was observed on the third day of shoot initiation.

A day after the shoot induction shoots had erected (Figure 5a). A number of callus with multiple shoots were observed after 3 weeks (Figure 5b). Two percent of CML 444 and 3% of CML 442 calli failed to shoot instead generated roots (Figure 5c). Green pseudo shoots that did not regenerate were observed during maturation.

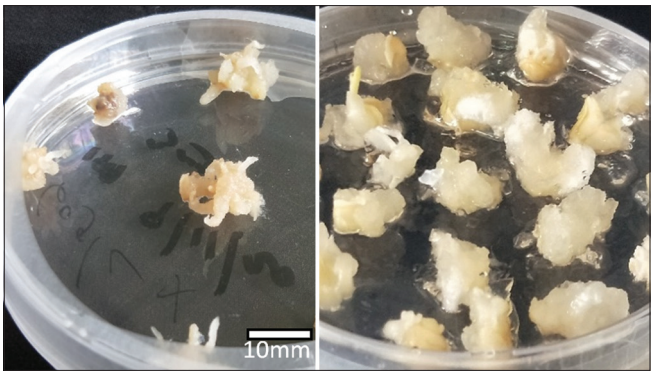

Figure 4: Maturation of callus showing somatic embryos and greening of somatic embryos during maturation

Cases of more rooting were observed from the 3rd day of shooting initiation with the appearance of adventitious roots being a major contributing factor. It was noted that plantlet regeneration was not relative to the capacity of callus induction since not all calli were converted into plantlets. Analysis of variance showed that the lines were significantly (p≤0.001) different for shooting induction (Table 6). The means of the lines and hormones (BAP and NAA) were not significantly (p≥0.05) different (Table 7). In the interaction between the lines and hormones, CML 442 had the highest mean for shoot induction compared to CML 444 when treated with both BAP and NAA. In both maize lines, BAP had the highest mean for shooting induction in comparison to NAA (Table 8).

Rooting induction

Shoots of about 3 cm that had not rooted were transferred to MS medium with 0, 0.2 and 0.3 mg L^{-1} IBA. Roots were observed one-week, (Figure 6) shows a plantlet with fully developed roots. Rooting had also been observed at the callus and shooting induction stages in MS without rooting hormone (adventitious roots) (Figure 5c). Analysis of variance showed that lines, IBA rates and lines × IBA rates interaction had significant (p≥0.05) effects on root induction (Table 9). Similarly, the means for the maize lines were significantly different for rooting induction. Conversely, all the IBA rate were significantly (p≤0.05) different for rooting induction. IBA rate 0 mg L^{-1} had the highest mean (38.333) followed by 0.3 mg L^{-1} (32.667) and 0.2 mg L^{-1} (25.500) (Table 10). The interaction between the lines and rates is highlighted in (Table 11). Line 442, had the highest mean at 0.3 mg L^{-1} (32.667) while CML 444 recorded the highest mean at 0 mg L^{-1} (45.000). Moreover, in both maize lines, IBA rate 0.2 mg L^{-1} had the lowest mean.

Acclimatization

Maize plants, 11 CML 444 and 9 CML 442 were successfully acclimatized and transferred to the greenhouse where there was 100% survival of the *in-vitro* regenerants (Figure 7).

DISCUSSION

Callus induction was seen to vary across different rates of plant growth regulators used; the same findings have been reported

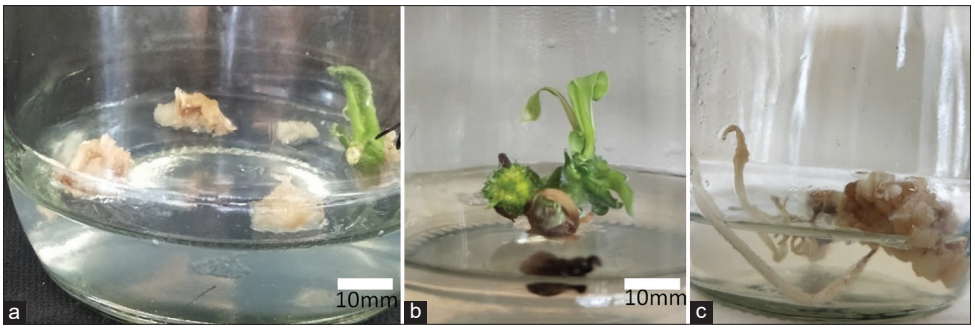

Figure 5: a) shooting onset (b) developed shoots (c) rooting under shooting conditions.

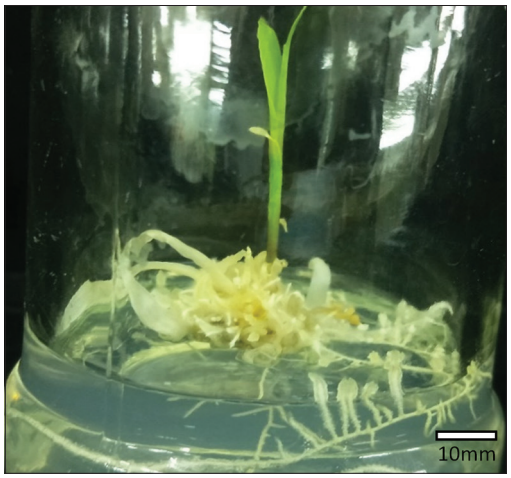

Figure 6: Fully developed plantlet with fully established shooting and rooting system

by Muppala *et al.* (2020). Callus induction was lowest at 0 g L^{-1} contrary to the findings of Malini *et al.* (2015), where callus formation was completely absent. Induction was highest at 2, 4-D 2 g L^{-1} followed by 3 g L^{-1} and 4 g L^{-1} produced the least. Such variation in induction percentages has been recorded in different lines/varieties (Muppala *et al.*, 2020). Both genotypes displayed significant differences in callus onset and induction which. Auxins at 2 g L^{-1} were found to be optimal for callus induction though a range between 1-3 g L^{-1} has been reported fundamental for embryogenic callus formation in cereal embryos was also seen in Bi *et al.* (2007) findings. The probability of callus formation during induction is not only dependent on physiochemical factors but rather on a wide range of physiological factors and the presence of endogenous plant growth hormones (Ikeuchi *et al.*, 2013) giving an explanation of callus presence at 0 g L^{-1} 2, 4-D.

Moreover, the formation of callus has been predicted to be genotypic dependent. Variation in homologous DNA sequences and Armstrong and Green (1985) findings suggest that RFLP markers associated with chromosomes 1, 2, and 9 in the long arm of maize chromosome 9 to be most responsible for callus formation. This serves as the background as to why there was a variation in the callus onset/induction in this study.

During callus induction, plant regulator 2, 4-D promotes callus formation by suppressing the effect of cytokinin to form chlorophyll in the callus. Additionally, it impacts the stability

Table 5: Mean for callus onset and callus induction in CML lines 442 and 444 at different 2, 4-D rates

Lines	2, 4-D rates	Callus					
		Onset			Induction		
		Mean	±	se	Mean	±	se
CML442	0	0.000	±	0.000	1.667	±	2.236
CML442	2	95.333	±	0.059	94.000	±	0.372
CML442	3	78.333	±	1.440	71.667	±	0.902
CML442	4	25.333	±	0.918	26.667	±	0.559
CML444	0	10.000	±	3.162	3.333	±	3.162
CML444	2	99.000	±	0.000	99.000	±	0
CML444	3	77.333	±	1.253	76.667	±	1.319
CML444	4	28.000	±	0.655	26.667	±	1.118

Se=Standard error

Table 6: Mean squares of the analysis of variance for shooting induction for maize lines CML 444 and CML 442 at different rates of BAP and NAA hormones

Source of variation	df	Shooting induction
Replicates	2	24.024
Lines	1	42.875***
Hormones	1	0.446
Line×Hormones	1	0.018
Error	36	1.719
R^2		0.599
CV (%)		16.634

***Significant at P≤0.001, CV=Co-efficient of variation.

Table 7: Maize lines, hormone interaction and different rates of 2, 4-D means during shooting induction

Maize lines	Shooting induction
CML442	8.905[a]
CML444	6.857[a]
$LSD_{0.05}$	0.821
Hormones	**Shooting induction**
BAP	8.00[a]
NAA	7.792[a]
$LSD_{0.05}$	0.829

Means followed by same letter are not significantly different at P≤0.05

of plant cells increasing their permeability against water and softening the cell walls thus decreasing the cell wall pressure to allow water surge in the cells leading to volume doubling (Saputro *et al.*, 2017). Cellular reprogramming of parenchyma

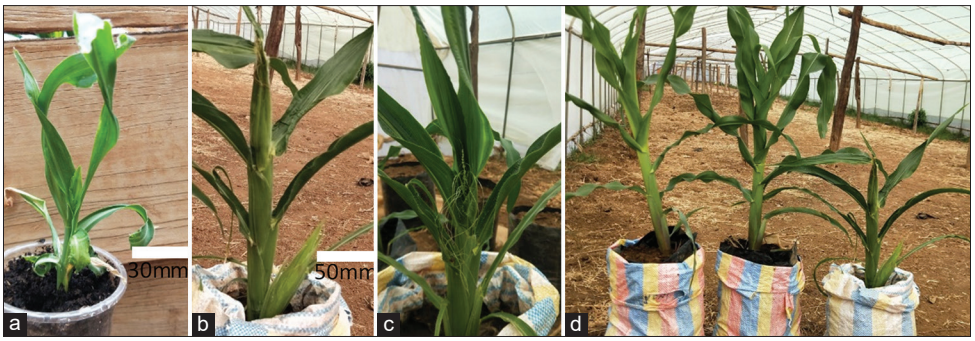

Figure 7: Survival of the in-vitro regenerants. a) Two weeks after introduction to hardening in peat moss (b, c) Soma clonal variants (d) Fully hardened plants in garden soil

Table 8: Means for shoot induction in CML 442 and CML 444 in BAP and NAA Plant growth regulators

Lines	Hormones	Shooting induction		
		Mean	±	se
CML442	BAP	9.000	±	0.408
CML442	NAA	8.833	±	0.347
CML444	BAP	7.000	±	0.866
CML444	NAA	6.750	±	0.772

se=standard error

Table 9: Mean squares of the analysis of variance for rooting induction for maize lines CML 442 and CML 444 at different rates of IBA hormone

Source of variation	df	Rooting induction
Replicates	2	23.167
Lines	1	220.500**
Rates	2	248.167***
Line×Rates	2	67.167*
Error	14	12.300
R^2		0.879
CV (%)		10.903

*Significant at P≤0.05, **Significant at P≤0.01, ***Significant at P≤0.001, Rates=IBA, CV=co-efficient of variation.

Table 10: Means for rooting induction at both genotypes, IBA interaction and response at different levels of IBA

Maize lines	Rooting induction
CML444	35.667[a]
CML442	28.667[b]
$LSD_{0.05}$	3.684
IBA rates	
0	38.333[a]
0.2	25.500[c]
0.3	32.667[b]
$LSD_{0.05}$	4.512

Means followed by same letter are not significantly different at P≤0.05

Table 11: Means for rooting induction in CML 442 and 444 maize lines under different IBA rates

Lines	IBA rates	Rooting induction		
		Mean	±	se
CML442	0	31.667	±	0.513
CML442	0.2	21.667	±	0.620
CML442	0.3	32.667	±	1.125
CML444	0	45.000	±	0.650
CML444	0.2	29.333	±	0.213
CML444	0.3	32.667	±	0.440

se=standard error

cells results in callus formation; this leads to a disorganized amorphic mass of rapidly dividing cells. Varying concentrations of endogenous plant growth regulators that occur in response to physical or chemical stimuli result in callus formation. Cellular reprogramming depends on a number of cascades/pathways; including a cytokinin-based route, an auxin-based route and a wound-induced route (Tuskan *et al.*, 2018).

Meristematic cells' presence in immature embryos is key to high cells division and callus proliferation. Cutting the germinations (plumule and radicle) increased callus formation as continued germination significantly reduced callus formation. Most germinated embryos in this study formed callus while those that did not form germinations had low quality callus contrary to Abebe *et al.* (2008). Two types of callus were recorded in both genotypes with type II callus being at a low frequency. The high frequency of Type I Callus and low frequency of Type II Callus generation observed in this work were similar to Manivannan *et al.* (2010) work. The types of callus formed during plant tissue culture is a result of different gene expression profiles as seen in *Arabidopsis* (Iwase *et al.*, 2011). Tomes and Smith (1985) reported that the control of genetic heritage involved in embryogenic callus formation in maize is the additive, with maternal effect and heterosis being much more influential. During somatic embryogenesis, gene expression patterns in maize is controlled by a gene or a block of genes (Willman *et al.*, 1989). In addition, Hodges *et al.* (1986) suggested that nuclear genes exhibiting dominance are important for the formation of somatic embryos and plantlet regeneration in maize plants. Furthermore, it has been proposed that the presence of a primary gene or genes in chromosomes 1, 2, and 9 are the underlying block for callus initiation and regeneration (Tomes & Smith, 1985).

According to Gao *et al.* (2019) studies on auxin-related pathways, in which the expression of auxin-responsive protein encoding gene *IAA18* and *IAA29* were up-regulated during the introduction of exogenous 2,4-D, the increased gene products of the *IAA18* and *IAA29* would then accelerate the release

of the ARFs through combination with different *TIR1/AFB* (transport inhibitor response 1/auxin-related F-box protein) and ARF proteins and resulting in activation of ubiquitinoylation, where the excited ARFs would up-regulate the expression of the downstream gene *CDK B2-234–36*. Besides, extremely up-regulated transcription factor *MYB15* has also been observed, indicating chances of the gene being involved in modulation of the cell de-differentiation as *MYB15* has the potential of modulating auxin-inducible genes through its interaction with *ARFs43*, thus, impacting plant growth and development. In the same study, it was revealed that *GH3* family protein encoding gene *GH3.1* was significantly up-regulated in callus, consistent with their previous study that *GH3* could maintain auxin homeostasis by coupling excessive indole-3-acetic acid to amino acids in the presence of excessive auxin.

The surge in the expression of the *MYB15*, *IAA18* and *IAA29* could promote auxin signaling pathways via related ARFs interactions; hence up-regulating *CDK B2-2* and *CYC D3-1*, involved in auxin and *CKs* signaling pathways, could have been core in callus induction of tea plant. The up-regulation of *GH3.1* and A-*ARR5* might be a response of tissues against high hormonal levels. After a change in the expression of genes related to signaling transduction, many downstream pathways such as DNA replication, zeatin biosynthesis, glutathione metabolism and photosynthesis were found to be modulated. Zeatin biosynthesis and DNA replication were modified in order to initiate cell division for callus formation. On the other hand, photosynthesis was changed due to the de-differentiation of the chloroplast. Up-regulation of glutathione metabolism is required for the initiation and proliferation of the callus as it provides antioxidant defence, nutrient acquisition, and regulation of cellular events.

Callus browning experienced in this study highlights the production of phenolic compounds by polyphenol oxsidase peroxidase or air explosion. Both abiotic and biotic factors have been found to influence phenolic compounds production. Further, it has been hypothesized that the surge in enzymes that regulate phenolic compounds could lead to a surge in their concentration while phenolic compounds' intensity depends on their enzyme hyperactivity. Moreover, browning can be due to chlorophyll degradation brought about by the loss of a phytol chain by chlorophyllase enzyme or klorofilin responsible for the green colour; which will be broken down into pheophorbites (brown coloured) and chlorine (colourless) (Saputro *et al.*, 2017). On the other hand, dark brown callus displays non viability and tissue autolysis (Gianazza *et al.*, 1992). Sucrose/high sucrose levels have been reported to promote maturation due to the creation of high osmotic stress from sugar according to Che *et al.* (2006a). The osmotic stresses serve to improve the expression of a greater percentage of genes encoding for protein proliferation and down-regulates expression of the cell nuclear antigen, nucleases, proteases and glucosidases leading to less cell division leading to increase in cell growth thus the expression of somatic bodies (Ombori *et al.*, 2008).

During regeneration, auxins can be excluded or introduced at low levels. Shooting can also be achieved without plant growth regulators and this was consistent with our findings and that of Anami *et al.* (2010) which was contrary to Manvannan *et al.* (2010). The potency of plants to develop shoots in the absence of synthetic hormones is due to the existence of endogenous plant growth regulators. It has been reported that the expression of cytokinin genes is high in developing kernels than in other maize tissues according to Brugière *et al.* (2003). And this could as well be the reason callus were shooting in zero hormonal conditions. The course of maize regeneration falls into two pathways; organogenesis or somatic embryogenesis. Organogenesis involves the direct induction of shoots and roots from the explant. On the other hand, indirect organogenesis is done via callus formation on the explant. Somatic embryogenesis involves the formation of scutella like structures from the explants or callus. This pathway is the most widely employed in plant regeneration. The embryogenic callus develops through organogenic stages to the plantlet stage aiding in the formation of highly developed plantlets.

Plantlet regeneration was not relative to the capacity of callus induction since not all calli were converted into plantlets according to Krakowsky *et al.* (2006), there is a lack of complete clarity on the genetics behind regenerative calluses. According to Abebe *et al.* (2008) failure to shoot could have been due to poor or completely no expression of genes responsible for plant regeneration. It has been hypothesized that regeneration from immature embryos lies on a few gene clusters or nuclear genes. Embryogenesis related genes are the basis for somatic embryogenesis and this could be the reason for the differences in different maize line/varieties responses to *in vitro* regeneration. This could be due to the increased synthesis of stress related, transporter encoding, chloroplast component and photosynthetic encoding proteins (Ombori *et al.*, 2008). The shooting was observed from day one up to a month, this duration of time before shooting has also been concluded in Binott *et al.* (2008) and Jiao *et al.* (2020) findings. The authors discovered that there exists 11 QTLs in 8 chromosomes, with 8 QTLs showing the main effect and 3 highlighting an epistatic interaction. In a study by Che *et al.* (2006b), the role of ERF/AP2 transcription factor RAP2.6L (a B-4 subfamily member), encoded by a gene that was specifically up-regulated during shoot regeneration was analysed. T-DNA knockdown mutations in *RAP2.6L* negatively impacted shoot development and shoot meristem-specifying genes expression. Concluding that cytokinin signaling is involved in events in shoot development. Moreover, some AP-2 sub-family transcription factors have been shown to affect shoot regeneration such as enhancer of shoot regeneration1.

According to Gao *et al.* (2019) study on *Arabidopsis* it was noticed that during root induction the signal from exogenous auxin could have been transmitted through interactions among the key regulators *ARF6* (*Auxin Response Factor 6*) and *ARF8* (*AUXIN RESPONSE FACTOR 8 WOX11/12* (*Wuschel-Related Homeobox* 11 and 12) and auxin. The regulators were up-regulated by the hormone from competent cells to root founder cells. Further, it has been observed that auxins promote *ACS4* gene encoding 1-aminocyclopropane-1-carboxylate (ACC) synthase expression to induce ethylene biosynthesis leading to

enhanced ethylene level up-regulating expression of ethylene-responsive transcription factor. Ethylene could influence auxin level facilitate auxin signaling pathway downstream through modulating the expression of the weak ethylene insensitive 2 and 7 (*WEI2* and *WEI7*) which encodes tryptophan biosynthesis rate-limiting enzyme anthranilate synthase. This results in up-regulating expression of *ARF18* and *ERF RAP2-12*, and down-regulation of *IAA18* and *IAA29* expression might be essential for root induction, as well as changes in gene expression of some downstream pathways, such as cutin, suberine, wax, and phenylpropanoid metabolism which is required to form the specialized cell wall through activating the pathway related to biosynthesis of the cutin, suberine, wax, and phenylpropanoid.

Analysis of variance did not reveal genotypic differences. The difference in terms of the plants regenerated 11 maize plants of CML 444 compared to 6 CML 442 which aligns with Li *et al.* (2019) findings where maize regeneration is always presumed to vary in different lines, that genotype effect plays an important role in plant tissue culture response that may be due to innate capacity a factor that seems to have a role during regeneration. Grando *et al.* (2013) reported that not all tropical genotypes that initiated embryogenic calli regenerate and also under their findings, some genotypes were classified as non-embryogenic. This concluded that such a classification does not accurately predict the regenerative potential of callus from a given genotype. Thus, providing a platform that can be reached through both embryogenic and non-embryogenic genotypes when optimal tissue culture conditions are availed. The variation in rooting was also due to endogenous plant regulator activities that are likely to cause higher root induction from 30 days of callus induction. Auxins alone play a major role in adventitious root development. The synergistic action of endogenous plant growth regulators artificially provided auxins is the foundation of the significant difference in rooting at the callus induction and shooting, thus arriving at the conclusion that there was more rooting in the hormonal devoid media.

Somaclonal Variation

This aspect of variation as highlighted in (Figure 6 d, c) occurs due to the new traits acquired during plant tissue culture with respect to the maintenance of genetic integrity to the parent explant. Somaclonal variation has been reported at various levels; morphological, cytological, cytochemical, biochemical, and molecular during plant micropropagation. Epigenetic somaclonal variation in regenerated plants are more frequent and variable than genetic somaclonal variation. Almost all the *in vitro* plants are produced via a process similar to development. Both the DNA methylation pattern and the chromatin state are known to be modulated during plant development and organ or tissue differentiation; development programs reset epigenetic patterns. However, precision lacks during the resetting of the epigenetic patterns outside the normal meiosis and fertilization. This, together with factors such as tissue culture stress and/or multicellular origin, media components, regeneration systems, and culture cycle durations, number of culture cycles and the genotype may account for the higher frequency of epigenetic somaclonal variation (versus genetic somaclonal variation) in regenerants (Wang & Wang 2012; Leva *et al.*, 2012).

CONCLUSION

The two maize lines had no significant difference in the number of regenerated plants. This indicated that genotypic variation did not play a role in regeneration. The protocol reported here was quick, efficient and highly reproducible and could be used downstream in transformation studies. The two genotypes in this study responded well in callus induction and plant regeneration though with a very low regenerative ability. This could provide a platform for genetic transformation to improve key preferred traits such as enhanced pest and drought tolerance and/or resistance. The present investigated method takes a range of 2-4 months to obtain a plantlet.

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