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# 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.)

**Alok Abhishek, Chikkappa G. Karjagi*, Ravindra Nath[1], Meenakshi Bhardwaj, Pramod W. Ramteke[2], Pradyumn Kumar, Sain Dass and R. Sai Kumar**

Directorate of Maize Research (ICAR), Pusa Campus, New Delhi; [1]Indian Council of Medical Research, New Delhi; [2]Sam Higginbottom Institute of Agriculture, Technology and Sciences (SHIATS) (Formerly Allahabad Agricultural Institute), Allahabad

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**Abstract**

**The study was undertaken to elucidate the effect of genotypes and age of immature embryo on callus induction, embryogenic type II calli production and regeneration from immature maize embryos. The immature embryos were excised from a selfed ear of ten elite Indian maize inbred lines on 6, 12 and 18 days after pollination. The mean callus induction, embryogenic type II calli production, and regeneration was highest across all the genotypes in 12 days old immature embryos and were 44.25, 26.12, and 11.20 per cent respectively. The significant differences were observed with respect to callus induction, embryogenic type II calli production and regeneration capacity between genotypes. The highest regeneration capacity was observed by immature embryo at 12 days after pollination (DAP) of genotype HKI1105 and CM300.**

**Key words:** Explants, embryogenic type II callus, genotype, maize and regeneration

## Introduction

Maize (*Zea mays* L.) is one of the important food crops in the World with highest production and productivity as compared to other cereal food crops. In India it is being cultivated on 8.71 mha, which produces 22.26 mt of maize with an average productivity of 2.56 t/ha (DAC NET. 2014), however the 2.56 t/ha is quite low as compared to global average maize productivity of >5 t/ha [1, 2]. Further, the demand for maize in India is increasing continuously across the world, and more predominantly in Asia. The main driving force for increased maize demand in recent years is due to its diverse uses [3]. Therefore to meet the increased demand for maize, India has made considerable progress in developing high yielding cultivars especially single cross maize hybrids in the last ten years, but the effort to decrease the losses caused due to pests and diseases are not satisfactory. The major insect pests which reduce the maize yield are *Chilo partellus, Sesamia inferans, Atherigona soccata*. In order to reduce the yield losses, there is need to develop insect pest resistant cultivars to achieve potential productivity of cultivars. In this regard the successes through conventional plant breeding methods were not quite successful to develop insect resistant cultivars.

The application of molecular biology tools and techniques like genetic transformation of maize with genes conferring resistance to different kinds of insects will not only overcome the barriers of conventional breeding but also will help in developing resistant cultivars. Regeneration of maize is pre-requisite for executing genetic transformation of maize with genes of interest. Therefore there have been continuous concerted efforts by many laboratories to develop or identify maize inbred lines which are able to produce embryogenic callus with good regeneration ability. Two types of embryogenic callus namely Type I - slow growing, compact/non-friable, harder with characteristic yellow colour and Type II - fast growing, friable, soft with characteristic white colour are being

*Corresponding author's e-mail: chikkappagk@gmail.com
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obtained by *in-vitro* culture of different explants. Type II callus is most suitable for obtaining large number of callus by rapid sub-culturing due to its friable nature thus helps to meet the demand of large number of calli required for routine transformation experiment especially in the absence of adequate greenhouse facility.

Several tissue culture techniques/regeneration protocols for maize have been developed by using different ex-plants *viz.,* immature embryos [4-7], mature embryos [8-9], nodal regions [10], leaf tissues [11], anther [12] tassel and ear meristem [13], protoplast [14] and shoot meristem [15]. But, most of the above studies used temperate lines which were adapted to temperate conditions and less attention was given for assessing the regeneration potential of maize germplasm adapted to the tropical and subtropical climate especially the Indian subcontinent. Because in India, most of the maize germplasm being cultivated were adapted to tropical conditions. Therefore, to harness the benefits of genetic transformation there is need to develop regeneration protocols to successfully execute genetic transformation by using suitable genes of interest suited to maize germplasm adapted to tropical and sub-tropical climate. In this context screening of elite Indian maize inbred lines/genotypes for *in-vitro* whole plant regeneration is the first logical step to identify donor genotype for genetic transformation. Therefore the present investigation was formulated with the objective to identify right stage/age of an immature embryo across different genetic background of tropical maize inbred lines/genotypes with high response to regeneration.

## Materials and methods

### *Plant material*

Ten elite Indian maize inbred lines *viz*., HKI193-1, HKI335, HKI1025, HKI1105, HKI1126, HKI1128, LM5, LM13, CM124 and CM300 developed from different maize research centers in India were used in the study. The selected lines are the parental lines of many promising Indian maize hybrids with diverse genetic background. The inbred lines were sown @ of 10 seeds per line in greenhouse at Directorate of Maize Research, New Delhi at regular interval of 25 days during *kharif* 2010 to *kharif* 2011. The individual plants of all the inbred lines allowed to grow until flowering and were self-pollinated.

### *Immature embryo culture and callus induction*

The immature embryos from the self pollinated ears were collected at different interval that is at 6, 12 and 18 days after pollination (DAP) to explore the right stage of immature embryos with optimum response for regeneration. Kernels aseptically extracted and surface-sterilized under laminar flow with sodium hypochlorite (0.6%) for 20 min and rinsed five times with sterilized distilled water. Immature embryos of 1.0-2.0 mm size were placed with scutellar side up and flat embryo axis side surface down in contact with callus induction medium N61 solidified with 0.3% gelrite. The maize callus initiation and maintenance media were based on N6 medium and vitamins [6, 16] with added 2.76 mg $L^{-1}$ L proline; 36.7 mg $L^{-1}$ FeNaEDTA; 0.1 g $L^{-1}$ myo-inositol; 0.1 g $L^{-1}$ casein hydrolysate; 2 mg $L^{-1}$ 2, 4-D; 3% sucrose; 0.3% gelrite and 0.015 g $L^{-1}$ silver nitrate autoclaved and adjusted to pH 5.8. The plates were wrapped with parafilm and foil and incubated at 27$^{o}$C in darkness for 4 weeks [17]. After 4 weeks, type II embryogenic calli from each genotype were scored by recording number of embryo cultured, total number of embryos in which callus induced and number of embryogenic type II callus induced.

### *Somatic embryo formation and callus culture maintenance*

After 4 weeks the embryogenic type II calli were transferred to maturation medium which was essentially the same as the callus induction media except that it was devoid of hormones and had 60 g$L^{-1}$ of sucrose. The maintenance of calli cultures were carried out by transferring onto fresh maturation medium and kept for incubation under darkness at 27°C for 3 weeks. The embryogenic competence for each genotype was evaluated by recording number of calli that converted into somatic embryos. However, to avoid contamination calli sub-culturing was done every week by transferring onto fresh maturation medium and incubating under darkness at 27°C.

### *Regeneration and acclimatization of plants*

Matured calli were transferred on MS media [18] with minor modification by addition of shooting supplement *i.e.,* BAP (1 mg $L^{-1}$) and IAA (0.5 mg $L^{-1}$) for proper shooting. Regenerated shoots were transferred to MS medium supplemented with NAA (1 mg $L^{-1}$) for rooting. Then the plantlets with healthy roots were transferred to ½ strength MS solution for hardening process for overnight. The well rooted plantlets were then

transferred to pots containing sterilized mixture of 3:1 vermiculate and soil and grown in culture room under humid condition for two weeks to acclimatize in growth chamber. They were further transferred to 12 inches pots in green house and grown up to maturity. Culture room conditions were maintained at 25±3°C with 16 hr photoperiod provided by cool white fluorescent bulbs (50 $\mu$mols $s^{-1}m^{-2}$). The plantlets were then transferred into small containers containing peat covered with a polythene bag and kept in a growth chamber for two week for adaptation before transferring to the green house. They were further transferred to 12 inches pots in green house and grown up to maturity.

### *Experimental design and statistical analysis*

The experiment was conducted in Petri plates and pot culture at laboratory and green house respectively. Three sets (replications) of experiment were carried out separately resembling a completely randomized design. The raw data was obtained by counting the number of embryos showing callus induction (CI) and were expressed as percentage. Similarly percentage of embryogenic calli induction (EC) and regeneration (R) were calculated by taking the ratio of calli to embryogenic calli and embryogenic calli to regenerated plants respectively. Since the data was generated in terms of percentage which varied from 10 to 73 per cent an *arc sine* transformation of means of CI, EC, and R was carried out. The transformed data was subjecting to analysis of variance (ANOVA) by using PROC GLM of SAS to test the statistical significance of differences among the genotypes and explants age of immature embryo and the interaction between genotypes and age of immature embryos. The differences in treatment means of CI, EC and R were compared across genotypes and age of immature embryos by following Duncan's Multiple Range Test by using MEANS model of SAS at 95% confidence level. The similar comparison was also made for days after pollination (DAP) with respect to age of immature embryos.

## Results and discussion

### *Analysis of variance in CI, EC and R*

The result of analysis of variance (ANOVA) presented in Table 1 has indicated the presence of highly significant differences existed among genotypes, among different ages of immature embryos and interaction between genotypes and age of immature embryos with respect to callus induction, embryogenic calli production, and regeneration. It is quite interesting that for regeneration, the sum of squares (SS) value of interaction between genotypes and age of immature embryos was quite higher (509.5) than the SS value of individual factors *viz.,* genotypes (371.5) and age of immature embryos (387.3). The possible reason is that the tropical maize is highly recalcitrant to *in-vitro* regeneration and the frequency of genotypes with good regeneration frequency is also very low [19]. Similar kind of results of strong interaction between genotypes and age of immature embryos were also reported previously [20-21]. Therefore may be the age of immature embryo is the main determinant factor for higher regeneration.

**Table 1.** Analysis of variance (ANOVA) in callus induction (CI), embryogenic calli production (EC) and regeneration (R)

| Source of variation | DF | Mean square | | |
|---|---|---|---|---|
| | | CI | EC | R |
| Model | 31 | 292.8** | 181.3** | 40.9** |
| Rep | 2 | 1.5NS | 0.7 NS | 0 NS |
| Gen | 9 | 592.5** | 234.5** | 41.3** |
| Dap | 2 | 1750.6** | 1314** | 193.7** |
| Gen × Dap | 18 | 13.4** | 48.9** | 28.3** |
| **Error** | 58 | 0.7 | 1.1 | 0.8 |

### *Response of genotypes for callus induction, embryogenic calli production, and regeneration*

The overall means of callus induction, embryogenic calli production and regeneration irrespective of the age of explant has shown significant differences across different genotypes (Table 2). The present result shows the weighted means after arcsine transformation of data. Irrespective of the age of immature embryos the genotypes differed significantly with respect to callus induction, embryogenic calli production, and regeneration. The range in mean percentage of callus induction (CI), embryogenic calli production (EC) and regeneration (R) are 25.42 (HKI1025) to 48.42 (HKI335), 9.19 (HKI1025) to 23.44 (HKI1126), and 5.96 (LM13) to 12.31 (HKI 1105) respectively (Table 2). The genotype HKI1105 alone was grouped in 'A' consistently in CI, EC, and R and did not differ significantly with the genotypes having highest mean. The genotype CM300 has shown second best mean with respect to CI, EC, and R as compared to other genotypes. The result of the present study is in conformity with the earlier report that CM300 performed

**Table 2.** Duncan's Multiple Range Test for callus induction (CI), embryogenic calli production (EC) and regeneration (R) over different ages of immature embryos.

| Days after pollination | Callus induction | | Embryogenic calli production | | Regeneration into whole plant | |
|---|---|---|---|---|---|---|
| | Mean | Duncan grouping* | Mean | Duncan grouping* | Mean | Duncan grouping* |
| HKI1105 | 47.87 | A | 23.22 | AB | 12.31 | A |
| CM300 | 44.12 | B | 22.27 | BC | 11.87 | A |
| HKI335 | 48.42 | A | 17.62 | E | 6.96 | D |
| CM124 | 35.87 | D | 21.74 | C | 8.27 | B |
| HKI1126 | 37.31 | C | 23.44 | A | 6.50 | DE |
| HKI1025 | 25.42 | I | 9.19 | G | 7.29 | CD |
| HKI1128 | 31.60 | F | 19.61 | D | 8.11 | BC |
| HKI193-1 | 28.26 | H | 16.70 | E | 7.37 | BCD |
| LM13 | 29.79 | G | 11.00 | F | 5.96 | E |
| LM5 | 34.94 | E | 22.43 | ABC | 8.08 | BC |

*Means with the same letter are not significantly different; DAP-days after pollination i.e. age of immature embryo

best among other lines with respect to regeneration [6]. Genotype plays an important role in tissue culture response across crop plants. Differential responses of genotype for regeneration have also been reported by various authors [5, 19, 22]. However, in the present study HKI1105 is found far better than the CM300 which gives an opportunity to use HKI1105 as ex-plant source. In addition CM300 is the white line, whereas in public sector not many white maize hybrids are being cultivated. Therefore HKI1105 being yellow line and also it is being used as one of the parent in HM4, HM8, HM9, Malviya Hybrid Makka can serve as new donor line after transformation.

Further, overall significant differences were also observed with respect to CI, EC, and R among different ages of immature embryos (Table 3). The callus induction percentage varied from 29 (18 DAP) to 44.25 (12 DAP). Irrespective of the genotypes the highest embryogenic calli production (26.12) and regeneration (11.20) was observed in an immature embryo extracted from 12 days old selfed ears. Whereas the lowest embryogenic calli production (13.35) and regeneration (6.64) was observed in an immature embryos extracted from 18 days old selfed ears. The results suggest the change in the metabolite compositions during immature embryos might influence the response to regeneration by different stages/aged immature embryos. There are no conclusive evidences with respect to influence of specific metabolites on regeneration but it was hypothesized that some endogenous hormones play an important role, which are responsible for differential response to regeneration.

### *Effect of immature embryo's age and genotype response*

The age of immature embryo has strong influence on CI, EC, and R. Irrespective of the genotypes, the mean percentage of CI, EC and R were highest in 12 days old immature embryos (Table 4). The results again reaffirm that particular stage/age of embryo is critical for optimum regeneration irrespective of the type of genotype.

### *Callus induction*

The per cent callus induction ranges with immature embryos harvested after 6, 12 and 18 days after pollination (DAP) are 19.0 (HKI1025) to 39.3 (HKI1105), 32.5 (HKI1025) to 58.2 (HKI335) and 24.7 (HKI1025) to 49.7 (HKI335) respectively with overall range of 39.2 *i.e.* from 19.0 (6 DAP) to 58.2 (12 DAP). The highest percentage of callus induction observed in HKI335 followed by HKI1105 (56.8, 12 DAP), CM300 (52.7, 12 DAP). The genotype HKI335 and HKI1105 have shown better response for callus induction as compared to other genotypes by standing first and second respectively (Table 2).

The overall mean responses of all genotypes irrespective of the age of immature embryo (Table 2) and overall mean responses of particular age immature embryos irrespective of the genotypes (Table 3) have showed significant difference. The results of mean response of genotypes for callus induction, embryogenic calli production, and regeneration indicated the presence of significant differences among genotypes as genotypes responded differently. The differential response of genotypes was very much evident that the ranking of genotypes changed between callus induction, embryogenic calli production and regeneration. The genotypes HKI335 (58.2, 12 DAP), CM 300 (36.9, 12 DAP) and HKI1105 (20.9, 12 DAP) has shown highest percentage of callus induction, embryogenic calli production and regeneration respectively (Table 4). The Duncan's Multiple Range Test indicated no significance difference among some genotypes especially genotypes with highest response, for example the genotype HKI1105 has shown no significant differences with HKI335 and CM300 with respect to callus induction and embryogenic calli production respectively (Table 2). In fact HKI1105 is the only genotype which was grouped under group 'A' by Duncan's Multiple Test Range Test for callus induction, embryogenic calli production and regeneration. The responses of all ten genotypes for callus induction and embryogenic callus production were highest when an immature embryo was 12 days old. However, some genotypes (HKI1025, HKI1126, HKI193-1 and LM13) have shown marginally but not significant better regeneration response with either or both 6 and 18 days old immature embryos (Table 4). It was quite evident as the Duncan's Multiple Range Test has shown that the callus induction, embryogenic calli production and regeneration was highest at 12 days old immature embryo across genotypes thus indicating the highest suitability of 12 days old immature embryo for whole plant regeneration (Table 3).

**Table 3.** Duncan's Multiple Range Test for callus induction (CI), embryogenic calli production (EC) and regeneration (R) over different genotypes

| Days after pollination | Callus induction | | Embryogenic calli production | | Regeneration into whole plant | |
|---|---|---|---|---|---|---|
| | Mean | Duncan grouping* | Mean | Duncan grouping* | Mean | Duncan grouping* |
| 6 | 35.84 | B | 16.70 | B | 7.00 | B |
| 12 | 44.25 | A | 26.12 | A | 11.20 | A |
| 18 | 29.00 | C | 13.35 | C | 6.64 | B |

*Means with the same letter are not significantly different

**Table 4.** The mean response of genotypes with respect to callus induction (CI), embryogenic calli production (EC) and regeneration (R) across different age of immature embryos

| Genotypes | Callus Induction* (%) | | | Embryogenic calli* (%) | | | Regeneration* (%) | | |
|---|---|---|---|---|---|---|---|---|---|
| | 6 | 12 | 18 | 6 | 12 | 18 | 6 | 12 | 18 |
| CM124 | 27.7 | 42.4 | 37.5 | 19.0 | 29.2 | 16.9 | 7.1 | 11.6 | 6.1 |
| CM300 | 38.3 | 52.7 | 41.4 | 18.4 | 36.9 | 11.5 | 7.1 | 20.4 | 8.1 |
| HKI1025 | 19.0 | 32.5 | 24.7 | 8.60 | 11.3 | 7.70 | 7.5 | 6.9 | 7.5 |
| HKI1105 | 39.3 | 56.8 | 47.5 | 18.7 | 35.2 | 15.7 | 7.6 | 20.9 | 8.5 |
| HKI1126 | 27.4 | 46.6 | 37.9 | 19.7 | 29.6 | 21.0 | 7.5 | 7.4 | 4.6 |
| HKI1128 | 23.2 | 39.9 | 31.6 | 20.3 | 21.5 | 17.0 | 7.5 | 9.3 | 7.5 |
| HKI193-1 | 23.1 | 34.3 | 27.4 | 17.4 | 21.2 | 11.5 | 7.5 | 7.1 | 7.5 |
| HKI335 | 37.4 | 58.2 | 49.7 | 15.1 | 24.9 | 12.9 | 5.5 | 8.9 | 6.5 |
| LM13 | 26.0 | 35.7 | 27.7 | 9.20 | 16.5 | 7.30 | 7.5 | 6.2 | 4.2 |
| LM5 | 28.5 | 43.3 | 33.0 | 20.5 | 34.7 | 12.0 | 5.1 | 13.2 | 5.9 |
| Mean | 29.0 | 44.2 | 35.8 | 16.7 | 26.1 | 13.3 | 7.0 | 11.2 | 6.6 |

***Embryogenic calli production***

The overall range in per cent embryogenic calli production was relatively narrow (29.58) as compared to callus induction (39.2). It ranged from 8.6 (HKI1025) to 20.5 (LM5), 11.3 (HKI1025) to 36.9 (CM300), 7.3 (LM13) to 21.0 (HKI1126) in immature embryos extracted from selfed ears harvested at 6, 12 and 18 days after pollination respectively (Table 4). The genotype showing highest percentage of embryogenic calli production (CM300) was followed by HKI1105 (35.2), LM5 (34.7), HKI1126 (29.6), and CM124 (29.2). In the present experiment the embryogenic responses by different genotypes was expressed as the percent of embryogenic type II calli at the eleventh day culture (approximately 15 days after culture initiation). In the present study also the total number of calli formed was determined on 45$^{th}$ day after culture initiation [23]. Five out of ten genotypes namely CM300, HKI1105, LM5, HKI1126 and CM124 have shown higher embryogenic type II response, out of them CM300 and HKI1105 showed highest regeneration capacity (Table 4). All the lines that developed type II callus proceeded to form many shoots, the small plantlets were separated and sub cultured until 4-5 leaves were formed when they were transferred to the rooting media. The losses at shoot or root induction stage were not significant in fact very meager and/or almost nil. The most important and critical stage is the friable type II callus production. The genotype HKI1105 was exceptional which has shown embryogenic response beyond 45 day sub-culturing. In fact none of the remaining genotypes advanced beyond the 45 day sub-culturing steps for the formation of the type II callus.

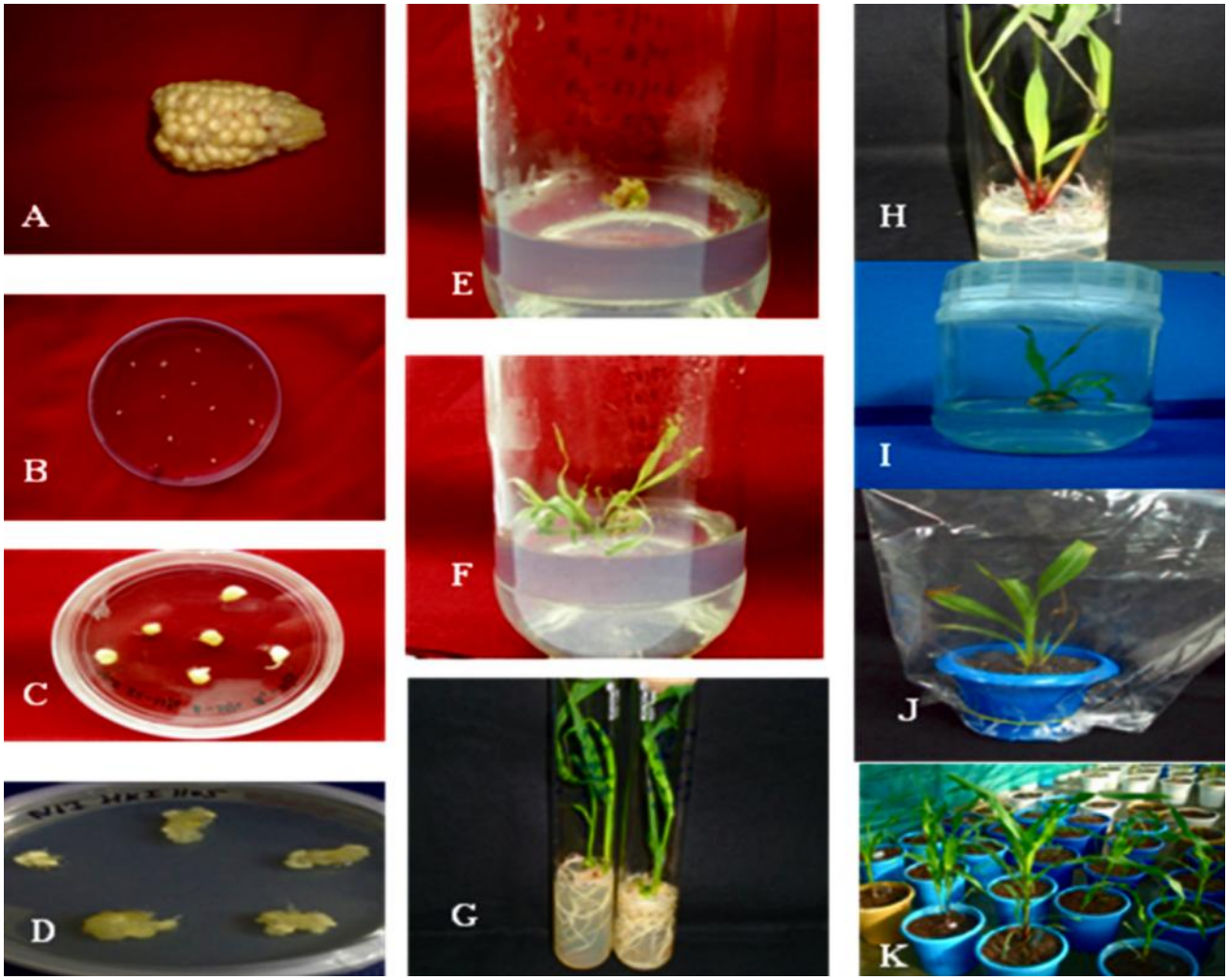


**Fig. 1. A=Harvested cob for immature embryo, B=inoculation of embryo, C-D=Embryogenic calli on N6I medium, E=Callus on regeneation medium, F-H=Various growing stage of calli to plantlets in regeneration medium, I=Hardening in ½ MS solution, J=Acclimatization in growth chamber and K=Regenerated plantlet in glass house**

These calli were non embryogenic, slow growing, hardened and turned dark brown may be due to cell death. The observation in the experiment suggests that very low probability of getting genotypes with robust regeneration capacity. In the present study the genotypes which were considered elite with respect to their *per se* performance were used for the study. The observation suggests that genetic background is an important factor in callus induction, the formation of the type II callus and regeneration [20, 24]. Only a few tropical genotypes have been shown to be capable of initiating type II callus [25, 26]. Type II callus has been found to be more responsive for regeneration than type I [24, 27].

### *Regeneration*

The whole plant regeneration varied from 4.2% (LM13, 18 DAP) to 20.9% (HKI1105, 12 DAP). The range was further narrowed down to 16.71 as compared to callus induction (39.2) and embryogenic calli production (29.5). In immature embryos harvested at 6 and 18 days after pollination, the regeneration percentage was very poor, in fact in many genotypes there was no regeneration at all. However, in an immature embryos harvested at 12 days after pollination, the regeneration was quite high and it was ranged from 6.2 (LM13) to 20.9 (HKI1105). The typical flow of immature embryos to whole plant regeneration which was observed in the present experiment was shown in Fig. 1. The constant growth conditions and media compositions were maintained while sub-culturing of embryonic calli. These embryogenic calli cultures were remained viable over many months while sub-culturing. Several factors *viz.,* genotype, choice of tissue as ex-plant, age of *ex-plant*, developmental stage of plant, culture media, culture room environment at different growth stages determine the callus induction to regeneration [28]. The minor factors like placement of embryos on medium also determine the success of callus induction [29, 4]. In the present research it was found that genotype plays an important role in callus induction to regeneration. Because, some genotypes have shown higher percentage of callus induction as compared to other genotypes, further, it was also found that there was strong interaction between genotype and age of immature embryo and similar findings were also reported elsewhere [20, 21]. The present study identified HKI1105 genotype as most promising for callus induction to embryogenic calli induction to regeneration. The other genotype was CM300 which was also has shown good response for regeneration.

## Conclusion

The immature embryos were isolated at 6, 12 and 18 days after pollination to identify and establish the right stage of immature embryos for achieving optimum regeneration across different genetic background. The results indicated 12 day old immature embryo is most suitable for callus induction, embryogenic calli production and regeneration across all ten tropical Indian inbred lines tested. Among different genotypes HKI1105 has shown better response as compared to other genotypes with respect to CI, EC and R. The present study is the first step in successful development of transformation protocol.

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