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EFFECT OF SILVER NITRATE AND CARBON SOURCES ON CALLUS INDUCTION AND REGENERATION IN MAIZE (*Zea mays* L.)

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ABSTRACT

To evaluate the efficacy of silver nitrate and various carbon sources on callus induction and regeneration, four maize varieties of Bangladesh *viz.*, 'Barnali', 'Mohar', 'Shuvra' and 'Khoi bhutta' were evaluated. Four carbon sources either singly or in combination forming seven treatments were tested. The highest callusing (67.1%) was recorded in treatment wherein 2.0% sucrose was used for 'Mohar'. Study on the impact of seven different doses of $AgNO_3$ revealed approximately 1.5-fold higher callus induction in 'Mohar' when 9 mg L^{-1} $AgNO_3$ was added to N6 medium. For regeneration, 3-4 weeks old calli were transferred to plant regeneration medium where IAA 0.5 mg L^{-1} + BAP 1.0 mg L^{-1} were used. Of the four genotypes, 'Mohar' showed highest regeneration (62.1%) in MS medium. Maximum shoot elongation and well-developed root formation were observed in 'Mohar' (15.75 cm) and 'Khoi bhutta' (82.2%), respectively. Higher doses of $AgNO_3$ were found effective in callus induction. Analysis of variance showed significant differences in genotypes with respect to callusing and regeneration efficiency.

Key words: Callus induction, carbon sources, *in vitro* regeneration, silver nitrate, maize

INTRODUCTION

Maize (*Zea mays* L.) is the 3rd most important cereal crop in the world as well as in Bangladesh. It is used as human and animal feed and serves as raw material for the manufacture of a large number of industrial products. Maize is considered a potential biofuel and forage crops. The demand for maize is increasing across the world, especially in Asia (Wada *et al*., 2008). Maize is considered as a complete source of nutrition than other cereals. First successful regeneration through *in vitro* culture of maize was reported by Green and Phillips (1975). Then this technique has been employed to improve the component of variation that can't be found in nature, thereby changing the characteristics of cultivars (Ahlowalia, 1986). By using tissue culture methods successful regeneration of maize has been achieved from immature and mature embryos (Huang and Wei, 2004; Al-Abed *et al*., 2006; Rakshit *et al*., 2010; Dhillon and Dosal, 2013). Good regeneration has also been achieved through embryogenic and organogenic callus culture approaches in maize (Pathi *et al*., 2013; Morshed *et al.*, 2014). Embryogenic and non-embryogenic callus induction is independent of genotypes when immature embryos are used in maize and wheat tissue culture (Akoyi *et al*., 2013; Saha *et al.*, 2015). However, good regeneration capability from embryo derived

calli depends on suitable maize genotype. The effect of media and plant growth regulators (PGRs) on callus induction and its subsequent regeneration has been described as critical factors to regenerate plants *in vitro* (Bennici *et al*., 1988; Khatun *et al*., 2012; Siddique *et al*., 2014; Islam and Bhattacharjee, 2015). Effect of different carbon sources i.e. fructose, sucrose, glucose, sorbitol and maltose has been examined to enhance somatic embryogenesis to maize and other crops e.g. citrus (Kochba *et al*., 1982), banana (Hossain *et al*., 2009), apple (Bahmani *et al*., 2009), alfalfa (Strickland *et al*., 1987), barley (Haque and Islam, 2015) and maize (Swedlund and Locy, 1993). The role of D-sorbitol on growth was reported in maize (Jain et al., 2010). An improved somatic embryogenesis has been reported from immature embryos using silver nitrate as stress factor in maize and other cereal crops (Arora *et al*., 2008; Dhillon and Gosal, 2013; Haque *et al*., 2015; Barbasz *et al*., 2016). To ensure sufficient food production by improving maize cultivar, it is imperative to adopt effective and efficient biotechnological techniques. Perusal of literature reveals that there is no report on improved regeneration testing the effect of different carbon sources along with silver nitrate as chemical pre-treatment factor for maize improvement. Therefore, the present investigation was undertaken to assess the effect of various carbon sources and silver nitrate in media on somatic embryogenesis and their subsequent regeneration using important maize cultivars in Bangladesh.

MATERIALS AND METHODS

Plant materials

Mature seeds of four maize varieties *viz.*, 'Barnali', 'Mohar', 'Shuvra' and 'Khoi bhutta' were collected from Bangladesh Agriculture Research Institute (BARI), Gazipur, Bangladesh for the present study.

Seed sterilization

The seeds were surface-sterilized with 70% (v/v) ethanol for 1.5 min. and in sodium hypochlorite for 5 min. Then the seeds were sterilized by 0.1% (v/v) mercuric chloride for 5 min. and after washing with sterile distilled water inoculated on callus induction media (CIM).

Callus induction

The sterilized seeds were inoculated on three types of CIM *viz.*, MS (Murashige and Skoog, 1962), N6 (Chu, 1975) and MSR (Islam *et al.*, 2001) and individually supplemented with 1.5 mg L^{-1} 2,4-D + 2.87 mg L^{-1} L-proline + 0.1 g L^{-1} casein hydrolysate + 2% sucrose. Inoculated petri-dishes were sealed with paraflim and incubated at 26±2ºC in dark for callus induction. Ten days old calli were sub-cultured upto 3-4 weeks using the same medium. To test the effect of carbons, four types of carbons were added to CIM (N_6 + 1.5 mg L^{-1} 2, 4-D + 2.87 mg L^{-1} L-proline + 0.1 g L^{-1} casein hydrolysate) that divided into two groups: i) single uses: 1% sucrose (C_1), 2% sucrose (C_2), 4% sucrose (C_3), 8% sucrose (C_4) and ii) combined uses: 2% sucrose + 1% glucose (C_5), 2% sucrose + 1% sorbitol (C_6) and 2% sucrose + 1% manitol (C_7). For evaluating the effect of silver nitrate ($AgNO_3$) an abiotic stress factor, seven concentrations of $AgNO_3$ i.e. 3, 6, 9, 12, 15, 18 and 21 mg L^{-1} were added to CIM where 2% sucrose was constant. A treatment without $AgNO_3$ was taken as control.

Plant regeneration

To observe regeneration efficiency, 3-4 weeks old calli were transferred to three regeneration media i.e. MS, N6 and MSR individually supplemented with IAA 0.5 mg L^{-1} + BAP 1.0 mg L^{-1} + 2.0% sucrose. To examined the efficiency of shoot elongation, regenerated shoots length of 2 cm were transferred to MS medium supplemented with three combinations of plant growth regulators

(PGRs), such as 1.0 mg L^{-1} IAA + 1.5 mg L^{-1} BAP, 0.5 mg L^{-1} IAA + 1.0 mg L^{-1} BAP and 1.5 mg L^{-1} IAA + 2.0 mg L^{-1} BAP. After 4 weeks of culturing, the length of shoots was measured, and the elongated values were determined by deducting of 2 cm (the previous length).

Rooting and acclimatization

The shoots length of 3-4 cm were placed on MS medium supplemented with three hormonal combinations T_1 (0.5 ml L^{-1} IAA + 0.5 mg L^{-1} BAP), T_2 (0.5 mg L^{-1} IAA + 0.1 mg L^{-1} BAP) and T_3 (1 mg L^{-1} NAA + 0.5 mg L^{-1} BAP). Plants with sufficient and well-developed roots were washed in tap water and kept in culture bottle at 20±2°C for 5-10 days. These were then transferred to the pot and kept under natural condition for further study and seed collection.

Data recording and statistical analysis

The mean values were computed from four replicates with standard error (SE) where every replication carried out 10-12 explants (seed/callus). The experiments were laid out in a completely randomized design. Analysis of variance, Duncan's multiple range test (DMRT) and least significant differences (LSD) were performed using SPSS16.0 software. The seed derived calli without any pretreatment were considered as control.

RESULTS AND DISCUSSION

Effect of media on callus induction

Among three media tested *viz.*, N6, MS and MSR, the highest callusing was found in N6 for 'Mohar' (68.6%) and lowest (40.2%) in MSR for 'Shuvra' (Fig. 1b; 2). Among the media tested, N6 proved most effective medium for all the responding genotypes; while lowest response was observed in MSR. Guruprasad *et al.* (2011) reported 85% callus induction using MS medium in maize and Kumar *et al.* (2013) recorded 97.3% in *Simmondsia chinensis*. Khatun *et al.* (2012) examined the effect of five different media in rice and found SK3 as the best medium for callus induction. They also reported that the response of N6 was very poor among the media tested, and no response was found for Z1 medium. Present study showed dissimilarities with previous reports and claims N6 as the best medium for callus induction in maize. It seemed that the dissimilarities might be due to the differences in explant material, PGRs and the genotypes. However, previously it has been reported that in a culture medium, PGRs and their concentration are the critical factors. Generally high concentration of auxins and low concentration of cytokinins promote abundant cell proliferations in formation of callus (Bennici *et al.*, 1988). In another report, better callusing has been found by optimal combination of auxin (NAA) and cytokinin (BAP) in *Astemisia annua* (Mohamad *et al.*, 2014). In present study, 1.5 mg L^{-1} auxin (2,4-D) was used singly with three basal media (MS, N6 and MSR), and good callusing was observed in all the tested genotypes. Hence, it could be suggested that N6 with 1.5 mg L^{-1} 2,4-D can be used for better *in vitro* callus induction in maize.

Effect of carbon sources on callus induction

Considering the best performance of N6 medium, the effect of carbon source was examined in the said medium. Four types of carbons divided into seven groups either single or in combination (C_1-C_7) were tested for callusing, and the results varied amongst the studied genotypes. The highest frequency (67.1%) was noticed for 'Mohar' when 2% sucrose was used, followed by 59.7, 54.1 and 51.8% for 'Khoi bhutta', 'Barnali' and 'Shuvra', respectively (Table 1). The lowest frequency was found 'Shuvra' (15.2%) when 2% sucrose + 1% D-manitol were added in callus induction medium.

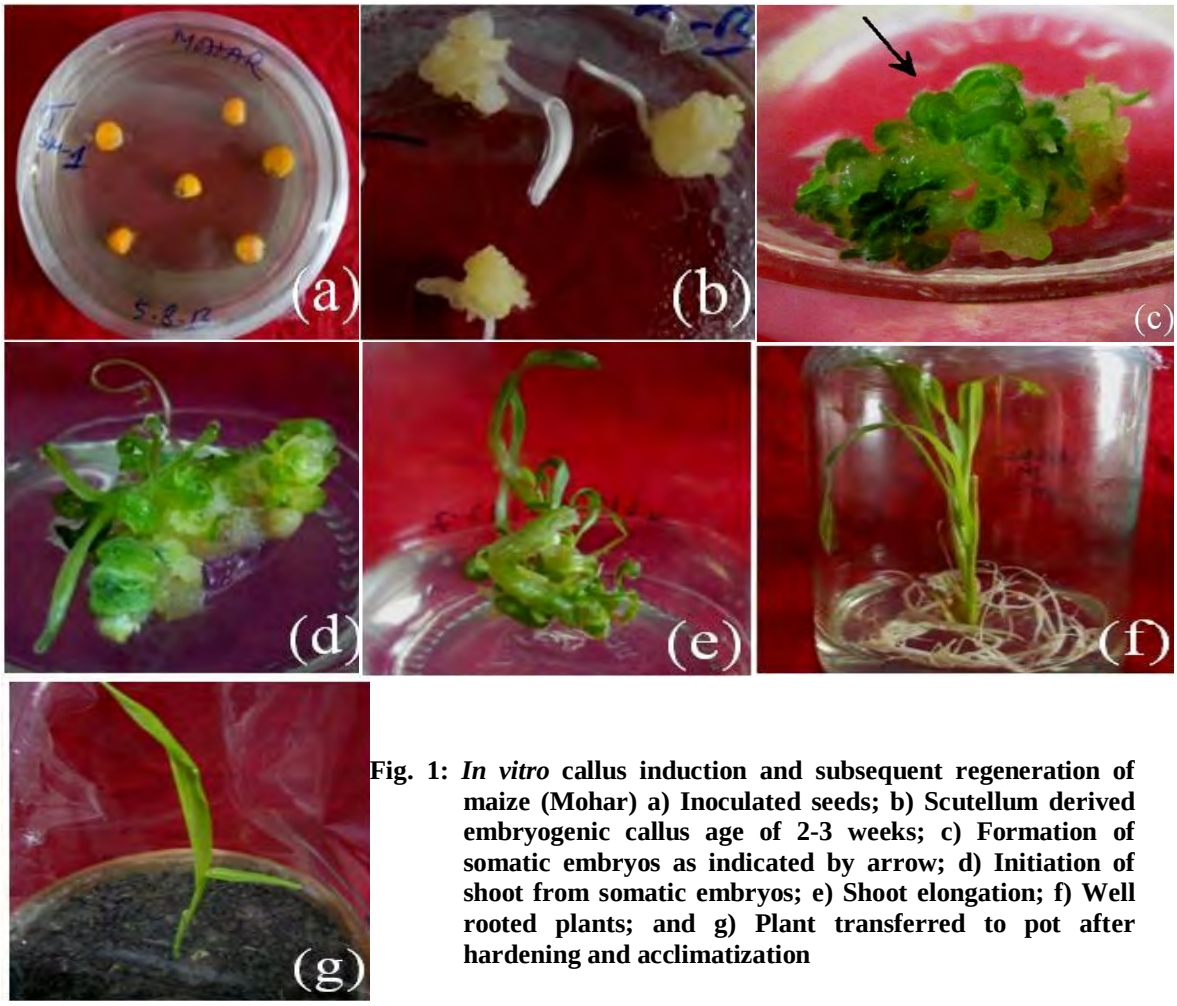

Fig. 1: *In vitro* callus induction and subsequent regeneration of maize (Mohar) a) Inoculated seeds; b) Scutellum derived embryogenic callus age of 2-3 weeks; c) Formation of somatic embryos as indicated by arrow; d) Initiation of shoot from somatic embryos; e) Shoot elongation; f) Well rooted plants; and g) Plant transferred to pot after hardening and acclimatization

Significant differences were observed amongst the varieties as well as in carbon sources (Table 3).

In study on the effect of carbon by addition of seven different combinations of carbons, N6 and 2% sucrose was found the best carbon source to enhance callusing (67.1%). Previously, the effects of fructose, glucose and D-sorbitol have been tested and maximum efficiency has been observed in sucrose (Swedlund and Locy, 1993). However, in the present study no fructose was used. The reports reveal that by using 3% sucrose, glucose and sorbitol singly or in combinations, glucose performed better in banana (Hossain *et al.*, 2009). Highest number of callus induction (81%) was found when 3% sucrose and 1% manitol was used (Hutami *et al.*, 2008). However, the

Table 1: Effect of carbon sources on callus induction in studied genotypes (% ± SE)

Treatment (carbons)	Variety			
	Barnali	Mohar	Khoi bhutta	Shuvra
1% sucrose	45.24 ± 2.36^{bc}	55.35 ± 1.14^{d}	53.51 ± 2.02^{b}	37.48 ± 2.33^{c}
2% sucrose	54.14 ± 1.11^{a}	67.09 ± 1.75^{a}	59.72 ± 1.06^{a}	51.76 ± 1.42^{a}
4% sucrose	48.12 ± 2.33^{b}	65.61 ± 2.17^{b}	59.21 ± 0.62^{a}	46.73 ± 1.80^{b}
8% sucrose	46.08 ± 1.75^{b}	60.39 ± 1.10^{c}	51.33 ± 1.66^{c}	35.81 ± 1.12^{c}
2% sucrose + 1% glucose	46.00 ± 0.57^{b}	47.45 ± 1.71^{e}	45.31 ± 2.34^{d}	29.20 ± 1.57^{d}
2% sucrose + 1% sorbitol	42.17 ± 2.22^{c}	46.98 ± 1.81^{e}	40.16 ± 1.79^{e}	21.60 ± 0.68^{e}
2% sucrose + 1% manitol	38.90 ± 1.78^{d}	37.65 ± 1.27^{f}	38.00 ± 1.73^{f}	15.20 ± 0.64^{f}

Basal N6, 1.5 mg L^{-1} 2,4-D, 2.87 mg L^{-1} L-proline and 0.1 g L^{-1} casein hydrolysate was constant. In each column the mean values followed by the same letter are not significantly different at $P \leq 0.05$ as per DMRT.

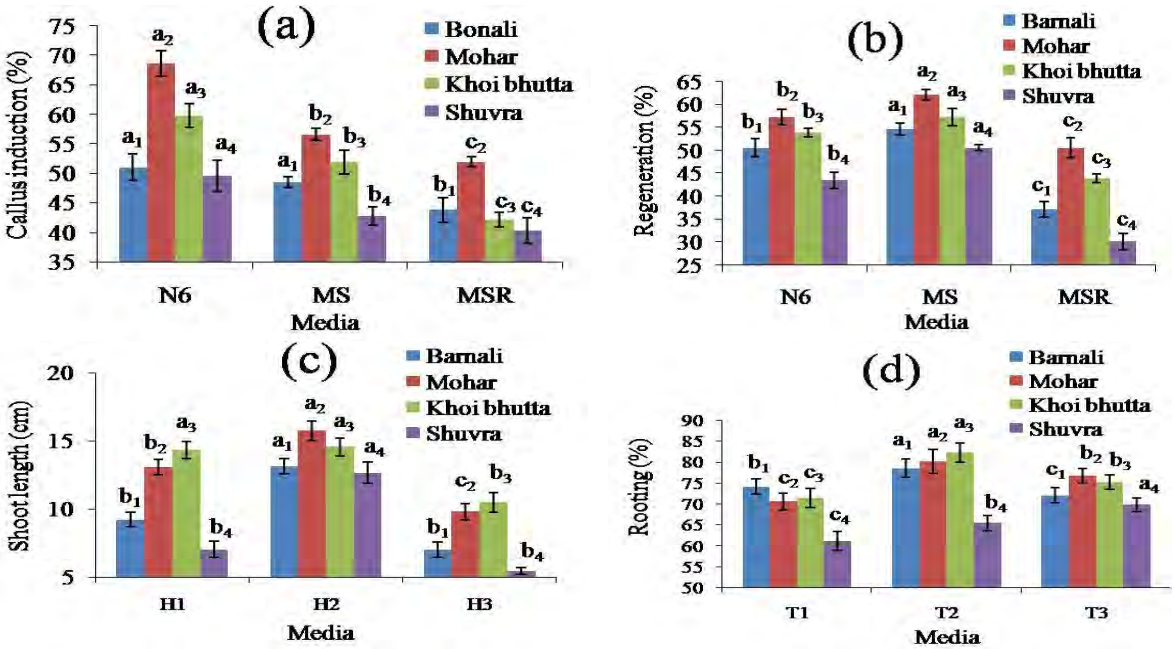

Fig. 2: a) Effect of media on a) Callus induction; b) Plant regeneration; c) Average shoot length after transfer of plantlets on shoot elongation media; d) Root formation of four maize genotypes. Different letters with same suffix indicate significant difference at P≤0.05 as per DMRT analysis

effect of maltose and 2,4-D on callusing was evaluated and found the clear relationship between them (Jia, 2008). In this investigation callusing range was 15.2-67.1% where 2% sucrose singly performed best to enhance callus induction in maize. The results are approximately similar to some previous reports, yet dissimilarities are also been found. It might be attributed to the differences in media including carbon sources, PGRs and/or genotypic variations. Genotypic effect on callusing was reported in maize (Akoyi *et al*., 2013). Significant effect of genotypes, age of embryos on callus formation has also been investigated (Ombori *et al*., 2008). They obtained 70% callus induction using 3% sucrose along with 10 mg L^{-1} silver nitrate. In some reports approximately 90% callus induction was reported in maize (Huang and Wei, 2004; Al-Abed *et al*., 2006; Dhillon *et al*., 2012; Pathi *et al*., 3013).

Effect of silver nitrate on callusing

The study on the effect of silver nitrate on callus induction revealed that out of the 7 concentrations of $AgNO_3$ used, maximum callusing was in 9 mg L^{-1} added in N6 medium for all the responding genotypes with maximum in 'Mohar' (78.5%), followed by 'Khoi bhutta' (74.5%), 'Barnali' (70.7%) and 'Shuvra' (62.6%) [Table 2]. In addition of 6 mg L^{-1} $AgNO_3$ in MS, 'Mohar', 'Khoi bhutta', 'Barnali' and 'Shuvra' gave their maximum callusing of 71.2, 68.4, 65.1 and 58.3%, respectively. In comparison to controls, the values were 1.19-1.34 and 1.20-1.33 fold higher in case of N6 and MS media, respectively. The differences were significant for media, varieties and $AgNO_3$ concentrations at P≤0.01 (Table 3). Applying seven doses of $AgNO_3$ in MS and N6 media individually gave 1.20-1.33 and 1.19-1.34 folds higher callus induction in 9 mg L^{-1} $AgNO_3$ as compared to the control, respectively (Table 2); hence it might be expressed that enhanced callus induction. Through use of 10 mg L^{-1} $AgNO_3$ enhanced callusing has previously been reported (Varhanikova *et al*., 2013). By testing three concentrations (2.5, 10.0 and 20.0 mg L^{-1}) of $AgNO_3$ 1.2-1.3 folds increase has been described by Dhillon (2012) which is in agreement with our findings. However, 56-80% callus induction was reported by using 15 mg L^{-1} $AgNO_3$ in N6 and 89-91% from 1.0 mg L^{-1} $AgNO_3$ (Zhu *et al.*, 2011). In this study, it is has been established that an optimal dose of $AgNO_3$ (9.0 mg L^{-1}) is effectively and positively enhanced callus induction in maize genotypes.

Table 2: Effect of silver nitrate on callus induction on N6 and MS media (% ± SE) in maize

Media	Silver nitrate (mg L^{-1})	Varieties			
		Barnali	Mohar	Khoi bhutta	Shuvra
N6	0.0	53.31 ± 1.62	65.72 ± 1.48	57.77 ± 1.13	46.72 ± 1.38
	3.0	62.63 ± 1.44**	66.79 ± 2.01^{NS}	61.06 ± 1.13*	56.88 ± 0.89**
	6.0	68.03 ± 1.15**	72.53 ± 1.68**	69.79 ± 1.92**	62.02 ± 2.49**
	9.0	70.66 ± 1.17**	78.51 ± 2.15**	74.53 ± 2.31**	62.63 ± 0.87**
	12.0	65.72 ± 0.61**	70.93 ± 1.18*	68.03 ± 1.72**	57.88 ± 2.12**
	15.0	61.73 ± 1.09**	68.03 ± 1.74^{NS}	62.13 ± 1.73*	52.51 ± 2.53**
	18.0	52.03 ± 1.01^{NS}	68.03 ± 2.30^{NS}	53.93 ± 1.68*	52.67 ± 1.48**
	21.0	43.64 ± 1.05**	57.86 ± 1.17**	50.51 ± 1.45**	42.77 ± 0.89*
MS	0.0	50.51 ± 0.46	53.31 ± 2.17	56.93 ± 1.96	44.31 ± 2.26
	3.0	61.00 ± 2.30**	66.88 ± 1.60**	62.63 ± 2.18**	52.00 ± 0.58**
	6.0	65.13 ± 2.31**	71.25 ± 2.31**	68.43 ± 1.07**	58.26 ± 2.23**
	9.0	62.13 ± 2.83**	65.72 ± 1.18**	63.88 ± 3.09**	57.03 ± 2.89**
	12.0	51.03 ± 1.57^{NS}	57.13 ± 1.46**	54.06 ± 2.34*	53.93 ± 1.77**
	15.0	50.93 ± 0.92^{NS}	51.77 ± 2.01^{NS}	53.03 ± 0.60**	51.64 ± 0.51**
	18.0	50.64 ± 2.60^{NS}	51.64 ± 1.52^{NS}	53.95 ± 1.52*	48.51 ± 2.24**
	21.0	46.31 ± 1.20^{NS}	50.51 ± 0.66*	50.51 ± 1.03**	46.20 ± 1.15^{NS}

N6 and MS media both supplemented with 1.5 mg L^{-1} 2,4-D + 2.87 mg L^{-1}, L-proline + 0.1 g L^{-1} casein hydrolysate + 2% sucrose. Significance of mean difference from controls; P $\leq$0.05*, P $\leq$0.01** and NS = Non- significant as per LSD analysis.

Effect of media and genotypes on regeneration

For plant regeneration, three basal media *viz*., MS, N6 and MSR were tested; and among the four genotypes 'Mohar' depicted highest performance (62.1%) in MS (Fig. 1c-e, 2b). The other genotypes 'Khoi bhutta', 'Barnali' and 'Shuvra' gave 57.2, 54.6 and 50.5% regeneration in the same medium (MS), while lowest value was observed in 'Shuvra' on MSR (30.1%). For efficient elongation of shoots, three types of hormonal combinations were added to MS and the highest elongated shoot was found in 'Mohar' (15.75 cm) in 0.5 mg L^{-1} IAA + 1.0 mg L^{-1} BAP treatment

Table 3: Analysis of variances (ANOVA) on the effect of media, silver nitrate and carbon sources to callus induction and plant regeneration in four maize genotypes

Purpose	Factors	Source of variation	Sum of square	Degrees of freedom	Mean sum of square
Callus induction	Media	Media	334.599	2	167.300**
		Variety	366.970	3	122.323**
		Error	49.820	6	8.303
		Total	31373.583	12	-
	Silver nitrate	Variety	1062.755	3	354.252**
		Media	546.332	1	546.332**
		$AgNO_3$	2606.388	7	372.341**
		Error	579.856	52	11.151
		Total	222064.352	64	-
	Carbon sources	Carbon	1987.340	6	331.223**
		Variety	1579.413	3	526.471**
		Error	299.436	18	16.635
		Total	62764.187	28	-
Regeneration	Media	Variety	519.27	2	259.63**
		Media	377.85	3	125.95**
		Error	28.37	6	4.73
		Total	925.49	11	-

Significance: P $\leq$ 0.01**

(Fig. 2c). In the same hormonal combination, 13.17, 14.57 and 12.67 cm shoots were noticed in 'Barnali', 'Khoi bhutta' and 'Shuvra', respectively. The genotype 'Shuvra' gave lowest length (5.45 cm) in 1.5 mg L^{-1} IAA + 2.0 mg L^{-1} BAP treatment. For root formation, the highest frequency of rooting was found in 'Khoi bhutta' (82.2%) when grown on MS + 0.5 mg L^{-1} IAA + 0.1 mg L^{-1} BAP treatment (Fig. 2d). The lowest value was recorded for 'Shuvra' (61.2%) in MS + 0.5 mg L^{-1} IAA + 0.5 mg L^{-1} BAP treatment. Reports indicate 35-90% regeneration in maize on MS medium and 16-21% on LS medium (Jia *et al*., 2008; Dhillon and Gosal, 2013; Pathi *et al.*, 2013). Fourteen plantlets out of 17 have been regenerated through somatic embryogenesis (González *et al.*, 2012). Our findings argued with most of the previous reports and we claim that efficient regeneration could be gained in MS medium if supplemented with IAA 0.5 mg L^{-1} + BAP 1.0 mg L^{-1} + 2.0% sucrose. Hence, the dissimilarities appear due to the differences in media and hormones including maize genotype. The effect of genotype on regeneration has been proved in maize previously (Huang and Wei, 2004). However, the present study suggested MS medium with 0.5 mg L^{-1} IAA and 1.0 mg L^{-1} BAP gains efficient regeneration of Bangladeshi maize cultivars. The highest elongated shoot length of 15.75 cm was seen in 0.5 mg L^{-1} IAA + 1.0 mg L^{-1} BAP for 'Mohar'. Effect of BAP + GA_3 has been tested in *Murraya koenigii* and maximum elongation reported at 2.5 mg L^{-1} BAP + 0.4 mg L^{-1} GA_3 (Rani *et al*., 2012). Our study revealed that higher concentration of BAP (2.5 mg L^{-1}) was not suitable for shoot elongation in maize. The findings might differ because of GA_3 and IAA and/or genotypes.

Conclusion: For agriculture development in cereal crops, it is desired to improve maize varieties using advance biotechnological approaches. In present study 'Mohar' showed highest callusing (67.1%) and regeneration (62.1%) under Bangladesh conditions. Sources of various carbons affected callusing and 2% sucrose was able to enhance callus induction. Silver nitrate, an abiotic stress factor, was also able to enhance callusing. The basal medium N6 proved better in inducing callus from Bangladeshi maize cultivars while for regeneration MS medium proved promising over other genotypes. The investigated protocol of efficient callusing and regeneration may helpful for advanced biotechnological research to develop maize cultivars.

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