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In vitro regeneration of some Ethiopian maize (*Zea mays* L.) lines and varieties from immature embryos through callus induction

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Abstract

Maize is an important food crop in Ethiopia. Success in genetic transformation requires efficient in vitro regeneration protocols. However, inadequate information is available on *in vitro* regeneration of maize varieties/lines developed for the Ethiopian climate. Therefore, this study was initiated to optimize in vitro regeneration protocols for some inbred lines and open-pollinated maize varieties from immature embryos. Immature embryos evaluated for their ability to form callus were cultured in N6 medium and incubated at room temperature in the dark to initiate callus. Embryogenic calli were transferred from callus maintenance medium to embryo maturation medium supplemented with 2 mg/l glycine, 1 mg/l NAA and different levels of sucrose (55, 60, 65, or 70 g/l). Matured somatic embryos were subcultured in a shoot regeneration medium consisting of MS medium supplemented with 2 mg/l glycine, 2% sucrose and different levels (0, 0.1, 0.2 and 0.3 mg/l) of BAP. Roots were induced by subculturing individual shoots on half-strength MS medium supplemented with 2 mg/l glycine, 2% sucrose and different levels (0, 0.1, 0.2 and 0.3 mg/l) of NAA. Immature embryos were harvested between 16-20 days after pollination, depending on the variety for average callus induction. Better plant regeneration was obtained at basal (hormone-free) MS medium. Better root formation was at 0.1 mg/l NAA with an average of 2.82- 4.50 roots per shoot. Regenerated plantlets were successfully acclimatized in greenhouse and field conditions with survival rates of 83.7% and 75.6%, respectively. This study established a regeneration scheme for maize lines/ varieties via somatic embryogenesis from immature embryos.

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Introduction

Maize (*Zea mays* L.) is a diploid species (2n = 20), and herbaceous monocot plant with an annual life cycle (Singh, 2007). It belongs to the tribe Maydaea of the family Poaceae (Mosisa *et al.,* 2001). The center of origin of maize is the Mesoamerican region, probably in the Mexican highlands (Walter, 2008). Maize (*Zea mays* L.*)* is the third most important cereal crop after wheat and rice worldwide. It is a staple food grown in many parts of the world's rural population who cultivate many hectares of land for subsistence as well as for sale (Gonzalez *et al.,* 2012).

Adopting recent technologies like genetic transformation in our maize improvement program is considered among the leading viable solutions to further increase maize productivity. But Ethiopia's maize productivity is one of the lowest in Africa, even compared to some East African countries. Among the major factors contributing to this fact is the limited contribution of low potential maize growing areas to the national maize production because of recurrent drought; the absence of suitable and improved maize varieties for drought-prone areas has been documented (Mandefro *et al.,* 2001). Over the years, conventional breeding has been used as a tool to overcome these constraints. This has resulted in the development of modest increments in yields and agronomic characteristics such as disease resistance and drought adaptability to different agro-ecological zones (Werkissa and Temesgen, 2021).

Improvement of maize through classical breeding holds little promise. Therefore, molecular breeding methods involving marker-assisted selection and genetic transformation now provide viable alternatives in several crops, including maize. However, the prerequisite for crop genetic transformation is the existence of a reliable plant regeneration system (Sreenu *et al.,* 2016b). Maize tissue cultures have been initiated from virtually all plant parts, but these have been found to be largely non-regenerable. In cereals such as wheat, barley and maize, immature embryos have been the desired explants for *in vitro* culture and plant regeneration (Malini *et al.,* 2015).

The development of genetic transformation techniques for the major cereal crops has been relatively slow, mainly as a consequence of their limited susceptibility to *Agrobacterium* and their poor capacity to regenerate fertile plants (Sreenu *et al.,* 2016b). The first report on plant regeneration from maize embryo-derived callus cultures was by Green and Phillips (Green and Phillips, 1975). In later years, the regeneration of several inbreds adapted to tropical regions has been shown to produce embryonic calli (Tiwari *et al.,* 2015).

Given ascertain fact of genotypic dependent regeneration response, inadequate information is available on *in vitro* regeneration protocol optimization for Ethiopian maize lines/ varieties adapted to Ethiopian climatic conditions to implement such techniques. Therefore, as part of the country's ongoing preparation for safe adoption and application of biotechnology, it is imperative to optimize regeneration protocol for maize genotypes adapted to the Ethiopian climatic condition. In line with this, this study was initiated to optimize *in vitro* regeneration protocol for two inbred lines and five open-pollinated maize varieties from immature embryos.

Materials and methods

Plant materials

Seeds from five open-pollinated maize varieties (OPVs) (Argane, Morka, Awassa, Katumani and Gibe) and two male and female parental inbred lines of BH-660 were obtained from Ethiopian Seed Enterprise (ESE) and Jimma Agricultural Research Center (JARC).

Sample preparation

The seeds of Plant materials were grown at the Jimma University College of Agriculture and Veterinary Medicine (JUCAVM) Elladale Research and Production farm site from May 2020 to October 2021 on the staggered plantation to ensure the continuous supply of explants (immature embryos). To get

genetically uniform explants, the purity of maize lines/varieties was maintained through selfing by means of hand pollination. Cross-pollination was controlled by covering the sprouting ears with transparent paper bags.

Sterilization and embryo isolation

Dehusked ears were first washed under running tap water to remove any dust particles from their surface. Then the ears were surface sterilized by dipping them in 70% ethanol for 3 minutes, followed by soaking in local bleach (3% active ingredient) for 30 minutes after rinsing the alcohol three times using sterile distilled water. To enhance the efficiency of sterilant chemicals, two drops of Tween- 80 were added to the local bleach solution.

The ears were thoroughly washed three to four times with sterile distilled water under aseptic conditions before embryo extraction to remove the remaining traces of chemicals from the explant surface.

Immature embryos were isolated aseptically by cutting the tops of the kernel with a sharp scalpel blade without touching the embryos according to procedures (Jimenez and Bangarth, 2001). Then, the excised embryos were placed on a callus induction medium with the embryo axis in contact with the medium or the scutellum facing away. Five to eight explants were inoculated per petri dish containing 20-25 ml of N6 media.

Experiment one

Effect of DAP on callus induction efficiencyImmature embryos was cultured on the Chu and his colleagues' medium (N6 medium) with their embryo axis in contact with the callus initiation medium. This orientation helps to retard the germination of embryos and induce the proliferation of scutellar cells (Green and Phillips, 1975). To see the effect of embryo physiological stages on callus induction efficiency, ears were harvested at different physiological stages, namely 14, 16, 18 and 20 days after pollination (DAP) with embryo sizes ranging between 1-2 mm in length and utilized immediately for culture initiation (Oduor *et al.*, 2006; Gonzalez *et al.*, 2012).

Experiment two: Effect of 2, 4-D levels on callus induction:

This procedure was modified from Oduor *et al.* (2006.). Immature embryos were cultured with their flat embryo axis in contact with the callus induction medium and the rounded scutellar side exposed.

The Induction medium was composed of N6 media supplemented with 30 g/l sucrose, 2.9 g/ l proline, 2 mg/ l glycine, 8 g/l agar and 10 mg/l $AgNO_3$, and containing 2, 4-dichlorophenoxyacetic acid (2, 4-D) levels of 0, 1.0, 2.0, and 3.0 mg/l to determine its effect on callus induction efficiency. Two to three weeks later depending on callus induction rate, the calli were transferred to callus maintenance media. The maintenance medium was a callus induction medium but devoid of $AgNO_3$, and supplemented with 2 mg/l of 2, 4-D. Cultures were incubated at room temperature in the dark for about one month.

Experiment three: Effect of sucrose levels on embryo maturation:

Friable calli were transferred from maintenance medium to somatic embryo maturation medium according to Armstrong and Green (1985). Somatic embryos were matured when embryogenic calli were transferred to N_6 medium supplemented with 1 mg/l α-napthalene acetic acid (NAA), 2 mg/l glycine, 8 g/l agar and 55, 60, 65 or 70 g/l sucrose to determine the optimum concentration of sucrose for somatic embryo maturation.

Experiment four: Effect of BAP levels on plantlet regeneration

For plantlet regeneration embryogenic calli containing numerous matured somatic embryos were transferred to Murashighe and Skoog (Murashige and Skoog 1962) medium (MS media) supplemented with 20 g/l sucrose, 2 mg/l glycine, 8 g/l of agar and 6-Benzylaminopurine (BAP) in the range of 0, 0.1, 0.2, and 0.3 mg/l. After four to five weeks, the number of regenerated plantlets on the regeneration medium was determined.

Experiment five: Effect of NAA levels on root formation

Isolated shoots obtained from experiment four or regeneration media were cultured on half-strength MS medium supplemented with 20 g/l sucrose, 2 mg/l glycine, and 8 g/l agar, and NAA in the range of 0, 0.1, 0.2, and 0.3 mg/l. The numbers of roots per shoot were determined.

Acclimatization

After plantlets developed two to three leaves, they were transplanted to plastic pots filled with moist pot mix composed of sterile forest soil, compost and sand 2:1:1 ratio and covered with polyethylene plastic bags. The Plants were water sprayed regularly to maintain higher relative humidity in the greenhouse. After two to three weeks, acclimatized plants were transferred to larger buckets filled with forest soil, compost and sand in a 2:1:1 ratio and transferred to field for further development into matured plants (Gonzalez *et al,*. 2012).

Statistical analysis

Analysis of variance (ANOVA) and significant differences among the varieties on callus induction efficiency, somatic embryo maturation, plantlet regeneration and rooting abilities were performed using the statistical analysis system (SAS) software package, version 9.2. Based on the significant results obtained in ANOVA, means separation was employed using the Least Significant Difference (LSD) mean separation method and regeneration response of each variety was examined and better candidate varieties for *in vitro* regeneration were identified.

Results and discussion

Effect of days after pollination (DAP) on callus induction: Zygotic immature embryos responded to culture on induction medium by swelling to almost twice their initial size after four to six days of culture initiation (Fig.1 A and B). Callus induction was observed in all the maize lines/varieties included in this study. After four to six days of immature embryo culture on Callus induction medium (CIM), callus formation was started with the swelling of the embryos on the middle portion as well as the basal sides of the scutellum. This is due to the presence of meristematic cells in the scutellum. Zaharee *et al.* (2017) reported the presence of meristematic cells in the scutellum of immature maize embryos from which callus is induced.

The embryo physiological stage or DAP was found to be a major factor in determining the capacity of callus initiation from immature embryos. Calli were initiated from immature maize embryos at different DAP (Table 2). The ANOVA test for average callus induction rate revealed a very highly significant difference ($p < 0.0001$) among different levels of DAPs.

The interaction effects of varieties with DAP exhibited that KAT at 20 DAP with callus induction efficiency of 61.0% produced the maximum average callus induction rate followed by AMH-800 at 16 DAP with callus induction efficiency of 60.6% and 18 DAP with callus induction efficiency of 60.5% as compared to other combinations. The lowest average callus induction frequency of 31.2% was recorded for variety A-511 from immature embryos harvested at 14 DAP (Table 2). On the other hand, A-511, FP-BH-660, MP-BH-660, $UCBS_1C_2$ and MMRC-51 gave the maximum of 54.7%, 47.3%, 51.6%, 57.0%, and 55.3% average callus inductions when 18, 20, 20, 16 and 20 DAPs were respectively combined with them in the two-way interaction.

Overall, although there was a very highly significant difference ($p < 0.0001$) among the maize varieties tested for their average callus induction using immature embryos harvested at 16, 18 and 20 DAP, they were generally more responsive in these DAPs (16, 18, and 20) as compared to 14 DAP. The average highest callus induction rates differed from 16-20 DAPs depending on the variety. These findings are consistent with the findings of Oduor *et al.* (2006), where twelve Kenyan maize inbred lines tested showed that embryo physiological ages of 15-21 DAP were optimal for callus induction. Callus induction among the maize varieties tested in this experiment

ranged from a minimum of 31.2% at 14 DAP for variety AMH-800 to a maximum of 61.0% at 20 DAP for variety KAT. At 14 DAP, callus initiation was slow, and the induced calli often turned soft, loose, and browned and many of them gradually showed necrosis and died. This could be probably due to tissue death as immature embryos were exposed to a culture medium and other external factors; these cells could not be able to withstand the stress in the culture environment (Malini *et al.,* 2015).

Table 1. Key developmental features of the maize lines/varieties used in the study.

Variety Name	Type	Altitude (meters)	Year of release	Maintainer (breeder)	Maturity (Months)
AMH-800	OPV	1800-2500	2005	AHMRC	$5\text{-}5^{1/2}$
A-511	OPV	500-1800	1973	AwARC	4-5
FP-BH-660	INL	1600-2200	1993	BARC	4-5
MP-BH-660	INL	1600-2200	1993	BARC	$5\text{-}5^{1/2}$
$UCBS_1C_2$	OPV	1600-1800	2008	JARC	4-5
KAT	OPV	1000-1700	1974	BARC	3-4
MMRC-51	OPV	1000-1800	2001	BARC	4-5

AMH-800 = Argane maize variety, A-511 = Awassa maize variety, FP-BH-660 = Female parental line of BH-660 maize variety, MP-BH-660 = Male parental line of BH-660 maize variety, $UCBS_1C_2$ = Morka maize variety, KAT = katumani maize variety, MMRC-51 Gibe maize variety, INL = Inbreed line, OPV= Open pollinated variety, AHMRC = Ambo Highland Maize Research Center, AwARC = Awassa Agricultural Research Center, BARC = Bako Agricultural Research Center, JARC = Jimma Agricultural Research Center, EARO = Ethiopian Agricultural Research Organization.

Effect of 2, 4-D concentrations on callus induction: Callus induction response of the maize lines/varieties tested in this experimental condition was evaluated by using four different 2, 4-D concentrations (0, 1, 2 and 3) mg/l. Embryogenic callus induction from cereals embryo using auxins, especially 2, 4-D in the range of 1-3 mg/l is well documented (Oduor *et al.*, 2006).

In this study, it was found that different maize varieties produced different callus induction rates at different 2, 4-D concentrations. The overall average callus induction rate was 48.92%. Average callus induction differed according to type of variety in different 2, 4-D concentrations and ranged from a minimum of 0% at 0 mg/l 2, 4-D in all varieties to a maximum of 70.90% at 2 mg/l 2, 4-D in $UCBS_1C_2$ maize variety (Table 3). The higher average callus induction was obtained from $UCBS_1C_2$, A-511 and KAT maize varieties with 70.90%, 70.34% & 69.67% average callus induction efficiencies, respectively (ANOVA, $p = 0.05$).

There was no callus induction in medium devoid of 2, 4-D. Instead, immature embryos of all varieties were readily germinated within three to four days to form shoots and roots on the induction medium. In addition, rates of callus induction were relatively low at the low level of 2, 4-D (1 mg/l), while shoots and roots appeared at a relatively high frequency. Callus induction from immature maize embryos is therefore found to be 2, 4-D dependent (Table 3). Similar results were—reported on other maize lines by (Gonzalez *et al.,* 2012; Malini *et al.,* 2015), where selected Sudanese and Kenyan inbredline and open-pollinated maize varieties studied showed optimal callus induction frequencies in the presence of 2 mg/l 2, 4-D, and absence of callus induction in media free from 2, 4-D from immature embryos.

Tissue culture of maize from immature embryos typically produces two different types of calli mainly differentiated by their appearance and growth rate: Type I and Type II. Type I callus appears to be compact, organized and slow-growing. Type II callus has soft, friable appearance and is fast growing. Type II callus is the desired type of callus in maize tissue culture due to its high regeneration capacity (Rasha *et al.*, 2012). The production of type II callus arises at low frequency and often, it is genotype-dependent (Alok *et al.,* 2014). This observation suggests that

genetic background is an important factor in the formation of the type II callus. Phillips *et al.* (1984) reported that successful Type II callus formation depends on several factors among them, the genotype used, choice of tissue, developmental stage of the plant, culture media and the environment at each stage of the tissue culture process. Other major factors are related to the age of embryos, placement of embryos on medium and composition of the culture medium (Green and Phillips, 1975; Azad *et al.,* 2015).

Table 2. Effect of days after pollination (DAP) on callus induction efficiency in the maize varieties, % callus induction (Means± SE).

	Days after pollination (DAP)			
Variety	14	16	18	20
AMH-800	31.2±1.014^{n}	60.6±1.014^{a}	60.5±1.014^{a}	56.4±1.014^{bc}
A-511	35.6± 1.014^{m}	51.9± 1.014^{efgh}	54.7±1.014^{bcde}	49.9±1.014^{hi}
FP-BH-660	34.9±1.014^{m}	46.4±1.014^{j}	46.6±1.014^{j}	47.3±1.014^{ij}
MP-BH-660	34.9±1.014^{m}	50.9±1.014^{gh}	46.2±1.014^{j}	51.6±1.014^{fgh}
$UCBS_1C_2$	41.7±1.104^{k}	57.0±1.014^{b}	56.6±1.104^{bc}	52.2±1.014^{efgh}
KAT	38.7±1.014^{l}	54.6±1.0104^{bcde}	54.0±1.014^{cdef}	61.0±1.104^{a}
MMRC-51	37.5±1.104l^{m}	45.8±1.104^{j}	52.9±1.104^{defg}	55.3±1.014^{bcd}

Means followed by the same letter(s) within the rows are not significantly different from each other, LSD at p = 0.05.

The optimum 2, 4-D level for callus induction in all varieties employed in this study was found to be 2 mg/l (Table 3). Similar results have been reported by Gonzalez *et al.* (2012), where 2 mg/l 2, 4-D was optimal for callus induction from immature embryos in different maize lines. In addition to 2, 4-D, embryogenic callus initiation media contained other additives such as L- proline, casein hydrolysate, silver nitrate and Thiamine. The importance of these additives in developing embryogenic calli was reported by several authors including Ali *et al.* (2014); Caroline *et al.* (2021). Thus, embryogenic callus cultures remained viable and regenerative by using these additives over many months of subculturing. Parallel experiments in the present study by adding and without adding these additives revealed that the addition of these additives in callus initiation and maintenance media enhanced Type II callus induction from immature embryos of maize lines/varieties included in this study.

Table 3. Effect of 2, 4-D concentrations on callus induction of the maize varieties, % Callus induction (Means ± SE).

	2,4-D level (mg/l)			
Variety	0	1	2	3
AMH-800	0±0^{k}	38.19±72^{hi}	65.87±0.72^{b}	52.93±0.72^{d}
A-511	0±0^{k}	36.86±0.72^{ij}	70.34±0.72^{a}	37.07±0.72ij
FP-BH-660	0±0^{k}	36.13±0.72^{j}	58.90±0.72^{c}	36.86±0.72^{ij}
MP-BH-660	0±0^{k}	41.52±0.72^{fg}	59.42± 0.72^{c}	37.03±0.72^{ij}
$UCBS_1C_2$	0±0^{k}	42.00±0.72^{f}	70.90±0.72^{a}	42.68±0.72^{f}
KAT	0±0^{k}	43.43±0.72^{f}	69.67±0.72^{a}	42.24±0.72^{f}
MMRC-51	0±0^{k}	39.71±0.72^{gh}	54.62±0.72^{d}	50.83±0.72^{e}

Means followed by the same letter(s) within the rows are not significantly different from each other, LSD at p = 0.05.

Somatic embryo maturation: Like callus induction frequency, somatic embryo maturation was also genotyped dependent. Somatic embryos at the heart-shaped stage were observed by using hand lens. Non-embryogenic (Type I) calli accounted for 50.55% of calli formed, whereas embryogenic (Type II) calli accounted for 49.45% out of total calli examined for embryo maturation (Fig. 2). Somatic embryos were matured when embryogenic callus was transferred to N6 media with different concentrations of sucrose (55, 60, 65 and 70) g/l (Table 5). Very highly significant variations among varieties were obtained on average somatic embryo maturation ($p < 0.0001$). From the main effects of varieties, as indicated in Table 4 the optimum somatic embryo maturation was recorded from the variety KAT (40.91%). Conversely, the least somatic embryo maturation was obtained from MMRC-51 maize variety (28.67%).

Table 4. Varietal differences on somatic embryo maturation.

Variety	% Embryo maturation
AMH-800	39.63 ± 1.79^{a}
A-511	38.87 ± 1.79^{a}
FP-BH-660	36.73 ± 1.79^{ab}
MP-BH-660	31.70 ± 1.79^{c}
$UCBS_1C_2$	32.34 ± 1.79^{bc}
KAT	40.91 ± 1.79^{a}
MMRC-51	28.67 ± 1.79^{c}

Means followed by the same letter(s) within the columns are not significantly different from each other, LSD at $p = 0.05$.

The other maize lines/varieties were responded in between these two ranges. Different sucrose levels also showed slightly different responses for somatic embryo maturation in the maize lines/ varieties (Table 5). As a result, the highest somatic embryo maturation was obtained at 60 g/l (36.81%), whereas the lowest somatic embryo maturation attained was at 55 g/l (34.08%). High concentrations of sucrose have been reported to promote the maturation of somatic embryos in soybeans (Korbes and Droste, 2005) and maize (El-Itriby *et al.,* 2003; Malini *et al.,* 2015). This is due to the osmotic stress caused by the high concentration of sugar. An increase in sucrose concentration enhanced the somatic embryo maturation when the callus was transferred to the somatic embryo maturation medium.

The results of this study are also in agreement with those reported by Kamo *et al.* (1985), who reported enhanced somatic embryo production and their germination into plantlets with an increase in sucrose concentration from 2-6% in maize tissue culture.

Table 5. Effect of sucrose levels on somatic embryo maturation.

Sucrose concentration (g/l)	% embryo maturation
55	34.08 ± 1.79^{b}
60	36.81 ± 1.79^{a}
65	35.00 ± 1.79^{ab}
70	36.30 ± 1.79^{a}

Means followed by the same letter(s) within the columns are not significantly different from each other, LSD at $p = 0.05$.

According to Ratif *et al.* (2006), embryogenesis-related genes are involved in the somatic embryogenesis of maize plants which could be the reason for the genotypic differences observed in this study. Somatic embryos are responsible for being eventually differentiated into plantlets. Failure of some of the somatic embryos to regenerate plantlets has also been reported in other maize genotypes

(Bohorov *et al.,* 1995; Carvalho *et al.,* 1997; Huang, 2004). This could be due to the down regulation of genes controlling plant regeneration, as reported by Hodges *et al.* (1986), in which it was concluded that regeneration from immature embryo-derived callus was controlled by a few nuclear genes or a few gene clusters. In this study, non-embryogenic callus did not regenerate plantlets.

Table 6. Effect of BAP levels on plantlet regeneration in the maize varieties, average number of shoots (Means ±SE).

	BAP level (mg/l)			
Variety	0	0.1	0.2	0.3
AMH-800	5.48 ± 0.06^{b}	2.29 ± 0.06^{j}	1.8 ± 0.06^{mn}	1.64 ± 0.06^{no}
A-511	4.41 ± 0.06^{d}	2.66 ± 0.06^{i}	1.99 ± 0.06^{kl}	1.48 ± 0.06^{op}
FP-BH-660	3.75 ± 0.06^{f}	1.99 ± 0.06^{kl}	1.67 ± 0.06^{n}	1.24 ± 0.06^{q}
MP-BH-660	4.18 ± 0.06^{e}	1.99 ± 0.06^{kl}	1.72 ± 0.06^{mn}	1.44 ± 0.06^{p}
$UCBS_1C_2$	4.75 ± 0.06^{c}	2.99 ± 0.06^{h}	1.44 ± 0.06^{p}	1.72 ± 0.06^{mn}
KAT	5.75 ± 0.06^{a}	2.62 ± 0.06^{i}	2.13 ± 0.06^{jk}	1.86 ± 0.06^{lm}
MMRC-51	$3.54+0.06^{g}$	1.75 ± 0.06^{mn}	1.34 ± 0.06^{pq}	1.46 ± 0.06^{p}

Means followed by the same letter(s) within the rows are not significantly different from each other, LSD at p = 0.05.

The result obtained in this study is also agreeable with the reports of Jimenez and Bangerth (2006) which pointed out that non-embryogenic calli formed from six inbred maize lines were not proliferated to somatic embryos and eventually regenerated into plantlets.

Plantlet regeneration

As a general rule, the addition of cytokinins to *in vitro* plant cultures promotes shoot regeneration (Bhaskara and Smith 1990). However, the inclusion of 6-Benzylaminopurine (BAP) in shoot regeneration media of maize had little effect in promoting plant regeneration in this study. After maintaining embryogenic calli on embryo maturation medium for a period of three to four weeks depending on varietal response, matured embryos were transferred to shoot regeneration medium supplemented with different levels of BAP (Table 6). After embryogenic calli were transferred to regeneration media their surfaces turned green within the first week and plantlet regeneration occurred within four to five weeks. All plantlets regenerated in this study were obtained from embryogenic calli.

Table 7. Effect of NAA levels on root induction in maize varieties, the average number of roots (Means±SE).

	NAA level (mg/l)			
Variety	0	0.1	0.2	0.3
AMH-800	1.12 ± 0.05^{p}	2.82 ± 0.05^{e}	1.26 ± 0.05^{mnop}	1.13 ± 0.05^{p}
A-511	1.74 ± 0.05^{gh}	3.92 ± 0.05^{b}	1.99 ± 0.05^{f}	1.22 ± 0.05^{nop}
FP-BH-660	1.36 ± 0.05^{lmno}	3.14 ± 0.05^{cd}	1.42 ± 0.05^{klm}	1.25 ± 0.05^{nop}
MP-BH-660	1.72 ± 0.05^{gh}	3.03 ± 0.05^{d}	1.70 ± 0.05^{hi}	1.38 ± 0.05^{lmn}
$UCBS_1C_2$	1.37 ± 0.05^{lmno}	3.20 ± 0.05^{c}	1.88 ± 0.05^{fg}	1.55 ± 0.05^{ijk}
KAT	1.13 ± 0.05^{p}	4.50 ± 0.05^{a}	1.64 ± 0.05^{hij}	1.43 ± 0.05^{kl}
MMRC-51	1.23 ± 0.05^{nop}	2.82 ± 0.05^{e}	1.48 ± 0.05^{jkl}	1.21 ± 0.05^{op}

Means followed by the same letter(s) within the rows are not significantly different from each other, LSD at p = 0.05.

The ANOVA test for the average number of plantlet regeneration showed a very highly significant difference ($p < 0.0001$) among different concentrations of BAP. MS medium without BAP excelled in average shoot induction for all varieties tested in this study (Fig. 3). The maximum average number of plantlets regenerated per embryogenic callus was 5.75 in variety KAT in a media-free from any plant growth regulator, while the minimum average number of plantlets regenerated per embryogenic callus was 1.24 from the variety FP-BH-660 in MS media supplemented with 0.3 mg/l BAP (Table 6).

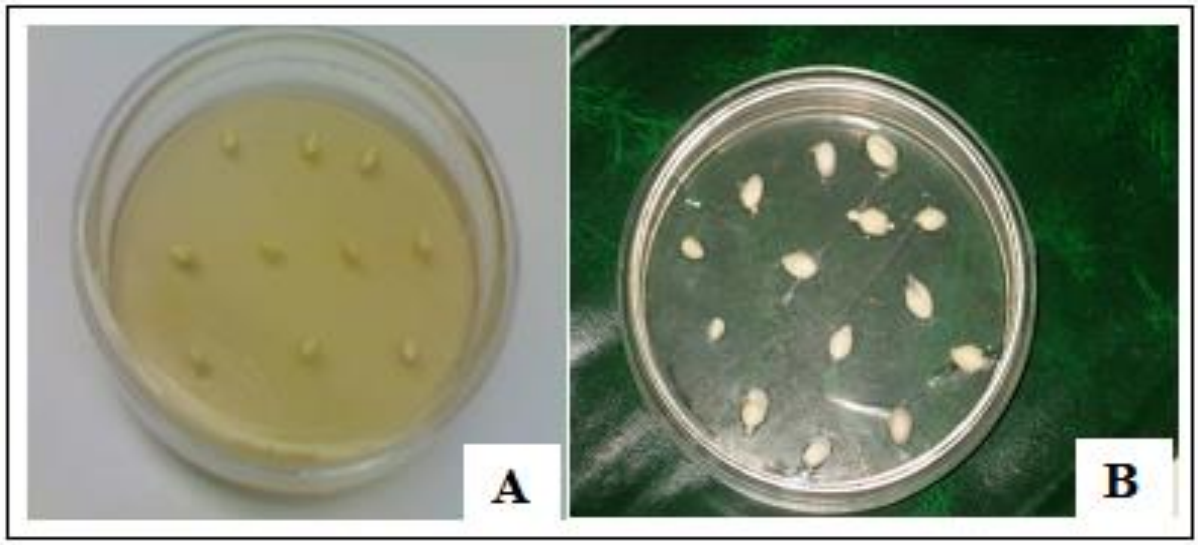

Fig. 1. Immature embryos in CIM. (A) Freshly isolated immature embryos cultured in callus induction medium containing 2 mg/l 2, 4-D, from $UCBS_1C_2$ (B) Swelling immature embryos after three days on callus induction medium containing 2 mg/l 2, 4-D from A-511.

In a medium without BAP, the average percentage number of shoots ranged from (36.67 to 63.64%) and the average number of shoots per embryogenic calli ranged from (3.54 to 5.75) in MMRC-51 and KAT maize varieties, respectively. When different levels of BAP were used in the regeneration medium, the percentage average numbers of regenerated shoots were ranged from 21.62 to 43.24% and the average numbers of shoots per embryogenic calli were ranged from 1.24 to 2.99 at 0.3 mg/l and 0.1 mg/l BAP concentrations in FP-BH-660 and $UCBS_1C_2$ maize varieties respectively. This shows that somatic embryos derived from embryogenic callus contained already predetermined cells that could easily be germinated into plantlets.

The two-way interaction effects of varieties with BAP concentrations revealed that KAT in MS medium with 0 mg/l of BAP produced the maximum number of shoots per embryogenic calli (5.75), followed by AMH-800 with an average number of shoots per embryogenic calli of 5.48 in the same medium (ANOVA, $p = 0.05$). On the other hand, MMRC-51 produced the least number of shoots (3.54) using the same medium composition that was proceeded by FP-BH-660 with a 3.75 average number of shoots per embryogenic calli (Table 6). While A-511, MP-BH-660 & $UCBS_1C_2$ maize varieties formed a reasonable number of shoots per embryogenic calli at 0 mg/l BAP. This result indicated that plantlet regeneration in maize tissue culture is genotype-dependent.

When embryogenic callus with matured somatic embryos was transferred into a regeneration medium, their surface became green and shoots were observed within four to five weeks (Fig. 3B). This could be due to the up-regulation of stress-related and transporter encoding genes followed by those encoding for photosynthetic and other chloroplast components (Malini *et al.,* 2015).

Fig. 2. Somatic embryos maturation from embryogenic callus after two weeks in maintenance medium from AHM-800 maize variety.

The results of this study showed that some of the somatic embryos were not regenerated to plantlets. This is probably due to the reality that not all embryogenic calli transferred to regeneration media induce plantlets.

The superiority of hormone-free MS media for plant regeneration from somatic embryos derived from immature embryos of maize has been reported previously (Gonzalez *et al.,* 2012).

The predetermined fate of somatic embryos to directly produce shoots and roots could be one possible explanation for this result (Huang and Wei, 2004). However, a higher callus or somatic embryo induction rate does not necessarily correlate with higher plant regeneration frequency.

Fig. 3. Plantlet regeneration from somatic embryos of maize varieties. (A) Multiple shoots on hormone-free MS medium two weeks after embryogenic calli transferred to regeneration medium from $UCBS_1C_2$, (B) Multiple shoots on hormone-free MS medium four weeks after embryogenic calli transferred.

This is mainly due to the fact that not all embryogenic calli are converted to plantlets. *Root formation:* Individual shoots (4-6 cm long) separated from clumps of embryogenic calli that regenerated them were cultured on half-strength MS media devoid of growth regulators associated with least root induction frequency in the varieties tested. This was very evident in all varieties which produced an average number of 1.38 roots per plant. The inclusion of NAA in the media enhanced the root induction capability of isolated shoots (Table 7). The numbers of shoots that produced roots were at least doubled with the addition of 0.1 mg/l of NAA to rooting media for all varieties. Consequently, the use of NAA resulted in an

average of 2.10 roots per plant (Fig. 4 A and B). On the contrary, the root induction capability of isolated shoots declined as the level of NAA concentration was increased to 0.2 mg/l and further reduction was observed with the increase of NAA level to 0.3 mg/l.

The decline in root induction rate associated with an increase in NAA level beyond 0.1 mg/l could be due to the inhibitory effect of NAA at a slightly higher concentration (Kumar, 2016).

ANOVA test for the average number of root formations showed a very highly significant difference ($p < 0.0001$) among different levels of NAA. The difference among varieties was also found very highly significant in root induction ($P < 0.0001$). The two-way interaction effect between the varieties and NAA concentrations revealed that the addition of NAA to MS media was most effective at 0.1 mg/l in all varieties of maize selected for this study, forming an average of 3.35 roots per plant. to regeneration medium from MMRC-51.

There was a lot of branching of roots when shoots were cultured on half-strength MS medium supplemented with 0.1 mg/l NAA. In addition, significant differences were obtained between varieties and different levels of NAA in other combinations too. As a result, variety KAT at 0.1 mg/l NAA concentration exhibited the highest average number in root formation per plant (4.50), followed by variety A-511 which induced an average of 3.92 roots per plant at 0.1 mg/l NAA. On the other hand, the least average number of roots (2.82) was obtained at the same concentration of NAA from AMH-800 and MMRC-51 maize varieties.

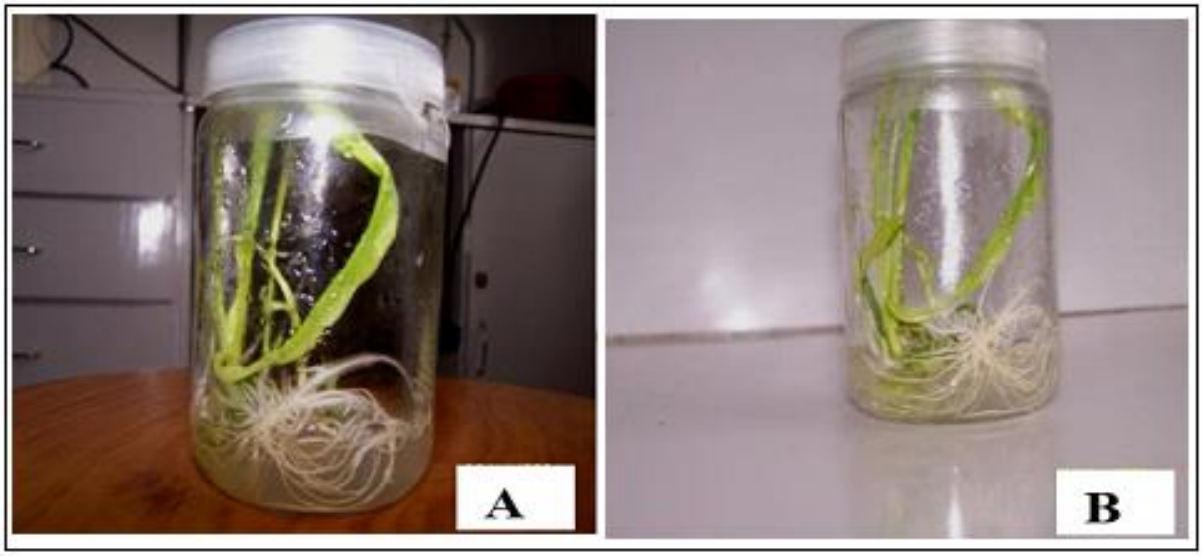

Fig. 4. Root induction from shoots transferred from regeneration medium to rooting medium. (A) and (B) Root formation on half-strength MS medium after three weeks in media containing 0.1 mg/l NAA from KAT and A-511 varieties, respectively.

There was non-significant variations for root induction with respect to interaction between varieties with NAA concentrations in FP-BH-660 at 0, 0.2 and 0.3; MP-BH-660 at 0 and 0.2 and MMRC-51 at 0 and 0.3 mg/l NAA. This result clearly shows that root formation was genotype-dependent in maize tissue culture. These findings are in agreement with the study conducted on four Kenyan maize genotypes by Hazarika (2003), where the studied genotypes showed significant variations for root number induction from plantlets obtained from embryogenic calli at different concentrations (0-0.6 mg/l) of NAA. Exogenously supplied auxin types like Indole 3-Butyric acid (IBA) and α-Naphthalene acetic acid (NAA) are fundamental factors in inducing rooting from *in vitro* grown plantlets (Hazarika 2003). The results of this study furthermore exhibited that use of NAA in minute concentration (0.1 mg/l) in culture

medium was essential for root induction in the maize lines/varieties adapted to different agro-ecological conditions.

Acclimatization of Regenerants: The success of the regeneration of normal plants is mainly affected by genotype, the type of explant materials employed and media composition (Sridevi *et al.,* 2020). The benefits of any tissue culture system can only be fully understood by the successful transfer of plantlets from the tissue culture vessel to ambient conditions found *ex vitro* (Hazarika 2003). In line with this, successful regeneration and acclimatization of normal fertile plants have been achieved in the present study.

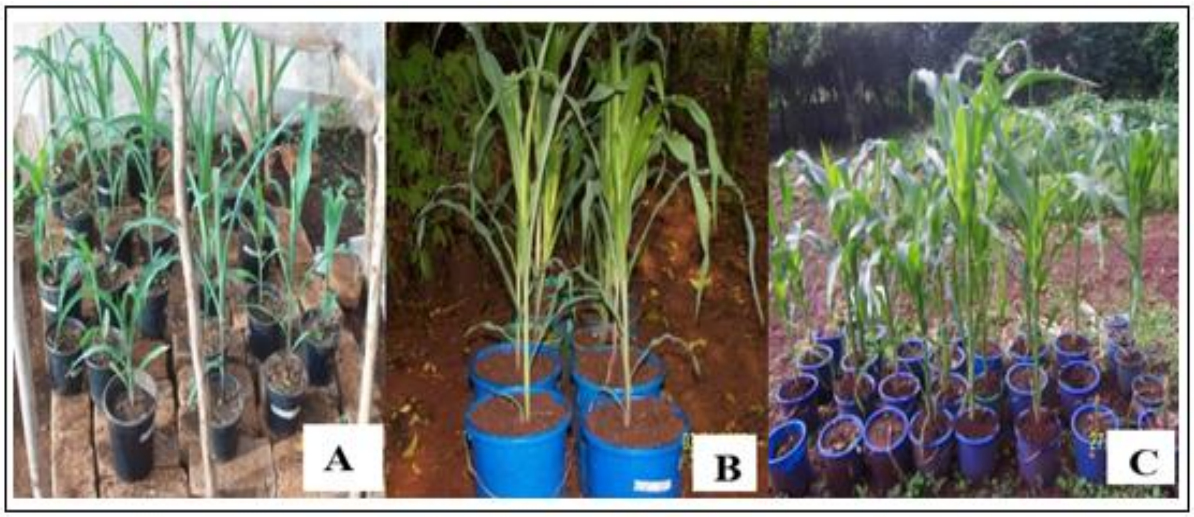

Fig. 5. Acclimatization of regenerants in pots. (A) Plantlets in pots containing sterilized forest soil, compost and sand in ratio of 2:1:1 respectively two weeks after the plantlets transferred to green house from *in vitro* condition, (B) Plantlets in pots after one week the plantlets transferred from green house to shade condition of the field, (C) Plants in pots after one month in an open environment.

Leftover media around the root of plantlets was carefully removed by washing them under tap water to reduce the decomposition of roots in the soil. Plantlets (12-15 cm high) with pairs of leaves and well-developed root systems from root initiation media were first hardened in the growth room in pots for five to seven days according to Oduor *et al.* (2006), before transferring to the greenhouse.

Then, washed plantlets were subsequently transferred to small plastic pots containing sterile forest soil, compost and sand in a 2:1:1 ratio respectively in the greenhouse. Pots were covered with polyethylene plastic bags and watered regularly to maintain higher relative humidity at the early stages of acclimatization.

Plantlets were then gradually exposed to greenhouse environmental conditions and transferred to buckets containing similar pot mix. Up to 83.7% survival rate was attained following acclimatization procedures described in the greenhouse (Fig. 5A). From *in vitro* regenerated (R_0) plantlets grown in the greenhouse, 75.6% of the regenerants survived in the field condition (Fig. 5B) and grew to normal plants (Fig.5C).

Conclusion

This study established a regeneration system for maize lines/ varieties through somatic embryogenesis from immature embryos. The study also established that the genotype, 2, 4-D and age of explant had significant effect on callus initiation and plantlet regeneration. The highest embryogenic callus induction and plant regeneration response were produced from KAT and UCBS1C2 maize varieties. Immature embryos harvested from 16-20 DAP depending on the varietal response rate were better for embryogenic callus induction. For the induction of calli in induction media, N6 media supplemented

with 2 mg/l 2, 4-D was the best. In regeneration of plantlets from the embryogenic calli, MS basal media had proved to be the best in giving higher number of shoots per embryogenic callus. In the current study, maize shoots cultured on half strength MS medium supplemented with 0.1 mg/l NAA produced optimal root system, i.e., containing several, long and more branching roots. Plantlets obtained in this *in vitro* regeneration protocol were successfully acclimatized in greenhouse as well as under open environmental conditions, and significant number of plants survived both in greenhouse and field conditions.

Recommendations

Only seven maize lines/varieties were tested for regenerability in this study. Hence, more Ethiopian maize varieties should be tested for their regeneration ability using immature embryos since maize regeneration from immature embryos is genotype-dependent. In the future line of work, different media comparisons and hormone combination effects on regeneration need to be done to enhance the present regeneration protocol. There should also be initiation to look into the transformation potentials of the maize lines/varieties used in this study.

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Conflict of interest declaration

The authors have declared that no conflict of interest exists.

References

Ali F, Ahsan M, Saeed N, Ahmed M, Ali Q, Kanwal N, Tehseen M, Ijaz U, Bibi I, Niazi N. 2014. Establishment and optimization of callus-to-plant regeneration system using mature and immature embryos of maize (*Zea mays* L.). International Journal of Agriculture and Biology **16,** 111-117.

Alok A, Chikkappa G, Ravindra, Meenakshi B, Pramod W, Pradyumn K, Sain D, Sai R. 2014. Differential effect of immature embryo's age and genotypes on embryogenic type II callus production and whole plant regeneration in tropical maize inbred lines (*Zea mays* L.) Indian Journal of Genet **74(3),** 317-324.
http://dx.doi.org/10.5958/0975-6906.2014.00849.9

Armstrong C, Green C. 1985. Establishment and maintenance of friable, embryogenic maize callus and involvement with L-proline. Planta **164,** 207-214.
http://dx.doi.org/10.1007/BF00396083

Bohorova N, Connor-Sánchez A, D'Amalto F. 1995. Cytogenetics of plant cell and tissue cultures and their regenerates. Critical Reviews in Plant Sciences, Boca Raton, **3,** 73-112.

Carvalho F, Van Osttveen A, Ammeren A. 1997. Comparative analysis of callus formation and regeneration on cultured immature maize embryos of the inbred lines A188 and A632. Plant cell tissue organ culture **50,** 57-65.

Caroline N, John M, Omwoyo O, Jacinta M, Ezekiel M. 2021. Banana Production Trends, Cultivar Diversity, and Tissue Culture Technologies Uptake in Kenya. International Journal of Agronomy.
https://doi.org/10.1155/2021/6634046

El-Itriby A, Assem A, Abdel-Galil M, Madkour P. 2003. Regeneration and transformation of Egyptian maize inbred lines via immature embryo culture and a biolistic particle delivery system. Plant, **39(5),** 524-531.
http://dx.doi.org/10.1079/IVP2003439

Gonzalez G, Pacheco G, Oneto C, Etchart V, Kandus M, Salerno J, Eyherabide G, PreselloD, Lewi D. 2012. Somatic embryogenesis and plant regeneration capacity in Argentinean maize (*Zea mays* L.) inbred lines. Electron. Journal of Biotechnolology **15(1),** 1-15.
http://dx.doi.org/10.2225/vol15-issue1-fulltext-7.

Green C, Phillips R. 1975. Plant regeneration from tissue culture of maize. Crop scicience **15,** 417-42. https://doi.org/10.2135/cropsci1975.0011183X0015000030040x

Hazarika N. 2003. Acclimatization of tissue-cultured plants, Department of Horticulture, Assam Agricultural University. Current Science India **85,** 1225- 1231.

Huang L, Wei D. 2004. The production of callus capable of plant regeneration from immature embryos of numerous maize (*Zea mays*) genotypes. Planta **165,** 322-333.

Jimenez L, Bangerth V. 2001. Hormonal status of maize initial explants and of the embryogenic and non-embryogenic callus cultures derived from them as related to morphogenesis *in vitro*. Plant Science, **160(2),** 247-257. http://dx.doi.org/10.1016/S0168-9452(00)00382-4

Kamo K, Hodges T, Malik T. 1985. Establishment and characterization of long term maize callusand cell suspension cultures. Plant Science **45,** 111-117.

Korbes C, Droste Y. 2005. Genotypes with high somatic embryogenesis and plant regeneration capacity obtained from tissue culture of Soya beans. Scientic Agriculture **57(4),** 717-722.

Kumar F. 2016. Plant regeneration through callus initiation from mature and immature embryos of maize (*Zea mays* L.) Indian Journal of Agricultural Research **50(2),** 135-138. http://dx.doi.org/10.18805/ijare.v0iOF.8435

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

Malini N, Anandakumar C, Hari Ramakrishnan S. 2015. Regeneration of Indian maize genotypes (*Zea mays* L.) from immature embryo culture through callus induction. Journal of Applied Natural Science **7,** 131-137.

Mandefro N, Hussein M, Gelana S, Gezahegne S, Yosef B, Hailemichael B, Aderajew S. 2001. Maize improvement for drought stressed areas of Ethiopia. Second National Maize Workshop of Ethiopia, Addis Ababa p 15-26.

Mosisa W, Hadji T, Mandefro N, Abera D. 2001. Maize Production Trends and Research in Ethiopia. Proceedings of the Second National Maize Workshop in Ethiopia. International Maize and Wheat Improvement Center, Addis Ababa.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum **15,** 473-497. https://doi.org/10.1111/j.1399-3054.1962.tb08052

Oduor R, Ndung'u S, Njagi E, Machuka J. 2006. In Vitro regeneration of dryland Kenyan maize genotypes through somatic embryogenesis. International Journal of Biotechnology **2,** 146-151.

Philippe V, Horth Y, Pascal F. 1988. Enhancement of production and regeneration of embryogenic type II callus in *Zea mays* L. by AgNO3. Plant tissue and organ culture **18,** 143-151.

Rasha A, Jonthan M, Steven R, Abdelbagi M, Eric K, Clet M, Charilis M, Jesse M. 2012. Effect of Auxin and Source of Explant on Callus induction of Tropical Maize. Biotechnology **11(4),** 225-231.

Ratif H, Furini A, Jewell D. 2006. Somatic embryogenesis and plant Regeneration from immature and mature embryos of tropical and subtropical maize (*Zea mays* L.) genotypes, Maydica, **39,** 155-164.

Singh S, 2007. Breeding of Field Crops. Indian Agricultural Research Institute, New Delhi, India, 22-30.

Sreenu P, Sridevi M, Kumar P, Reddy M, Sailaja D, Kumar G. 2016b. Optimization of SomeInfluential Factors In Maize Genetic Transformation Through Microprojectile Bombardment. International Journal of Recent Scientific Research **7,** 10517-10522.

Sridevi M, Pavan K, Sreenu P, Kodandarami R, Sateesh K, Premalatha D. 2020. Distinctiveresponse of maize (*Zea mays* L.) genotypes in vitro with the acceleration of phytohormones. Journal of Plant Biotechnology **47,** 26–39.

Tiwari S, Agrawal P, Pande V, Gupta H. 2015. Callus induction and whole plant regenerationin sub-tropical maize (*Zea mays* L.) using mature embryos as explants. Indian Journal of Genet and Plant Breed. **75,** 330-335. http://dx.doi.org/10.5958/0975-6906.2015.00052.8

Walter K. 2008. The biology of *Zea mays* L. office of gene technology regulatory, Department of health and ageing, version **1**.

Werkissa Y, Temesgen B. 2021. Plant tissue culture and its application in modern crop improvement. International Research Journal of Plant and Crop Science **6(4),** 201-210.

Zaharee A, Shaukat K, Jalal U, Gahulam M. 2017. *In Vitro* Plant Regeneration from Coleoptilar Node of Maize Seedling: a New Tool to Bioengineer the Maize Rapidly. Sarhad Journal of Agriculture, **33(4),** 606-614.