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Full Length Research Paper

Callus induction and regeneration of elite Indian maize inbreds

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Five elite Indian maize inbreds namely; HKI1105, HKI1105, HKI335, CM300 and LM5 were evaluated for callus induction and regeneration. Immature embryos obtained 14 days after pollination were used as explants. Genotype, medium, type of auxin and their concentrations influenced callus induction. N_6 medium supplemented with different concentration of 2,4-D (1, 2 and 3 mg/l) and Dicamba (1, 2 and 3 mg/l) were used for callus induction. N_6 supplemented with 2 mg/l of 2,4-D has shown highest percentage of embryogenic callus induction. Among the five genotypes tested, CM300 gave highest percentage of embryogenic calli. CM300 and LM5 both have shown higher regeneration percentage of 12.22%.

Key words: Maize, *in-vitro* culture and regeneration.

INTRODUCTION

Maize (*Zea mays* L.) is the most important cereal crop in the world in terms of global annual tons produced (Food and Agricultural Organization, 2009). Maize is raw material for a number of industrial products besides its uses as human food and animal feed. At present, the developed world uses maize more than the developing world, but forecasts indicate that by the year 2020, the developing countries will demand more maize than the developed world (Duvick, 1998). One of the strategies to mitigate various stresses in maize is development of transgenic maize. Genetic transformation of maize with genes conferring resistance to biotic/abiotic stresses is expected to address many of these issues synergistically with conventional breeding.

Green and Philips (1975) first reported regeneration of maize from immature embryos. Since then, maize regeneration has been reported from immature embryos (Duncan et al., 1985; Bohorova et al., 1995; Ishida et al., 1996; Aguado-Santacruz et al., 2007, Rakshit et al., 2010), mature embryos (Huang and Wei, 2004; Al-Abed et al., 2006), nodal regions (Vladimir et al., 2006), leaf tissues (Conger et al., 1987; Ahmadabadi et al., 2007), anthers (Ting et al.,1981; Barloy and Beckert, 1993), tassel and ear meristem (Pareddy and Petolino, 1990), protoplast (Morocz et al., 1990) and shoot meristem (Sairam et al., 2003). Immature embryos are predominantly used for establishing regeneration competent cells or callus cultures for genetic transformation (Ahmadabadi et al., 2007). Gordon Kamm et al. (1990) first developed transgenic maize for bialophos resistance. Koziel et al. (1993) developed insect-resistant transgenic maize with *Cry1Ab* for the first time. Monsanto has actively involved in transgenic research for drought tolerance in maize, and is scheduled to commence commercial sales of a transgenic drought tolerance product in 2012 (Edmeades, 2008). However, maize genotypes adapted to temperate regions have been used in these studies on regeneration and transformation (Prioli and Silva, 1989; Bohorova et al., 1995). To harness the benefits of genetic transformation in breeding programme under tropical and subtropical Indian climatic conditions, it is important to develop protocols of regeneration and transformation for Indian maize inbreds. Therefore, the objectives in the present study were to establish a reproducible regeneration protocol for well adapted Indian maize inbred lines and to compare the efficiency of different sources of

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Abbreviations: 2,4-D, 2,4-Dichlorophenoxyacetic acid; **Dicamba,** 2 methoxy-3,6-dichlorobenzoic acid; **BAP,** 6-benzylaminopurine; **NAA**, naphthaleneacetic acid; **IAA,** indole-3-acetic acid; MS, Murashige and Skoog medium.

Table 1. Characteristics of maize inbred lines.

S/N	Characteristics	HKI 1105	HKI 1126	CM 300	HKI 335	LM 5
1	Tassel: Time of anthesis	Medium	Late	Medium	Early	Late
2	Ear: Time of silk emergence	Medium	Late	Late	Early	Late
3	Ear:Anthocyanin colouration of silks	Absent	Present	Absent	Absent	Absent
4	Leaf:Anthocyanin colouration of sheath	Absent	Absent	Present	Absent	-
5	Ear: Shape	Cylindrical	Conical	Conical	Conical	-
6	Ear: Type of grain	Flint	Flint	Flint	Flint	Flint
7	Ear: Colour of top of grain	Orange	Yellow	White	Yellow	Yellow
9	Kernel: Row arrangement	Straight	Straight	irregular	Irregular	Straight
10	Kernel: Shape	Round	Round	Round	Toothed	-
11	Source	CCS HAU, Karnal	CCSHAU, Karnal	DMR, New Delhi	CCS HAU, Karnal	PAU,Ludhiana

auxins on callus induction and regeneration in Indian inbred lines.

MATERIALS AND METHODS

Plant materials

Five well adapted tropical Indian maize inbred lines namely: HKI1105, HKI335, HKI1126, LM5 and CM300 (Table 1) were used in the study. These lines are from diverse genetic background and are parental lines of many promising maize hybrids. These lines were planted in the green house, Directorate of Maize Research, New Delhi. Plants were self pollinated and the whole ears were collected 14 days after pollination. Immature kernels were extracted and washed with Tween-20 (1 - 2 drops) followed by surface-sterilization with sodium hypochloride (0.6%) for 20 min. Subsequently, immature kernels were washed with 70% ethanol for 30 sec and rinsed five times with sterile water. Immature embryos of 1.0 - 2.0 mm size were aseptically excised from surface sterilized kernels under laminar flow and placed with scutellar side up and flat surface down on the callus induction medium solidified with 0.8% agar.

Callus induction

N_6 medium was used for callus induction (Chu et al., 1975) supplemented with three levels (1, 2 and 3 mg/l) of 2,4-dichlorophenoxyacetic acid (2,4-D) and three levels of (1, 2 and 3 mg/l) Dicamba with pH adjusted to 5.8 prior to autoclaving at 121°C (108 kPa) for 20 min. Thirty explants per treatment were taken in three replications. Explants were incubated in dark for 24 h at 28°C. Then, these were transferred to 16 h photoperiod, 50 - 70 $\mu E/m^2/s$ light intensity, and 28°C. After two weeks, number of explants producing primary callus were recorded. Calli were sub-cultured onto fresh medium of the same composition after 15 - 20 days.

Regeneration

After one month, the embryogenic calli transferred onto R1 maturation medium (MS + Sucrose 60 gm/l) for three weeks. Every ten days, the regenerated calli were sub cultured on fresh medium. The calli were transferred on regeneration medium without any hormones for two weeks. After two weeks, the calli were transferred onto R2-1 shooting medium (MS + IAA 0.5 mg/l + BAP 1.0 mg/l). After 10 days they were transferred onto R2-2 rooting medium (MS + NAA 1 mg/l) for one week. Plantlets with well developed roots were transferred overnight to 1/2 strength liquid MS medium (pH 5.8) without sucrose and then transferred to pots containing sterilized soil (cocopeat, vermiculite and sand, 6:3:4) for acclimatization, under 16 hr photoperiod for seven days. Following acclimatization, plants were moved to the greenhouse for further growth.

Data analysis

Percent callus induction and regeneration was calculated. The percentage values transformed using arcsin transformation (Table 2). The callus induction and regeneration data was subjected to Analysis of Variance (ANOVA). The transformed values were used for Tukey's test (Freeman and Tukey, 1950; Compton, 1994).

RESULTS AND DISCUSSION

The present work focuses on devising a standard protocol for regeneration of tropical Indian maize inbreds. Although standard protocols for regeneration are available for temperate maize worldwide but not many reports are available for tropical maize. Since the pioneering work of Green and Phillips (1975), several protocols for *in vitro* culture of maize had been developed (Rice et al., 1978; Springer et al., 1979; Torne et al., 1980; Ting et al., 1981; Armstrong and Green, 1985; Green 1982; Lu et al., 1982, 1983; Rhodes et al., 1982, 1986; Sachs et al., 1982; Santos et al., 1984; Suprasanna et al., 1986; Conger et al., 1987; Pareddy and Petolino, 1990; Ray and Ghosh 1990; Songstad et al., 1992; Zhong et al., 1992; O'Connor-Sánchez et al., 2002; Zhang et al., 2002; Huang and Wei, 2004; Rakshit et al., 2010).

Maize genotypes have profound differences for *in-vitro* culture (Armstrong and Green, 1985) and only a small number of maize genotypes posses regeneration capacity. Hence, it becomes important to specify growth condition for specific genotypes under *in-vitro* culture to exploit

Table 2. Means percentage of callus induced by genotypes in different combination of auxin.

Genotype	N_6 1+ 2,4-D (1 mg/L)	N_6 1+ 2,4-D (2 mg/L)	N_6 1+ 2,4-D (3 mg/L)	N_6 2 + Dicamba (1 mg/L)	N_6 2 + Dicamba (2 mg/L)	N_6 2 + Dicamba (3 mg/L)
LM5	55.57 ± 5.50 (29.32)	85.57 ± 2.56 (44.22)	58.90 ± 4.78 (29.90)	51.10 ± 3.25 (25.85)	70.00 ± 5.03 (35.76)	58.90 ± 3.01 (29.89)
HKI1105	45.57 ± 5.74 (24.70)	76.67 ± 5.30 (39.36)	54.43 ± 5.26 (27.58)	42.23 ± 3.78 (21.28)	68.90 ± 3.64 (35.17)	44.43 ± 4.41 (22.42)
CM 300	62.23 ± 4.35 (31.65)	84.43 ± 3.84 (43.61)	63.33 ± 3.94 (32.24)	57.77 ± 2.71 (28.16)	77.77 ± 3.54 (39.95)	61.10 ± 3.33 (31.64)
HKI 335	48.90 ± 4.23 (24.13)	73.33 ± 3.09 (37.55)	51.10 ± 3.82 (25.85)	50.00 ± 4.62 (25.28)	68.90 ± 4.41 (35.17)	54.43 ± 2.09 (27.58)
HKI 1126	44.43 ± 2.65 (22.42)	67.77 ± 3.44 (34.58)	46.67 ± 5.06 (23.56)	41.10 ± 1.99 (20.71)	62.23 ± 4.36 (31.06)	47.77 ± 4.52 (22.99)

Values are mean ± SE. Values in parenthesis are transformed values.

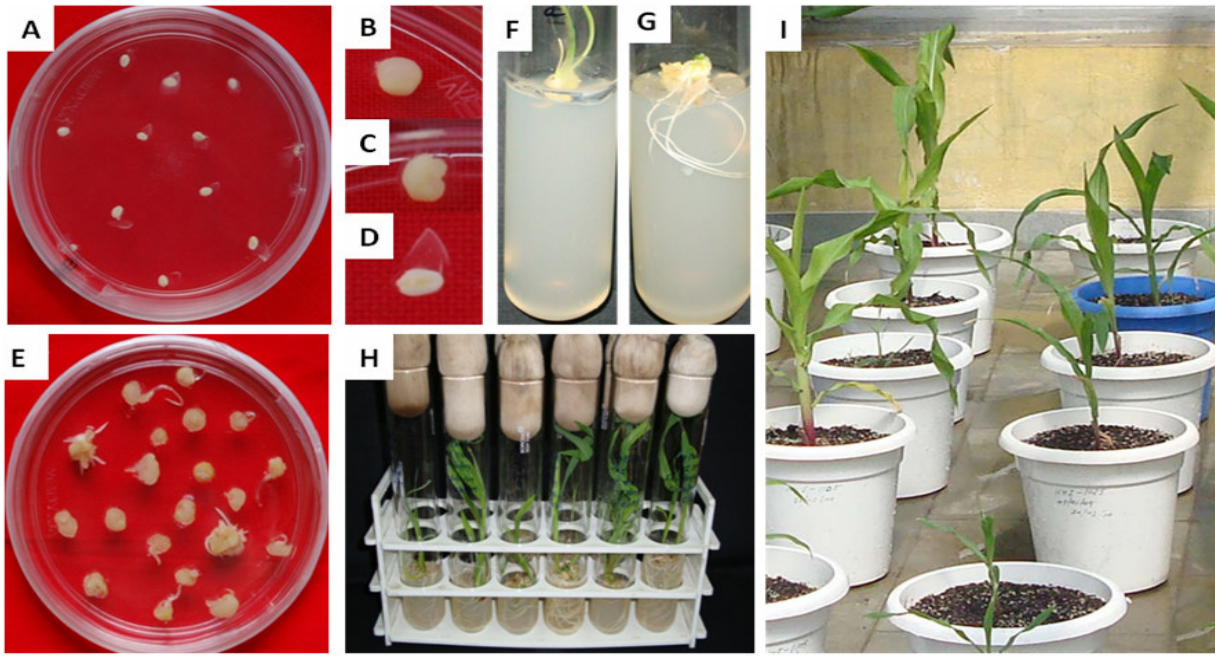

Figure 1. Callus formation and plant regeneration of elite Indian maize inbreds. **(A)** Explants of immature embryo extracted from Inbred CM 300 on N6 1 + 2,4-D (2 mg/l). **(B)** Globular embryo. **(C)** Heart shaped embryo. **(D)** Torpedo shaped embryo of CM 300. **(E)** Type I and Type II calli of LM 5 **(F)** Shoot induction of LM 5 onR2-1 media (MS+IAA 0.5mg/l + BAP 1.0 mg/l. **(G)** Root induction of LM 5 on R2-2 media (MS+NAA 1mg/l). **(H)** Fully regenerated plants with roots and shoots. **(I)** Regenerated plants in green house.

potential tools of *in-vitro* culture namely; doubled haploid, somaclonal variation, genetic transformation and somatic hybridization.

Callus Induction

All genotypes responded best at the concentration of N_6 1 + 2,4-D (2 mg/l) (Figure 1A), followed by N_6 2 + Dicamba (2 mg/l), N_6 2 + Dicamba (3 mg/l), N_6 1 + 2,4-D (3 mg/l), N_6 1 + 2,4-D (1 mg/l) and least at N_6 2 + Dicamba (1 mg/l). Auxin especially 2,4-D in the range of 1-3 mg/l, is essential for embryogenic callus induction from cereal embryos (Bhaskaran and Smith,1990). The result of this study showed that the 2,4-D at 2 mg/l concentration was best for embryogenic callus induction, which concurred with the findings of Armstrong and Green, 1985; Bohorova et al., 1995; Carvalho et al., 1997. Different morphological classes of somatic embryos such as globular, heart and torpedo was observed in all genotypes (Figures 1B - 1D). Immature embryos can initiate two types of callus cultures from their scutella surfaces; Type I and

Table 3. Analysis of variance of callus induction from immature embryos.

Source	DF	SS	MSS	F Value	Prob
Genotypes (G)	4	265.844	66.461	24.31**	0.00
Combination of Hormones (H)	5	910.889	182.178	66.65**	0.00
G X H	20	39.889	1.994	0.73 ^{NS}	
Error	60	164	2.733		
CV (%)	9.31				

** Significant at 1% level; NS= Not Significant.
Df = Degree of freedom; SS = sum of square; MSS = mean sum of square.

type II callus. Type I is compact and organogenic and easily obtained from immature embryo. On the other hand, type II is friable and embryogenic and is initiated at a lower frequency than type I (Carvalho et al., 1997). Only a few tropical genotypes have been shown to be capable of initiating type II callus (Oduor et al., 2006; Carvalho et al., 1997). Type II callus has been found to be more regenerable than type I (Armstrong and Green, 1985; Omer et al., 2008). Mixture of type I and type II calli (Figure 1E) was observed in LM5, HKI1105 and HKI335 genotypes.

Analysis of variance for percent callus induction revealed the genotypes and treatment varying significantly (Table 3). The effect of genotype and different combination of auxin treatments were highly significant ($P \leq 0.01$) indicating that the inbreds have genetic difference (genetic potential) for induction of somatic embryogenesis and the combination of auxin treatments affect the initiation of embryogenic callus. But the genotype-treatment (genotype X treatment) was not significant suggesting there is independent effect of treatment on genotypes.

Based on ANOVA, Tukey's test was conducted to compare all possible pairs of means at 5% significance level (Table 4). Based on Tukey's test, LM5 with $N_6$1 + 2,4-D (2 mg/l) and CM300 with N_6 1 + 2,4-D (2 mg/l) ranked first but other combinations did not show significant difference. All genotypes showed highest performance in N_6 1 + 2,4-D (2 mg/l) and least from N_6 2 + Dicamba (1 mg/l). In general, the callus induction was higher irrespective of genotypes in N_6 1 + 2,4-D (2 mg/l). And CM300 found to be more responsive to callus induction than other genotypes.

Regeneration

Embryogenic callus obtained from N_6 media was transferred for regeneration into R1 media (Maturation medium) which contains MS + Sucrose 60 mg/l for three weeks. Later, these calli were transferred to fresh medium for subculture. These calli were divided into two batches. First batch calli were transferred into MS medium without any hormone, here small regenerated green plantlets with light roots and shoots were observed. Second batch calli were transferred into R2-1 media (MS + IAA 0.5 mg/l + BAP 1.0 mg/l). Here, regenerated plantlets respond well and good shooting percentage (Figure 1G) was observed. This regenerated plantlets again transferred into R2-2 media (MS + NAA 1 mg/l) and good root development (Figure 1F) was observed. This inferred that the 0.5 Auxin: 1 Cytokinin (IAA: BAP) ratio is optimum for shoot development and NAA (1mg/l) for root development (Figure 1H). Similar results have been reported by Bohorova et al.,1995; Kennedy et al., 2001; Slater et al., 2004; Rakshit et al., 2010.

CM300 and LM5 showed a maximum of 12.22% of regeneration followed by HKI335 and HKI1105 (4.44%) and the least was in HKI1126 (3.33%) (Table 5). Analysis of variance revealed genotypic difference which was highly significant for regeneration (Table 6). This implies differential genetic potential for regeneration in tested genotypes. Carvalho et al., 1997; Binnot et al., 2008 reported that not all tropical genotypes that initiated embryogenic calli could regenerate plants and also some genotypes classified as non-embryogenic. They concluded that such a classification does not accurately predict the regenerative ability of a calli from a given genotype. This implies that plant regeneration is achievable in both embryogenic and non-embryogenic genotypes under appropriate tissue culture conditions. Comparing means of regeneration percentage with callus induction percentage (Table 7) showed that CM300 is best among the other geno-types, followed by LM5, HKI335, HKI1105 and HKI1126. The present study has confirmed the differential genetic potential of genotypes for callus induction, somatic embryo formation and regeneration capacity in the Indian maize inbreds.

Conclusion

CM300 is one of the parents of Ganga Safed-2, Ganga

Table 4 . Comparison of means percentage of callus induction by Tukey's test.

Number	Genotype treatment combination	Mean	Rank
1	LM5/ N_6 1 + 2,4-D (2 mg/l)	85.57 ± 2.56 (44.22)	A
2	CM 300/ N_6 1 + 2,4-D (2 mg/l)	84.43 ± 3.84 (43.61)	A
3	CM 300/ N_6 2 + Dicamba (2 mg/l)	77.77 ± 3.54 (39.95)	AB
4	HKI 1105/ N_6 1 + 2,4-D (2 mg/l)	76.67 ± 5.30 (39.36)	ABC
5	CM 335/ N_6 1 + 2,4-D (2 mg/l)	73.33 ± 3.09 (37.55)	ABCD
6	LM5/ N_6 2 + Dicamba (2 mg/l)	70.00 ± 5.03 (35.76)	ABCDE
7	HKI 1105/ N_6 2 + Dicamba (2 mg/l)	68.90 ± 3.64 (35.17)	ABCDEF
8	CM 335/ N_6 2 + Dicamba (2 mg/l)	68.90 ± 4.41 (35.17)	ABCDEF
9	HKI1126/ N_6 1 + 2,4-D (2 mg/l)	67.77 ± 3.44 (34.58)	ABCDEFG
10	CM 300/ N_6 1 + 2,4-D (3 mg/l)	63.33 ± 3.94 (32.24)	BCDEFGH
11	CM 300/ N_6 1 + 2,4-D (1 mg/l)	62.23 ± 4.35 (31.65)	BCDEFGHI
12	CM 300/ N_6 2 + Dicamba (3 mg/l)	61.10 ± 3.33 (31.64)	BCDEFGHI
13	HKI1126/ N_6 2 + Dicamba (2 mg/l)	62.23 ± 4.36 (31.06)	BCDEFGHI
14	LM5/ N_6 1 + 2,4-D (3 mg/l)	58.90 ± 4.78 (29.90)	CDEFGHIJ
15	LM5/ N_6 2 + Dicamba (3 mg/l)	58.90 ± 3.01(29.89)	CDEFGHIJ
16	LM5/ N_6 1 + 2,4-D (1 mg/l)	55.57 ± 5.50 (29.32)	DEFGHIJ
17	CM 300/ N_6 2 + Dicamba (1 mg/l)	57.77 ± 2.71 (28.16)	DEFGHIJ
18	HKI 1105/ N_6 1 + 2,4-D (3 mg/l)	54.43 ± 5.26 (27.58)	EFGHIJ
19	CM 335/ N_6 2 + Dicamba (3 mg/l)	54.43 ± 2.09 (27.58)	EFGHIJ
20	LM5/ N_6 2 + Dicamba (1 mg/l)	51.10 ± 3.25 (25.85)	FGHIJ
21	CM 335/ N_6 1 + 2,4-D (3 mg/l)	51.10 ± 3.82(25.85)	FGHIJ
22	CM 335/ N_6 2 + Dicamba (1 mg/l)	50.00 ± 4.62 (25.28)	GHIJ
23	HKI 1105/ N_6 1 + 2,4-D (1 mg/l)	45.57 ± 5.74 (24.70)	HIJ
24	CM 335/ N_6 1 + 2,4-D (1 mg/l)	48.90 ± 4.23 (24.13)	HIJ
25	HKI1126/ N_6 1 + 2,4-D (3 mg/)	46.67 ± 5.06 (23.56)	HIJ
26	HKI1126/ N_6 2 + Dicamba (3 mg/l)	47.77 ± 4.52 (22.99)	HIJ
27	HKI 1105/ N_6 2 + Dicamba (3 mg/l)	44.43 ± 4.41 (22.42)	IJ
28	HKI1126/ N_6 1 + 2,4-D (1 mg/l)	44.43 ± 2.65 (22.42)	IJ
29	HKI 1105/ N_6 2 + Dicamba (1 mg/l)	42.23 ± 3.78 (21.28)	J
30	HKI1126/ N_6 2 + Dicamba (1 mg/l)	41.10 ± 1.99 (20.71)	J

Values in parenthesis are transformed values. Mean ± SE followed by a letters are not significantly different at 5% level, according to Tukey's test.

Table 5. Mean percentage of regeneration capacity of genotypes.

Genotype	Mean of regeneration (%)
LM 5	12.22 ± 1.923 (19.23)
HKI 1105	4.44 ± 1.928 (11.41)
CM 300	12.22 ± 1.923 (19.23)
HKI 335	4.44 ± 1.928 (11.41)
HKI1126	3.33 ± 0.001 (10.02)

Values are expressed in mean ±SE; values in parenthesis are transformed values.

hybrid-4 and High Starch hybrid. It is tolerant to a number of foliar diseases and is a good pollen shedder. LM5 is a yellow flint type inbred, it is one of the parent of India's first released single cross hybrid Paras. HKI335 is a yellow flint inbred line with good ear traits. HKI1105 is a dual purpose inbred line; which can be used as both male and female parent. It is used as male parent for the Malviya hybrid 2 and HM8 and as a female parent in the HM9 hybrid. Thus, the established regeneration protocol for these lines might possibly be useful in developing

Table 6. Analysis of variance of regeneration capacity of genotypes.

Source	DF	SS	MSS	F value	Probability
Genotypes	4	250.87	62.718	15.28**	0.0008
Replications	2	0.28	0.138	0.03NS	0.967
Error	8	32.85	4.106		
CV %	14.21				
LSD	9.25				

** Significant at 1% level; NS= Not Significant
Df = Degree of freedom; SS = sum of square; MSS = mean sum of square; F = frequency.

Table 7. Comparison of means percentage of callus induction and regeneration.

Genotypes	Mean of callus induction (%)	Mean of regeneration (%)
CM 300	67.77 ± 3.54(34.58)	12.22 ± 1.923 (19.23)
LM 5	63.34 ± 3.95(32.25)	12.22 ± 1.923 (19.23)
HKI 335	57.77 ± 2.71(28.16)	4.44 ± 1.928 (11.41)
HKI1105	55.37 ± 5.26(27.59)	4.44 ± 1.928 (11.41)
HKI 1126	51.66 ± 3.25(25.85)	3.33 ± 0.001 (10.02)

Values are expressed in mean ±SE; values in parenthesis are transformed values.

transgenic maize.

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