

Efficient Plant Regeneration using Mature and Immature Embryos of Maize (*Zea mays* L.)

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Abstract – Under this study an efficient protocol on callus induction (CI) and plant regeneration (PR) was developed using immature (IE) and mature embryos (ME) of four maize varieties viz. Barnali, Mohar, Khoi bhutta and Shuvra. For callus induction three basal media viz. MS, N6 and 6N1 were used where different concentration of 2,4-D (0.5, 1.0, 1.5, 2.0 mg/l) were used. Among the tested varieties, highest 75.28 and 77.16% callus were recorded for Mohar in MS supplemented with 1.5 mg/l BAP using immature and mature embryos respectively. The lowest value (25.48%) was recorded for Khoi bhutta on 6N1 + 0.5 2,4-D in ME. For PR, 3-4 weeks old calli were transferred to regeneration media (MS, N6 and 6N1) with BAP (0.5, 1.0, 1.5, 2.0 mg/l) and/or IAA (0.0, 0.5, 1.0, 1.5 mg/l). The maximum regeneration (77.44%) was found in MS that supplemented with 1.0 BAP and 0.5 IAA for Shuvra by IE and from ME, Mohar showed 76.09% regeneration. In the same medium combination, the highest elongated shoot (20.47cm) was recorded for Shuvra in IE; and from ME the value was 22.45 cm for Mohar. The variety Mohar also exhibited the maximum rooting (82.84%) in MS supplemented with 1.0 BAP and 0.5 IAA when it was regenerated from ME derived calli. Analysis of variance (ANOVA) showed significant differences among genotypes, media and hormonal combinations for callus induction and regeneration at $P < 0.01$.

Keywords – Callus Induction, Immature and Mature Embryo, *In Vitro* Regeneration, Somatic Embryogenesis.

I. INTRODUCTION

Maize (*Zea mays* L.) belongs to the family Gramineae is a major and third important cereal food crop in the world and also in Bangladesh. It's used as human food, animal feed and raw material for manufacturing in large number of industrial products. It is considered as a complete source of nutrition than other cereals [1]. Across the world, more predominantly in Asia, demand of maize is increasing [2]. Hence, to ensure sufficient production by improving maize cultivar, it's time to embrace reliable, effective and fast techniques like biotechnology [3], [4]. Green and Phillips [5] regenerated maize through *in vitro* tissue culture. It was reported that embryogenic and non-embryogenic callus induction was independent on maize genotypes when immature and mature embryos were used for tissue culture [6], [7]. Besides, immature embryos showed relatively high rate of callus induction compared to mature [8]. By using immature embryos regeneration system has been reported for maize [9]-[13]. On the other hand, mature embryos were also used to regenerate plants [14]-[18]. Pathi et al. [19] reported highly efficient regeneration by using two different approaches i.e. embryogenic and organogenic callus culture. Some reports

mentioned that regeneration capability depends on genotype; such as maize [20], wheat [21], barley [22], rice [23], sorghum [24] and *Asterias annua* L. [25]. Plant regeneration also influenced by media composition and/or growth regulators, especially 2, 4-D, BAP and IAA [26]-[31].

Some studies investigated the factors which affect plant regeneration including media composition, genotype and environment [32]-[34]. In basil plants (*Ocimum basilicum* L.), BAP and IAA were used either individually or in combination for multiple shoot induction [35]. It was also reported that mature embryos showed better result than immature [36]. However, as far as we know, no enough reports are found on *in vitro* culture using Bangladeshi maize variety. Hence, this study has been conducted to develop better protocol for efficient callus induction and subsequent plant regeneration using immature and mature embryos as explants.

II. MATERIALS AND METHODS

Plant materials

For this study mature seeds of four maize varieties viz. Barnali, Mohar, Khoi bhutta and Shuvra were collected from Bangladesh Agriculture Research Institute (BARI), Gazipur, Bangladesh. For cultivation seeds were sown in the research field of Institute of Biological Sciences, University of Rajshahi, Bangladesh. From the plants grown in research field, the young and mature cobs were collected.

Methods

Sterilization of explants

The young cobs and mature seeds were used as the source of explants i.e. immature embryos (IE) and mature embryos (ME) respectively. Both types of explants were sterilized with 70% (v/v) ethanol for 1.5 minutes and in sodium hypochlorite (NaOCl) for 25 minutes. Then they were sterilized with 0.1% (v/v) mercuric chloride ($HgCl_2$) for 5 minutes and washed with double distilled water.

Culture media, inoculation and callus induction

After sterilization, immature and mature seeds were inoculated on callus induction media (CIM); and after sealing with parafilm, petri dishes were kept under dark conditions at $25 \pm 2^\circ C$. Three basal media, such as MS [37], N6 [38] and 6N1 [39] were individually supplemented with four different concentrations of 2, 4-D (0.5, 1.0, 1.5 and 2.0 mg/l) as shown in Table 1. The other supplements of CIMs were 2.87 mg/l L-proline, 0.1 g/l casein hydrolysate, 2% sucrose and 0.01 g/l silver nitrate

(AgNO₃) as constant. Eight percent agar was used as gelling agent and pH 5.8 was adjusted for all the media.

Maintenance and formation of embryogenic callus

After inoculation, 10 days (d) old calli were transferred to CIM for their maintenance, proliferation and embryogenic callus formation. The calli age of 3-4 weeks (w) after inoculation were visually observed and the qualitative features on color and texture were recorded; along with the weight was determined as quantitative features. The frequencies of callus induction (CI) were calculated by using the following formula.

$$\text{Callus induction (\%)} = \frac{\text{Number of induced callus}}{\text{Number of inoculated seed}} \times 100$$

Plant regeneration and shoot elongation

The calli age of 3-4 w, derived from immature and mature embryos were transferred separately to regeneration media (RM) for plant regeneration. Three basal media, such as MS, N6 and 6N1 were individually supplemented with different concentrations and combinations of IAA (0.0, 0.5, 1.0, 1.5 mg/l) and/or BAP (0.5, 1.0, 1.5, 2.0 mg/l) as shown in Table 3. Regenerated shoots, length of 2 cm were transferred to RM for shoot elongation. After 4 w of transfer, the lengths of shoots were measured and the shoot lengths were determined by deducting 2cm from the measured length.

Rooting and acclimatization

Regenerated shoots, height of 3-4 cm were transferred to root induction media (RIM). In this case, the basal media MS, N6 and 6N1 were individually supplemented with IAA (0.0, 0.5, 1.0, 1.5 mg/l) and/or BAP (0.5, 1.0, 1.5, 2.0 mg/l). The well rooted plantlets were transferred to plastic cups containing autoclaved soil and sealed with polythene bags. After acclimatization, plants were transferred to natural condition for further study and seed collection.

Data recording and statistical analysis

The average or mean values were computed from five replicates with standard error (SE) and each experiment was repeated thrice. Analysis of variance (ANOVA) and list significant difference (LSD) was done by SPSS 16.0 software.

III. RESULTS

Effect of media and growth regulators to callus induction

From immature embryos, the variety Mohar showed maximum CI (75.28%) in N6 + 1.5 mg/l 2,4-D; and the lowest value (29.48%) was recorded in 6N1+ 0.5 mg/l for the same variety (Table 1). Mohar also performed with the highest frequency of CI (77.16%) when mature embryos were cultured in the same medium and hormone level. The variety Khoi bhutta exhibited the lowest callusing from mature embryo in 6N1 + 0.5 mg/l. The effect media and hormonal levels on CI, the varieties showed significant difference at P<0.01. Callus fresh weights were determined; and the highest value was recorded in Mohar (15.74 g) when embryo derived calli were cultured for further proliferation in N6+1.5 mg/l 2,4-D (Table 2). It was also observed that approximately similar weight (15.14 g) was found in IE derived calli for the same

variety and same media with hormonal condition. On the other hand, the lowest weight of callus (2.58 g) was measured in 6N1 + 0.5 mg/l from ME for Shuvra. On qualitative point of view, the effect of different media and hormonal levels were observed on the morphology of induced calli. The calli of three different colors i.e. whitish, off-white and creamiest were produced by various media and hormonal combinations (Table 2). Besides, textures of calli were also varied, and two types of textures (friable and compact) were found in the developed calli. It was clearly observed that both explants i.e. immature and mature embryos, produced similar color of callus in the same medium with same 2,4-D concentration, except 6N1+1.5 mg/l. In this case, callus color was off-white for immature embryos and creamiest for mature.

Effect of media and growth regulators to plant regeneration

Efficient regenerations of four varieties were evaluated on three basal media; such as MS, N6 and 6N. The results showed that Shuvra performed with the highest frequency (77.44%) in MS + 1.0 BAP + 0.5 IAA, when the calli were derived from IE (Table 3, Fig. 1). The other genotypes Barnali, Mohar and Khoi bhutta exhibited 76.16, 73.65, and 72.62% regeneration in the same medium and hormone. The lowest value (20.77%) was recorded for Khoi bhutta in 6N1 + 0.05 BAP. To observe the effect of growth regulators on shoot elongation, four types of hormonal combinations were tested; and the highest length of shoot was recorded for Shuvra (20.47 cm) in MS + 1.0 mg/l BAP + 0.5 mg/l IAA (Table 3, Fig. 1g). In the same medium 18.14, 17.46 and 15.13 cm shoot lengths were recorded from IE in Barnali, Mohar and Khoi bhutta respectively. The variety Khoi bhutta gave the lowest length (2.63 cm) in 6N1 + 0.0 mg/l IAA + 0.5 BAP. For well root formation, three basal media with hormones were tested; and the highest frequency was obtained from the shoots developed from IE of Shuvra (76.07%) when those were placed on MS + 0.1 mg/l BAP + 0.5 mg/l IAA (Table 4, Fig. 1h). The lowest rooting (23.75%) was recorded for the same variety in 6N1 + 0.5 mg/l BAP. For the same explant, the highest root length was recorded in Mohar (22.88 cm) on MS + 1.0 mg/l BAP + 0.5 mg/l IAA; and the lowest value was measured in Khoi bhutta (2.95 cm) on 6N1 + 0.5 mg/l BAP. In case of ME, Mohar showed 82.84% rooting in MS + 1.0 BAP + 0.5 IAA and root length was 24.64 cm in 1.0 mg/l BAP + 0.5 mg/l IAA. Chronologically, in the same medium and hormonal combination, the minimum rooting was recorded in Barnali (25.65%) and root length was in Shuvra (3.78 cm). By using the calli derived from ME, the highest regeneration (76.09%) and shoot length (22.45 cm) was observed in Mohar on MS + 0.1 mg/l BAP + 0.5 mg/l IAA (Table 3). In the same medium and hormonal level, the recorded values on regeneration and shoot length of other varieties were Barnali (74.47% and 16.14 cm), Khoi bhutta (74.34% and 21.39 cm) and Shuvra (73.50% and 15.01 cm) as chronological order. The lowest value of regeneration was found in Shuvra (25.50%) and shoot length was 3.85 cm in Khoi bhutta on 6N1 + 0.0mg/l IAA + 0.5 mg/l BAP.

Survivability of regenerated plantlets

After acclimatization, the plants were cultured in the field of natural condition; and in the periods of culture, the rates of survival plants were decreased. The highest survival rates were recorded from the plantlets which developed by IE derive calli in Mohar as 67.15, 59.99 and 52.69% after 1st, 2nd and 3rd weeks of culture periods respectively (Table 5). On the other hand, for IE, the values were 58.96, 54.02 and 46.84% after 1st, 2nd and 3rd weeks in the same variety. As a whole, the plants produced from ME derived calli survived better than IE.

IV. DISCUSSION

Response to callus induction

Efficiency of IE and ME were evaluated on callusing; and considering the results it was observed that both factors influenced significantly. By analyzing the variance (ANOVA) significant effect of genotype and media including hormonal levels were found to CI (Table 6). The range of CI was 29.48-75.28% among three media for studied varieties; and Mohar showed maximum callusing in N6. Therefore, the medium N6 was best effective than MS and 6N1 to all the responding genotypes. However, Islam et al. [40] reported on significant effect of media and hormones in wheat. Such significance was also found in some previous reports. By using N6 medium 38.1-85% CI was recorded in maize [9], [11] [41]. Present investigation showed similarities with the previous reports, considering N6 medium as better. On the contrary, dissimilarities also found with the findings of Verma et al. [42]. They examined the effect of five media in rice and mentioned the best result in SK3 and MS with different concentrations of 2, 4-D; whereas in N6 the response was very poor. Furthermore, no response was found using Z1 medium for rice culture by Khatun et al. [26]. In culture medium, plant growth regulators and their concentration are the critical factors; generally high concentration of auxins and low concentration of cytokinins promote abundant cell proliferations to induce callus [30]-[31]. In our study, auxin (2,4-D) was used singly at various concentrations; and the level 1.5 mg/l of 2,4-D exhibited good callusing for studied varieties. Dhillon et al. [13] reported the highest frequency of CI (88.7%) on MS medium supplemented with picloram 10.0 mg/l and 2,4-D 3.0 mg/l. Our findings is that, 1.5 mg/l concentration of 2,4-D can performed with highest frequency. Jia et al. [7] recorded 38-43% callusing on MS supplemented with 3.0 mg/l 2,4-D. By using LS basal salts and B5, both supplemented with 3 mg/l 2,4-D, maximum 90.0 and 52.5 % CI were recorded respectively [43]. On addition of picloram, 2,4-D and BAP with MS, 42.06-88.06% CI was recorded in maize [44]. In another reports, using MS with 2,4-D the results were 85% and 97.30% in maize [45] and *Simmondsia chinensis* [46] respectively. The auxins, especially 2,4-D in the range of 1-3 mg/l are essential for better embryogenic callus formation from embryos of cereal crop plants [47]-[50]. Haque and Islam [22] mentioned the media effect on callusing in barley. Seventy percent of CI was recorded in N6 supplemented with 1.50

mg/l 2, 4-D, 3% sucrose, 10 mg/l silver nitrate, 2.87 mg/l L-proline and 0.1 g/l casein hydrolysate [51]. Ninety percent CI was mentioned at different media and hormonal combinations in maize by Pathi et al. [19]. Approximately same efficiency of CI was also described in some reports [14]-[16]. However, according to our findings, it could be suggested that single doses of 2, 4-D (1.5 mg/l) with MS, enhanced callus induction using ME in maize varieties. It was also observed that different media and 2,4-D levels including genotype affected callusing significantly. Genotypic effect along with the age of embryos and 2,4-D levels was tested on callusing [6], [51]. In 2014 Kaushal et al. [52] expressed highly significance of genotype to CI in rice. We observed such similar findings and claimed that a wide range of variations were expressed in the tested varieties, both in mature and immature embryos. It might be happened due to different genotypes along with the maturity and immaturity of the embryos. However, the tested factors affect callusing directly and their partial influence could be able to develop the wide range of difference on CI performance. Weight of callus was depended on the growth of callus; and was varied significantly in different media and hormonal levels. We measured the weights of calli as range of 2.58-15.74g. The highest value was on N6 + 1.5 2,4-D for Mohar in IE and MEs. By using MS with 2,4-D and kinetin, callus growth was promoted and gained increased weight of callus in rice [42]. Islam [21] reported on the effect of age of embryoids in wheat. Our suggestion is that, growth as well as the weight of callus partially depends on genotype, hormonal levels and maturity of embryos. It was observed that the morphology of induced callus was varied qualitatively. Three different colors i.e. whitish, off-white and creamiest were found in the calli developed in various media and hormonal combinations (Table 2). A specific concentration of 2,4-D produced a specific color to the calli derived from immature and mature embryos. Moreover, variations on the textures of calli were also found; and two types of textures i.e. friable and compact were recorded. Considering the features of our findings, no specific reason was assigned that affects the texture. In previous reports, the colors of calli were visualized as white, cream and light yellow; and two types of textures (compact and friable) were observed in maize [53], [54].

Response to plant regeneration

The calli derived from IE, showed significant variability on plant regeneration (Table 6). The range of regenerations was recorded as (20.77-77.44%) which expressed the wide range variations among the varieties (Table 3). Used media, BAP and IAA combinations and different varieties might develop the variability. Maximum performances of plant regeneration were recorded in MS supplemented with 0.5 mg/l IAA + 1 mg/l BAP. Hence, compared to other media, MS supplemented with 1 mg/l BAP + 0.5 mg/l IAA is regarded as optimal medium for regeneration of tested maize varieties. Pathi et al. [7] reported 35-90% plant regeneration by using IE in MS with growth regulators. Fourteen plantlets out of 17 have been regenerated in *Zea mays* L. through somatic embryogenesis [55]. The efficiency of plant regeneration

was examined by using ME derived calli; and the results were varied significantly among the varieties as range of around 25-75 percent. In previous reports, an efficient regeneration of 35–39% in MS [7] and 16-21% in LS [43] were reported by using ME for maize respectively. The range regeneration could be developed from various factors used including maize genotype. The effect of genotype on plant regeneration was expressed in maize [14] and wheat [21]. In 2014 Siddique et al. [23] reported that genotype affects to plant regeneration significantly in rice. Moreover, similar reports have been published for maize [9], [49], [53] and barley [22].

Elongation of shoot

By using IE derived calli, the highest and lowest shoot lengths were measured as 20.47 cm in MS + 1.0 mg/l BAP + 0.5 mg/l IAA for Shuvra and 2.63 cm for Khoi bhutta in 6N1 + 0.5 BAP respectively (Table 4). On the other hand, from ME, the highest elongated shoot length of 22.45 cm was found for Mohar in MS + 1.0 mg/l BAP + 0.5 mg/l IAA; and lowest was 3.85 cm in 6N1 + 0.5 BAP for Khoi bhutta. Previously, Rani et al. [56] examined the effect of BAP with GA₃ for the plant *Murraya koenigii* L. and showed that proliferated shoots were elongated highest in 2.5 mg/l BAP + 0.4 mg/l GA₃. Our findings argue with the report and stated that higher concentration of BAP (2.5 mg/l) was not suitable for shoot elongation in Maize. The dissimilarity might be happened due to different hormones of and /or different genotypes. Therefore, it could be noticed that different media and hormonal combinations together with the different genotype affected regeneration phenomena and created dissimilarities. For in vitro culture of maize, most reports express betterment of IE than ME. But availability of IE is limited to specific season in Bangladesh, while ME is needed to be used frequently. In this study, by using ME, highest 77.16% CI has been gained in N6 + 1.5 mg/l 2,4-D for Mohar; and on an average of four 2,4-D levels, ME also performed higher, comparison to IE in N6 (Table 1, Fig. 4a). Moreover, higher performance was found to plant regeneration, shoot elongation and root formation using ME in MS with suitable hormonal combinations as describe earlier (Table 3-4, Fig. 4c-e). The plants, developed from ME, survived with maximum rate of 52.69% after 3w of transplantation to field for Mohar (Table 5, Fig. 4f). Hence, it could be suggested that mature embryos might be used successfully for *in vitro* culture, especially for variety Mohar.

V. CONCLUSION

Food security is the burning issue in the world and also in Bangladesh. Hence, it is needed to improve food crops as well as maize cultivars, applying biotechnological approaches. This study has been developed an efficient system for *in vitro* culture of maize. Out of tested four varieties, Mohar showed the best callusing, and the medium N6 with 1.5 mg/l 2,4-D influenced callus induction more effectively than MS and 6N1. On the other hand, for plant regeneration MS + 0.5 mg/l IAA + 1 mg/l BAP is better than N6 and 6N1; especially for Shuvra (77.44%) from immature embryos, and Mohar (76.09%)

for mature embryo culture. The developed system might be helpful to *in vitro* culture of maize and related advance research on biotechnology.

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Table 1: Effect of 2, 4-D on callus induction of four varieties.

Embryos	Media	2, 4-D mg/l	Callus induction (% ± SE)			
			Barnali	Mohar	Khoi bhutta	Shuvra
Immature embryo	MS	0.5	43.14 ± 0.82	56.34 ± 0.70	56.34 ± 0.70	45.19 ± 0.67
		1.0	61.66 ± 0.79**	67.30 ± 0.82**	66.22 ± 0.99*	56.75 ± 0.89**
		1.5	66.72 ± 0.88**	73.12 ± 0.76**	68.21 ± 0.86*	61.36 ± 0.95**
		2.0	52.59 ± 0.95**	62.72 ± 0.82**	62.72 ± 0.82*	54.00 ± 0.96*
	N6	0.5	53.12 ± 0.92	62.48 ± 0.91	54.57 ± 0.97	44.83 ± 0.79
		1.0	69.97 ± 0.87**	72.33 ± 0.76*	69.84 ± 0.92**	57.58 ± 0.86**
		1.5	73.59 ± 0.79**	75.28 ± 0.79*	72.33 ± 0.76**	67.22 ± 0.76**
		2.0	60.28 ± 0.83*	66.31 ± 0.97 ^{NS}	63.31 ± 0.90*	53.92 ± 0.93*
	6N1	0.5	30.93 ± 0.77	29.48 ± 1.12	33.45 ± 0.89	36.68 ± 0.79
		1.0	42.91 ± 0.78**	45.01 ± 0.80**	48.47 ± 0.85**	49.87 ± 0.76**
		1.5	48.66 ± 0.92**	52.60 ± 0.78**	56.23 ± 0.92**	56.81 ± 0.73**
		2.0	35.82 ± 0.80**	36.89 ± 0.87**	38.03 ± 0.70*	43.34 ± 0.85**
Mature embryo	MS	0.5	41.75 ± 0.86	52.88 ± 0.95	41.63 ± 0.78	40.28 ± 0.91
		1.0	59.66 ± 0.89**	66.52 ± 0.96**	55.22 ± 0.91**	56.75 ± 0.89**
		1.5	64.74 ± 0.96**	72.49 ± 0.90**	68.87 ± 0.76**	59.52 ± 0.94**
		2.0	50.43 ± 0.97**	59.69 ± 0.99**	52.75 ± 0.85**	51.78 ± 0.98**
	N6	0.5	51.93 ± 0.99	64.36 ± 0.99	46.18 ± 0.22	46.03 ± 0.80
		1.0	67.30 ± 0.98**	74.04 ± 0.81**	56.93 ± 0.29**	60.15 ± 0.80**
		1.5	71.57 ± 0.88**	77.16 ± 0.93**	71.08 ± 0.71**	69.16 ± 0.91**
		2.0	58.14 ± 0.96**	68.36 ± 0.85*	52.95 ± 0.75*	62.67 ± 0.92**
	6N1	0.5	28.52 ± 0.90	25.64 ± 0.94	25.48 ± 0.77	29.25 ± 0.94
		1.0	40.42 ± 0.97**	40.87 ± 0.84**	40.23 ± 0.77**	43.78 ± 0.88**
		1.5	48.66 ± 0.92**	49.92 ± 0.96**	43.29 ± 0.92**	49.36 ± 0.80**
		2.0	33.47 ± 0.96**	44.92 ± 0.90**	36.18 ± 0.78*	37.59 ± 0.82**

Concentration of 0.5 mg/l 2,4-D considered as control. Significance of mean difference from controls: p < 0.05*, p < 0.01** and NS = Non significant according to LSD analysis.

Table 2: Effect of 2, 4-D on callus weight, color and texture of four maize varieties.

Embryos	Media	2, 4-D (mg/l)	Fresh weight of callus (g)				Texture of the callus	Color of the callus
			Barnali	Mohar	Khoi bhutta	Shuvra		
Immature embryo	MS	0.5	8.97 ± 0.84	9.13 ± 0.83	7.87 ± 0.82	7.90 ± 0.78	Friable	Creamiest
		1.0	11.51 ± 0.95**	12.18 ± 0.85*	10.91 ± 0.94*	10.78 ± 1.01*	Compact	Creamiest
		1.5	12.53 ± 0.92**	13.06 ± 0.78**	12.86 ± 0.85**	11.86 ± 0.90*	Compact	Creamiest
		2.0	10.00 ± 0.80*	11.20 ± 0.72*	9.40 ± 0.79 ^{NS}	9.31 ± 0.92 ^{NS}	Friable	Off-white
	N6	0.5	9.30 ± 0.81	9.57 ± 0.81	8.83 ± 0.76	8.77 ± 0.89	Compact	Off-white
		1.0	12.11 ± 0.96*	12.33 ± 0.90**	10.67 ± 0.88*	11.58 ± 0.97*	Compact	Creamiest
		1.5	13.88 ± 0.76**	15.14 ± 0.75**	13.77 ± 0.96**	12.60 ± 0.99**	Compact	Creamiest
		2.0	10.93 ± 0.95*	11.13 ± 0.92*	9.73 ± 0.88 ^{NS}	9.53 ± 0.85 ^{NS}	Compact	Whitish
	6N1	0.5	5.65 ± 0.75	6.55 ± 0.92	4.21 ± 0.55	3.18 ± 0.83	Friable	Whitish
		1.0	9.40 ± 0.74**	10.23 ± 0.76**	8.77 ± 0.98**	6.86 ± 0.76*	Friable	Off-white
		1.5	11.93 ± 0.91**	12.13 ± 0.86**	10.58 ± 0.86**	8.06 ± 0.90**	Friable	Off-white
		2.0	7.24 ± 0.70*	8.18 ± 0.73*	7.05 ± 0.89*	5.96 ± 0.95*	Friable	Whitish
Mature embryo	MS	0.5	9.03 ± 0.83	8.73 ± 0.70	7.63 ± 0.81	6.90 ± 0.67	Compact	Creamiest
		1.0	12.04 ± 0.86*	11.84 ± 0.90*	10.78 ± 0.98*	9.05 ± 0.99*	Compact	Creamiest
		1.5	13.06 ± 0.87**	13.39 ± 0.67**	13.39 ± 0.77**	10.60 ± 0.96**	Compact	Creamiest
		2.0	11.20 ± 0.81*	12.06 ± 0.75*	9.98 ± 0.86*	7.65 ± 0.74 ^{NS}	Friable	Off-white
	N6	0.5	10.30 ± 0.87	10.02 ± 0.79	9.10 ± 0.87	9.10 ± 0.87	Compact	Off-white
		1.0	13.24 ± 0.89*	12.60 ± 0.90 ^{NS}	11.20 ± 0.90*	12.18 ± 0.85*	Compact	Creamiest
		1.5	15.39 ± 0.99**	15.74 ± 0.90**	14.44 ± 0.88**	13.21 ± 0.74**	Compact	Creamiest
		2.0	11.73 ± 0.96 ^{NS}	13.53 ± 0.84*	10.06 ± 0.88 ^{NS}	10.26 ± 0.83*	Compact	Whitish
	6N1	0.5	6.25 ± 0.70	3.90 ± 0.71	3.13 ± 0.67	2.58 ± 0.63	Friable	Whitish
		1.0	10.18 ± 0.80**	7.19 ± 0.97**	6.48 ± 0.81*	7.19 ± 0.67**	Friable	Off-white
		1.5	12.46 ± 0.90**	10.73 ± 0.98**	7.22 ± 0.77**	9.17 ± 0.81**	Compact	Creamiest
		2.0	8.04 ± 0.75*	9.03 ± 0.65**	6.18 ± 0.74*	4.88 ± 0.75*	Friable	Whitish

Concentration of 0.5 mg/l 2,4-D was considered as control. Significance of mean difference from controls: p < 0.05*, p < 0.01** and NS = Non significant according to LSD analysis.



Table 3: Effect of three basal media and growth regulators on plant regeneration and shoot elongation.

Media	BAP	IAA	Plant regeneration (% ± SE)				Length of shoot (cm ± SE)			
			Barnali	Mohar	Khoi bhutta	Shuvra	Barnali	Mohar	Khoi	Shuvra
MS	0.5	00	64.00±0.99	61.14±0.88	56.18±0.83	66.05±0.82	12.70±0.98	11.07±0.93	8.13±0.93	12.21±0.83
	1.0	0.5	76.16±0.90	73.65±0.84	72.62±0.85	77.44±0.70	18.14±0.88	17.46±0.78	15.13±0.92	20.47±0.99
	1.5	1.0	73.23±0.97	70.27±0.96	64.82±0.91	75.18±0.94	15.10±0.85	15.23±0.78	13.37±0.84	17.44±0.85
	2.0	1.5	68.71±0.91	64.07±0.99	61.05±0.71	68.96±0.94	13.03±0.89	13.42±0.64	11.93±0.99	16.61±0.75
N6	0.5	00	43.73±0.94	44.11±0.84	43.67±0.84	57.10±0.84	10.30±0.93	9.02±0.99	6.50±0.88	10.87±0.92
	1.0	0.5	66.26±1.05	61.41±0.91	59.99±0.84	72.91±0.73	15.22±0.96	15.27±0.86	12.18±0.97	17.27±0.99
	1.5	1.0	58.73±0.95	57.08±0.88	51.46±0.97	68.35±0.71	14.22±0.80	13.94±0.84	9.71±0.85	15.90±0.86
	2.0	1.5	50.86±0.74	52.69±0.97	47.53±0.95	62.12±0.95	13.04±0.96	12.40±0.90	7.30±0.86	13.70±0.84
6N1	0.5	00	28.05±0.94	30.65±0.88	20.77±0.92	32.14±0.76	6.48±0.93	7.06±0.97	2.63±0.65	5.45±0.93
	1.0	0.5	58.31±0.91	51.12±0.78	46.15±0.99	57.65±0.73	11.75±0.77	12.2±0.97	9.88±0.78	9.15±0.70
	1.5	1.0	51.74±0.97	43.11±0.99	42.72±0.87	53.41±0.95	9.68±0.87	10.80±0.98	7.65±0.89	7.13±0.71
	2.0	1.5	46.40±0.88	37.85±0.80	34.41±0.77	40.28±0.78	7.70±0.80	8.37±0.93	6.43±0.61	6.08±0.98
Immature embryo derived calli										
MS	0.5	00	58.73±0.95	64.22±0.97	59.93±0.76	60.17±0.81	11.00±0.98	14.12±0.84	12.77±0.78	10.27±0.90
	1.0	0.5	74.47±0.95	76.09±0.93	74.34±0.98	73.50±0.94	16.14±0.88	22.45±0.99	21.39±0.91	15.01±0.89
	1.5	1.0	70.06±0.86	73.50±0.88	69.75±0.90	69.05±0.84	13.24±0.96	20.75±0.89	17.74±0.95	13.77±0.90
	2.0	1.5	66.66±0.96	70.86±0.97	65.10±0.91	63.44±0.84	12.60±0.83	18.04±0.98	16.12±0.73	11.75±0.97
N6	0.5	00	42.74±0.89	52.94±0.96	45.59±0.94	41.63±0.80	8.20±0.87	11.05±0.89	9.85±0.84	7.75±0.76
	1.0	0.5	60.76±0.84	67.15±0.90	62.35±0.98	60.76±0.84	12.72±0.80	19.67±0.98	16.99±0.91	13.12±0.67
	1.5	1.0	51.16±0.82	61.24±0.91	58.96±0.84	51.32±0.98	10.21±0.93	17.53±0.89	14.53±0.89	11.71±0.90
	2.0	1.5	45.24±0.90	56.27±0.85	52.96±0.97	46.85±0.96	9.35±0.87	14.03±0.95	10.87±0.82	10.38±0.95
6N1	0.5	00	24.95±0.89	30.80±0.88	26.21±0.97	25.50±0.80	5.45±0.85	5.12±0.90	3.85±0.53	6.15±1.11
	1.0	0.5	53.16±0.82	54.02±0.96	52.71±0.67	56.23±0.93	11.02±0.97	12.45±0.99	10.77±0.95	10.68±0.99
	1.5	1.0	46.04±0.93	46.84±0.99	44.82±0.94	48.68±0.98	9.25±0.80	10.02±0.98	8.31±0.89	8.91±0.75
	2.0	1.5	35.30±0.74	38.80±0.92	37.21±0.83	40.41±0.98	6.77±0.79	7.90±0.91	7.77±0.87	7.10±0.97

Mature embryo derived calli

Values are average of five replicates (percentage); SE= Standard error; BAP= 6-benzul aminopurine; IAA= Indole-3-acetic acid; MS= Murashige and Skoog, 1962; N6= Chu, 1978; 6N1= Genovesi, 1990.

Table 4: Effect of three basal media and growth regulators on root formation

Embryos	Media	BAP	IAA	Root formation (% ± SE)			
				Barnali	Mohar	Khoi bhutta	Shuvra
Immature embryo regenerated plants	MS	0.5	00	41.28 ± 0.89	49.54 ± 0.95	52.90 ± 0.86	47.54 ± 0.95
		1.0	0.5	71.97 ± 0.90	75.90 ± 0.86	72.00 ± 0.91	76.07 ± 0.83
		1.5	1.0	64.84 ± 0.92	67.52 ± 0.66	65.44 ± 1.00	69.40 ± 0.90
		2.0	1.5	56.08 ± 0.98	60.07 ± 0.82	56.44 ± 0.97	59.25 ± 1.01
	N6	0.5	00	41.86 ± 1.01	52.62 ± 0.97	39.97 ± 0.69	40.05 ± 0.96
		1.0	0.5	65.33 ± 0.96	60.25 ± 0.88	64.84 ± 0.92	66.10 ± 0.94
		1.5	1.0	56.61 ± 0.90	57.64 ± 0.83	55.78 ± 0.88	58.52 ± 0.97
		2.0	1.5	52.33 ± 0.93	54.56 ± 1.15	52.93 ± 0.86	52.93 ± 0.86
	6N1	0.5	00	25.93 ± 0.83	27.75 ± 0.84	25.44 ± 0.90	23.75 ± 0.92
		1.0	0.5	51.29 ± 0.82	48.75 ± 0.85	52.29 ± 0.93	54.56 ± 0.76
		1.5	1.0	40.86 ± 0.96	39.59 ± 0.73	38.87 ± 0.91	40.42 ± 0.82
		2.0	1.5	36.29 ± 0.84	33.85 ± 0.71	34.87 ± 0.99	37.47 ± 0.97
Mature embryo regenerated plants	MS	0.5	00	45.81 ± 0.97	52.13 ± 0.90	42.41 ± 0.87	50.92 ± 0.90
		1.0	0.5	75.23 ± 0.92	82.84 ± 0.94	73.86 ± 0.95	76.10 ± 0.83
		1.5	1.0	67.61 ± 0.89	71.73 ± 0.87	68.06 ± 0.81	68.94 ± 1.00
		2.0	1.5	58.39 ± 0.96	64.86 ± 0.92	56.08 ± 0.98	60.07 ± 0.82
	N6	0.5	00	43.72 ± 0.80	42.66 ± 0.98	41.83 ± 0.96	42.80 ± 0.88
		1.0	0.5	68.15 ± 0.93	75.03 ± 0.92	67.17 ± 0.91	65.77 ± 0.89
		1.5	1.0	58.03 ± 0.87	67.62 ± 0.85	57.02 ± 0.84	58.52 ± 0.97
		2.0	1.5	54.25 ± 0.80	56.26 ± 0.92	52.93 ± 0.86	53.12 ± 0.84
	6N1	0.5	00	25.65 ± 0.84	26.03 ± 0.99	27.31 ± 0.81	27.20 ± 0.70
		1.0	0.5	56.69 ± 0.77	50.54 ± 0.81	55.57 ± 0.84	51.29 ± 0.82
		1.5	1.0	42.58 ± 0.90	41.28 ± 0.95	40.26 ± 0.87	42.12 ± 0.76
		2.0	1.5	38.89 ± 0.96	35.22 ± 0.92	39.39 ± 0.86	36.11 ± 0.69

Values are average of five replicates (percentage); SE= Standard error; BAP= 6-benzul aminopurine; IAA= Indole-3-acetic acid; MS= Murashige and Skoog, 1962; N6= Chu, 1978; 6N1= Genovesi, 1990.

Table 5: Survival percentage of calli regenerated plantlets in the pots under natural conditions.

Source explant	of Variety	NPTS	Survival rate of plantlets in different period of transplantation (% ± SE)		
			After 1 st week	After 2 nd week	After 3 rd week
Immature embryo	Barnali	50	48.68 ± 0.98	45.59 ± 0.94 ^{NS}	38.80 ± 0.92**
	Mohar	50	58.96 ± 0.84	54.02 ± 0.96*	46.84 ± 0.99**
	Khoi bhutta	50	56.23 ± 0.93	53.16 ± 0.82 ^{NS}	41.63 ± 0.80**
	Shuvra	50	52.94 ± 0.96	47.53 ± 0.95**	44.11 ± 0.84*
Mature embryo	Barnali	50	57.08 ± 0.88	56.18 ± 0.83 ^{NS}	43.11 ± 0.99**
	Mohar	50	67.15 ± 0.90	59.99 ± 0.84**	52.69 ± 0.97**
	Khoi bhutta	50	62.35 ± 0.98	58.73 ± 0.95*	50.86 ± 0.74**
	Shuvra	50	51.12 ± 0.78	46.04 ± 0.93*	42.74 ± 0.89*

NPTS: Number of plantlets transplanted in the soil. After 1st week was considered as control. p < 0.05*, p < 0.01** and NS = Non significant according to LSD analysis.

Table 6: ANOVA on the effect of variety, media, hormonal level to callus induction, weight of callus, plant regeneration, shoot length, root formation and survival rate of transplantation in maize.

Experiment (Source of data)	Explants	Source of variation	DF	Mean sum of square
Callus induction (Table 1)	Immature embryo	Variety	3	107.82**
		Media	2	1942.32**
		2, 4-D level	3	793.76**
		Error	39	16.403
	Mature embryo	Variety	3	186.79**
		Media	2	2419.64**
		2, 4-D level	3	926.85**
		Error	39	11.86
Callus weight (Table 2)	Immature embryo	Variety	3	9.36**
		Media	2	51.14**
		2, 4-D level	3	51.82**
		Error	39	0.38
	Mature embryo	Variety	3	17.78**
		Media	2	97.89**
		2, 4-D level	3	54.90**
		Error	39	0.80
Regeneration (Table 3)	Immature embryo	Variety	3	256.68**
		Media	2	2744.13**
		BAP + IAA level	3	792.29**
		Error	39	11.33
	Mature embryo	Variety	3	66.35**
		Media	2	2871.43**
		BAP + IAA level	3	821.97**
		Error	39	10.69
Shoot length (Table 3)	Immature embryo	Variety	3	30.34**
		Media	2	171.64**
		BAP + IAA level	3	77.13**
		Error	39	1.56
	Mature embryo	Variety	3	42.36**
		Media	2	210.48**
		BAP + IAA level	3	89.53**
		Error	39	2.02
Root formation (Table 4)	Immature embryo	Variety	3	10.59 ^{NS}
		Media	2	2300.20**
		BAP + IAA level	3	1240.32**
		Error	39	8.72
	Mature embryo	Variety	3	30.14*
		Media	2	2374.32**
		BAP + IAA level	3	1598.88**
		Error	39	7.66

Survival rate of transplantation (Table 5)	Variety	3	117.67**
	Explant (IE or ME)	1	147.76**
	PT	2	282.24**
	Error	17	8.03

DF = Degrees of freedom, PT = Periods of transplantation, IE= Immature embryo, ME= Mature embryo, Significance: $p < 0.05^*$, $p < 0.01^{**}$ and NS = Non significant.

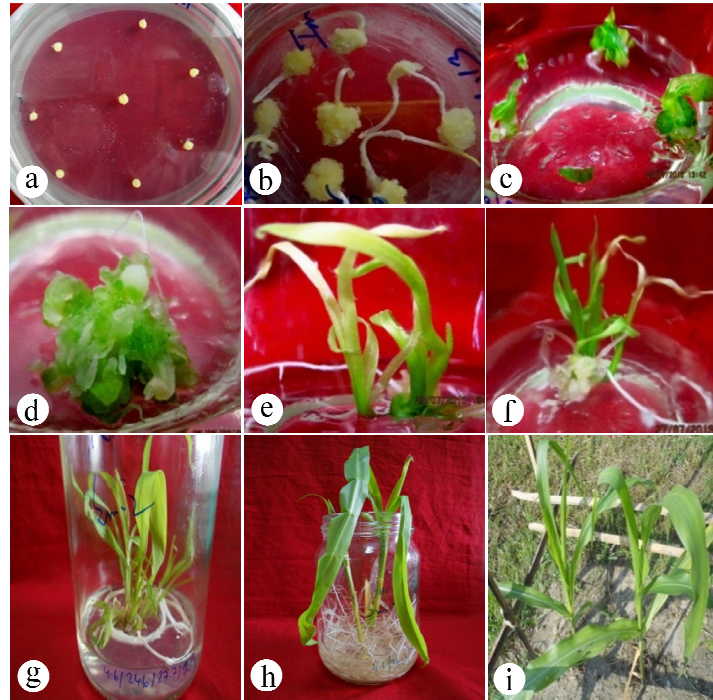


Fig.1. Callus induction and regeneration from immature embryos: (a) Inoculation of embryos (b) Scutellum derived embryogenic callus age of 2-3 weeks, (c-d) Formation of somatic embryos, (e-f) Shoot formation, (g) Shoot elongation, (h) Well rooted regenerated plantlet, (i) Plant transferred to pot after hardening and acclimatization.

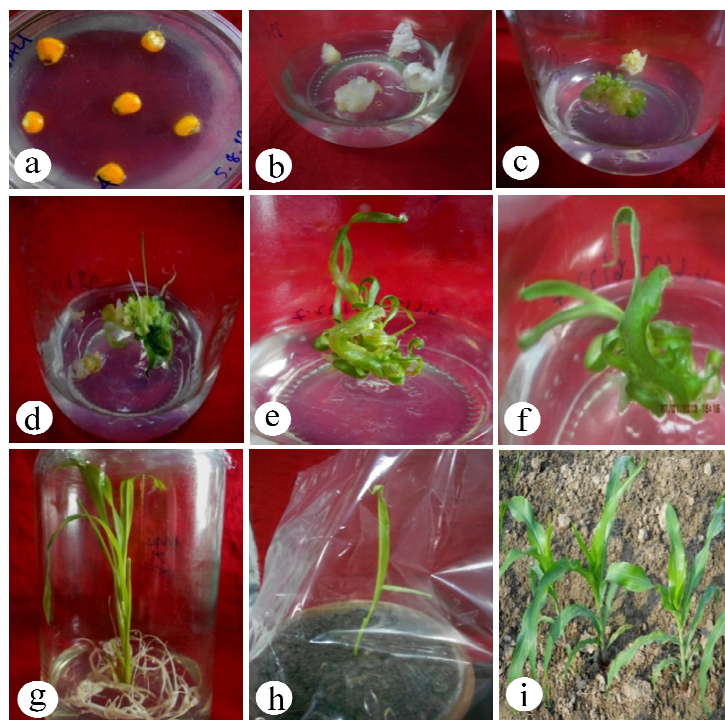


Fig.2. Callus induction and plant regeneration from mature embryos: (a) Inoculation of embryos (b) Scutellum derived embryogenic callus age of 2-3 weeks, (c-d) Formation of somatic embryos, (e-f) Shoot formation, (g) Shoot elongation with well rooted regenerated plantlet, (h) Plant transferred to pot, (i) Field culture after hardening and acclimatization.

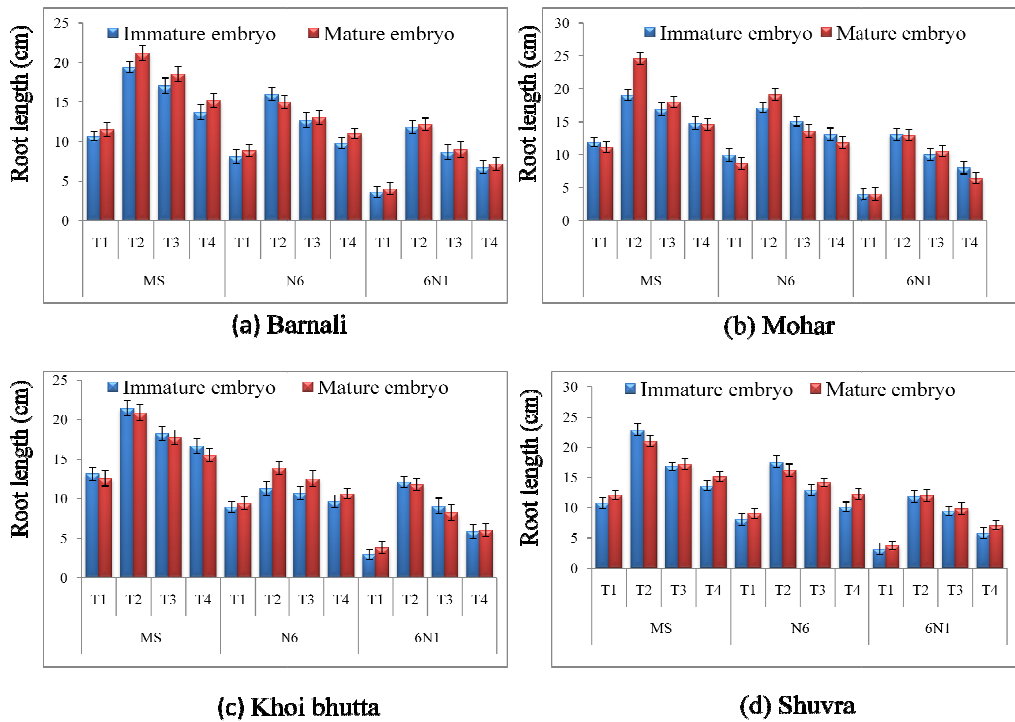


Fig.3. Comparison of immature and mature embryos on root length in studied varieties. T1= 0.5 mg/l BAP + 0.0 mg/l IAA, T2= 1.0 mg/l BAP + 0.5 mg/l IAA, T3= 1.5 mg/l BAP + 1.0 mg/l IAA, T4= 2.0 mg/l BAP + 1.5 mg/l IAA.

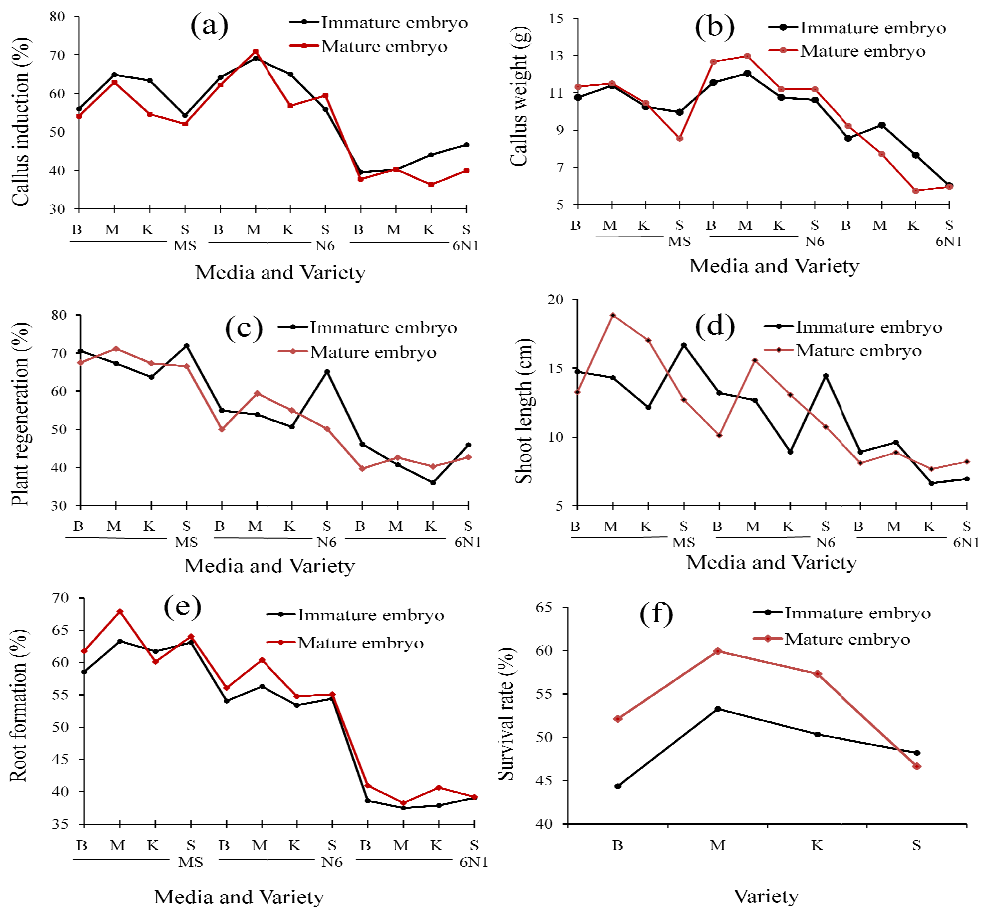


Fig.4. Comparison of immature and mature embryos on callus induction (a), weight of callus (b), plant regeneration (c), length of shoot (d) and root formation (e) and survival rate of plants after transplantation to culture field (e). B= Barnali, M= Mohar, K= Khoi bhutta, S= Shuvra