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Comparison Between Maize Inbred and Hybrid Lines for Their Amenability to *Agrobacterium* Mediated Transformation and *In Vitro* Plant Regeneration

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Abstract

The field of genetic engineering and molecular breeding utilizes maize immature embryos for transformation studies. However, there are hinderances such as the unavailability of immature embryos throughout the year and their low transformation frequencies which make them unsuitable for use at a commercial level. Among cereals, maize has prime importance due to its productivity, industrial products, animal feed and fodder. Regeneration capability in maize transformation studies varies due to differences in genetic makeup and explant source. In this study, we evaluate mature embryos as explants for in vitro plant regeneration. Six varieties including 3 hybrid lines (Pioneer 3025, SG 2002 and Neelam) and 3 inbred lines (NCML 107, CML 161 and FBF 3368) were screened for *Agrobacterium* mediated transformation amenability using mature maize embryos. The two inbred lines NCML 107 and CML 161 performed best showing 100% callus induction frequency, 93.33% and 86.66% transient GUS expression, and 43.75% and 13.3% regeneration frequency, respectively. Using split seed as an explant, better regeneration was observed in NCML 107 (14.86%) and CML 161 (7.5%) as compared to mature embryos, NCML 107 (10%) and CML 161 (2%). Here we report successful plant regeneration (regeneration medium supplemented with Kinetin + BAP) using split seeds as an explant. Our results demonstrate the capability of two elite maize inbred lines (NCML 107 & CML 161) for their amenability to *Agrobacterium* mediated transformation and better tissue culture response; hence can be selected in maize transformation studies for improving crop varieties for enhanced nutritional content and better adaptation.

Keywords: maize tissue culture; *Agrobacterium* mediated transformation; GUS reporter gene; split seed; mature embryos

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Introduction

Maize (*Zea mays*. L), the 'Queen of cereals' is ranked third most important cereal crop in the world following rice and wheat [1]. The demand for maize has increased over the years primarily as animal feed therefore contributing to the global agri-food systems and food security. Annually, it is the second highest cultivated crop worldwide on approximately 197 million hectares of land after wheat and is expected to surpass wheat by the year 2030 [2]. In Pakistan, the area for maize cultivation is 0.9 million hectares and annual production reaches to 1.3 million metric tons [3]. Maize is mostly grown in the monsoon season, but it can be sown from March to October. Moisture and warmth are important factors from germination stage to flowering. Maize is of great importance as it has a variety of uses. It is used as feed for monogastric animals, humans and also serves as a raw material in industries [4].

Due to its immense use *Zea mays* has been highly investigated and serves as an ideal model plant for the study of plant molecular biology and genetics. It is very necessary to maintain and enhance the *Zea mays* germplasm as it is of immense importance to the Pakistan economy as well [5]. Enhancing the germplasm means producing genetically modified maize which is an important step in terms of promoting sustainable agricultural practices [6]. Maize is the only crop with the most biotechnology traits available in the market since the first transgenic Bt maize products released in the 1990s [7]. Maize biotechnology has gained immense importance in Pakistan over the past years as it is an agriculturally and environmentally important crop. The agriculture sector in Pakistan contributes 22.35% to GDP [8]. There have been maize yield losses of upto 87% due to drought and 42% due to heat [9]. There is a need for biotechnological intervention to produce biotic and abiotic stress tolerant, and nutritionally improved maize in Pakistan.

Monogastric animals such as poultry and fish that consume maize seeds do not have sufficient phytase activity to breakdown phytate and utilize phosphorus for their normal growth and development. Thus, undigested phytate is released into the environment and leads to eutrophication of water [10]. As a solution to this was the production of exogenous microbial phytase and mixing it with the poultry feed [11] to make phosphorus available

for digestion but this is not feasible as enzyme costs involve about 45% of the total feed cost in the poultry industry [12, 13]. A sustainable strategy to meet the phosphorus needs of monogastric animals as well as to reduce phosphorus load on the environment was production of transgenic plants expressing phytase in the seeds [14].

It is very necessary that high efficiency transformation should be carried out so that a large number of transgenic events can be generated using elite maize genotypes. The *Agrobacterium* mediated transformation protocol [15] that uses the 'super binary vector' system was a major breakthrough in maize transformation for transgenic event generation and since then has been adopted throughout the world [16, 17]. Successful transformation of inbred lines A188 [18], Hi-II [19] and Hi-II/A188 hybrids [20] using the *Agrobacterium* method were reported.

Maize immature embryos are an optimal choice for maize tissue culture owing to their high transformation efficiency. Due to the unavailability of immature embryos all year round, maize mature embryos are selected [21]. A recent technique [22] known as the split-seed method exposes the three different seed tissues simultaneously thus reducing tissue culture time period and enhancing the number of shoots. The split seed keeps the natural nutrients and hormones of the endosperm intact while exposing majority of the cells of the shoot apical meristem, coleoptilar ring and the scutellum. Treating maize as a dicotyledonous seed upon splitting, high regeneration frequencies i.e., upto 28 shoots per callus in 2-3 weeks were reported.

Callus induction and regeneration capability in maize is highly genotype dependent [23] and affected by composition of culture medium [24]. Most of the maize genotypes either do not produce embryogenic calli or the embryogenic calli produced do not regenerate into plants in an efficient manner [25]. Therefore, it is necessary to evaluate maize genotypes and explant sources to select the best performing ones for maize transformation studies. For this purpose, we have evaluated six different maize genotypes (three hybrids and three inbreds) for their transformation amenability and in vitro plant regeneration using maize mature embryos and split seeds as explant sources.

Materials and methods

Plant materials

Seeds of three maize inbred lines were obtained from National Agricultural Research Centre (NARC), Islamabad and Four Brothers (4B) Group, Pakistan, and seeds of the three maize hybrid lines were obtained from the local market. Seed surface sterilization was done by using 70% ethanol for 30 seconds and 50% Clorox (bleach) for 15-20 minutes with continuous shaking and finally 5-6 washings with autoclaved distilled water. The seeds were soaked in autoclaved distilled water for 48 h. For maintaining sterile conditions all the steps were carried out in a laminar flow cabinet.

Comparison of selected three maize inbreds and three maize hybrid lines using mature embryos

a) Callus induction: Mature embryos of inbred lines (CML 161, NCML 107 and FBF 3368) and hybrid lines (SG 2002, Neelam and Pioneer 3025) were aseptically excised from the sterilized seeds using forceps and scalpel fitted with a blade. These mature embryos were placed on Callus Induction Medium (CIM) i.e. MS medium [26] supplemented with 1 mg/L 2,4-Dichlorophenoxyacetic acid for the purpose of callus induction and incubated in a box in the dark at a temperature of 22 ± 1 ^{o}C in the climate control room for two weeks.

The callus induction frequencies were calculated as:

Callus induction frequency (CIF)= (No. of calli formed/No. of embryos placed on CIM) × 100

b) *Agrobacterium* strain and plasmid: The *Agrobacterium* tumefaciens strain (AGL1) used for transformation of maize calli of 6 different varieties harboring the binary plasmid pGA482 contains GUS with intron [27]. The plasmid also contains nptII gene for selection.

*Ag*robacterium culture was grown in 10 mL LB medium [28] supplemented with kanamycin (50 mg/L) and rifampicin (100 mg/L). The secondary culture was placed on a shaking incubator (N-Biotek Catalog No. NB-205LF) at 28 ^{o}C and 150 rpm for 2 days. At an OD600 of 0.4-0.6, 160 mg/mL acetosyringone was added to the culture and it was further placed on the shaking incubator for 2 h and used for infection.

c)Infection and co-cultivation: Two weeks old embryogenic calli were arranged in the centre of the petri plate and sprinkled with 500 µL of Agrobacterium culture per plate. The petri plate was covered and placed in the dark for 20 minutes after which the calli were shifted to an autoclaved filter paper to remove the excess culture and were shifted onto co-cultivation medium [MS medium + 1 mg/L 2,4-D + acetosyringone (160 mg/L)]. The calli were co-cultivated in the dark at 22 ± 1 ^{o}C for 3 days [15].

d) Suppression of *Agrobacterium* overgrowth and regeneration: For GUS analysis 30% calli (co-cultivated

Table 1. Callus Induction Response in Maize inbred and hybrid lines from mature embryos

	Name of Variety	No. of Embryos on CIM	% of Calli formed	Quality of callus	% of true callus
Inbred maize lines	CML-161	50	100%	Good & healthy	100%
	FBF-3368	50	100%	Good & healthy	94%
	NCML-107	50	100%	Good & healthy	100%
Hybrid maize lines	Pioneer 3025	50	94%	Good & small size	94%
	SG-2002	50	100%	Fleshy with shoots	68%
	Neelam	50	48%	48% good & 52% false/bad. Callus formation was very slow	48%

for 24 h) were used for GUS assay. For regeneration purposes 70% calli (co-cultivated for 72 h) were shifted to MS medium + 1 mg/L 2,4-D containing timentin (160 mg/L) for suppressing the overgrowth of Agrobacterium and kept under the same conditions as before. After 2 weeks, the calli were shifted on MS medium containing 1 mg/mL Kinetin and 2 mg/mL BAP for regeneration purposes and kept in the tissue culture room at 25 ± 1 °C and 25-27 µmol m-2 s-1 photon intensity. The regeneration frequencies were calculated as:

Regeneration frequency = (No. of calli regenerated / No. of calli placed on RM) × 100

e) Histochemical GUS assay: Each of the 30% calli co-cultivated for 24 h was placed in an eppendorf tube containing GUS solution (1M Na_2HPO_4 + 1M NaH_2PO_4 + 0.5M EDTA + 1% Triton X-100 + 0.1M X-gluc + 20% Methanol + Ultrapure H_20). The samples were incubated at 37 °C for 2-3 days [29]. Transient GUS expression was calculated as:

Transient GUS expression= (No. of calli turned blue/No. of calli placed in GUS solution) × 100

Comparison of selected two inbred maize lines using mature embryos and split seeds

Upon screening of the 6 maize varieties the two best performing ones were selected for further analysis using different explant sources. The selection of the two inbred lines, NCML-107 & CML-161 was done based on their successful in vitro regeneration response.

a) Using mature embryos as explants: Callus induction of inbred maize lines (NCML 107 and CML 161) was carried out by following the method stated in the section above.

b) Using split seeds as explants: The sterilized seeds of inbred maize lines (NCML 107, CML 161) were soaked in germination media (CIM with 2,4-D 3 mg/mL) for 3-5 days. The seeds were then split longitudinally and placed on germination media and kept under light conditions in the tissue culture room for 3-5 days. Split seeds were shifted to CIM supplemented with 2,4-D (1 mg/mL) and kept in the dark at 22 ± 1 °C for two weeks. Prior to infection the seed part was separated from the developed callus and callus was used for transformation. Transformation and regeneration were carried out as stated in the section where mature embryos were used. After 2 weeks, the calli were shifted on MS medium containing 1 mg/mL Kinetin and 2 mg/mL BAP for regeneration purposes and kept in the tissue culture room at 25 ± 1 °C and 25-27 µmol m-2 s-1 photon intensity. Regenerated plants were shifted to jars on rooting medium containing IBA (0.6 mg/mL) till healthy roots developed. The plant with well-developed roots and shoots was transferred to a plastic pot containing a mixture of peat moss, vermiculite, and perlite in the ratio 2:1:1 and shifted to the climate room. Plants were supplemented with Hoagland's solution [30] weekly for provision of nutrients.

Statistical analysis

The quantitative data was analyzed statistically using Tukey's HSD test at 5% level of significance ($p<0.05$) using SPSS version 18.0.

Results

Callus Induction Frequencies of maize inbred and hybrid lines

Callus induction frequencies (CIF) among all six maize varieties ranged from 48-100%. CIF of hybrid and inbred maize lines have been listed in the **Table-1**. In general, highest embryogenic CIF (100%) were observed in CML 161 (**Figure-1A**), NCML 107 (**Figure-1B**) and FBF 3368 (**Figure-1C**) where FBF 3368 exhibited only 94% true calli. Neelam exhibited the lowest CIF of 48% (**Figure-1F**). Significant difference was observed in CIF when all three hybrid varieties were compared ($p<0.05$). Among inbreds CML 161 showed significant difference with all maize varieties (hybrid and inbred) except for NCML 107. FBF 3368 showed a significant difference in CIF in comparison to all other varieties except Pioneer 3025. CIF was observed to be less in maize hybrids as compared to maize inbreds.

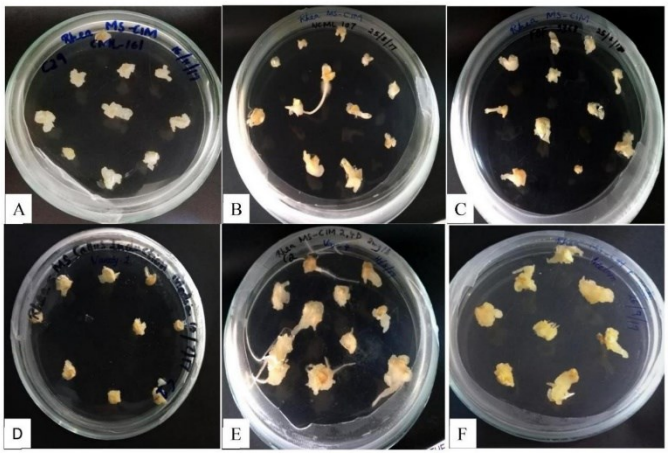

Figure 1. Two weeks old callus from maize mature embryos Row 1 (inbreds) A- CML 161 B- NCML 107 C- FBF 3368; Row 2 (hybrids) D- Pioneer 3025 E- SG 2002 F- Neelam

Transient GUS Expression in maize inbred and hybrid lines

Transient GUS expression among all six maize varieties ranged from 80-100% and is exhibited in Figure-2. Most of the calli exhibited blue dots concentrated in the central region of the calli. Highest transient GUS expression was observed in NCML 107 (93.33%) (**Figure-2B**), while lowest was observed in Pioneer 3025 (**Figure-2D**) and Neelam (80%) (**Figure-2F**) as blue dots on the edges of calli. In comparison with SG 2002 (**Figure-4**) all other maize varieties showed significant difference in transient GUS expression ($p<0.05$). Among other five varieties results showed non-significant difference.

Figure 2. Transient GUS expression in different maize varieties after 24 hours of co-cultivation; A- CML 161 (inbred) B- NCML 107 (inbred) C- FBF 3368 (inbred) D- Pioneer 3025 (hybrid) E- SG 2002 (hybrid) F- Neelam (hybrid) G- Control calli

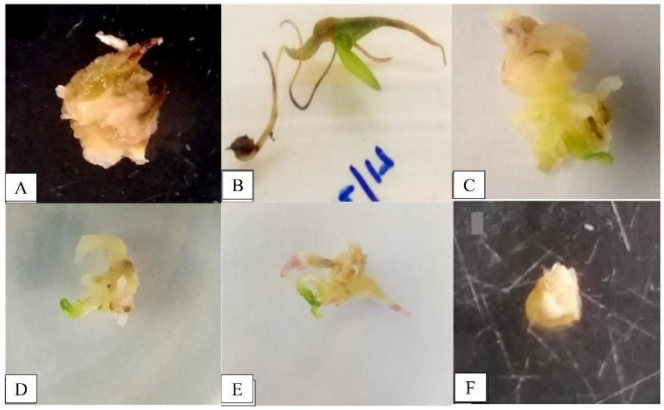

Figure 3. Regeneration at 3 weeks stage in different maize varieties, Row 1 (Inbred lines) A- CML 161 B- NCML 107 C- FBF 3368; Row 2 (Hybrid lines) D- Pioneer 3025 E- SG 2002 F- Neelam

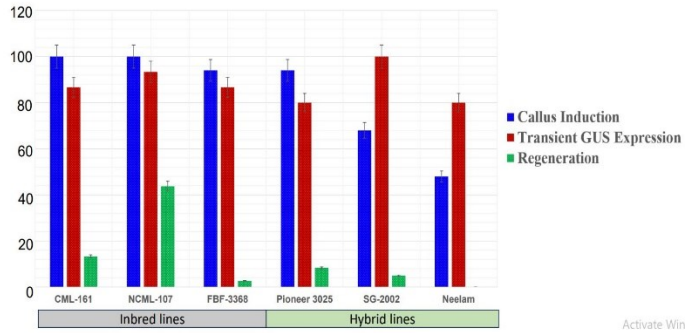

Figure 4. Comparison of Callus Induction, Transient GUS Expression and Regeneration among maize inbreds (CML-161, NCML-107, FBF-3368) and hybrids (Pioneer 3025, SG-2002, Neelam) using mature embryos as explant

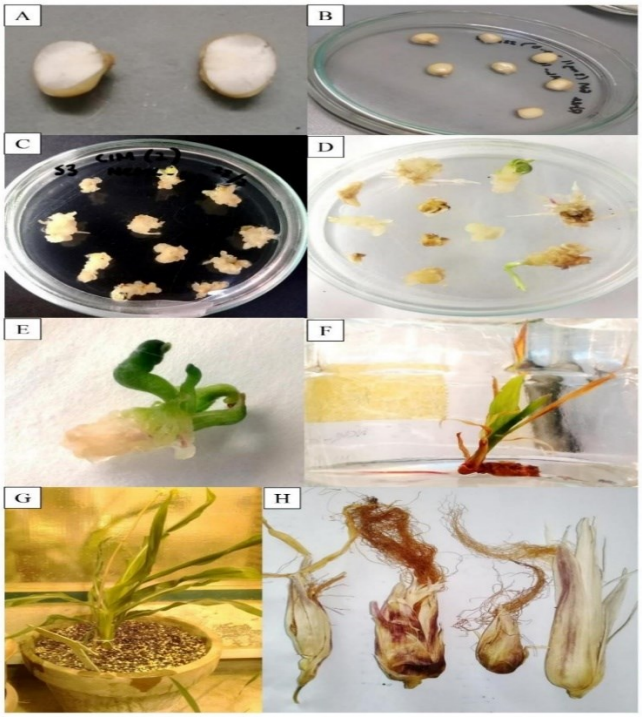

Figure 5. Steps from callus induction to regeneration using split seeds of NCML 107 A- Seed split longitudinally of inbred maize line NCML 107 B- Split seeds placed on germination media C- Two weeks old calli D- Calli shifted to regeneration media E- Shoot emergence from callus F- Root emergence on rooting medium G- Acclimatized plant shifted to pot in climate control room H- Harvested ears from the NCML-107 regenerated plant

Regeneration Frequencies of maize inbred and hybrid lines

For the 6 maize varieties, highest regeneration frequency recorded was 43.75% (NCML 107) (**Figure-3B**) while the minimum was 2.7% (FBF 3368) (Figure-3C). Rooting was observed only in NCML 107 (**Figure-5F**). Only Neelam (**Figure-3F**) showed 0% regeneration response. Significant difference was observed in 6 varieties except FBF 3368 which showed a non-significant difference only when compared to SG 2002 (**Figure-4**). Regeneration frequencies were higher among maize inbred lines as compared to maize hybrids.

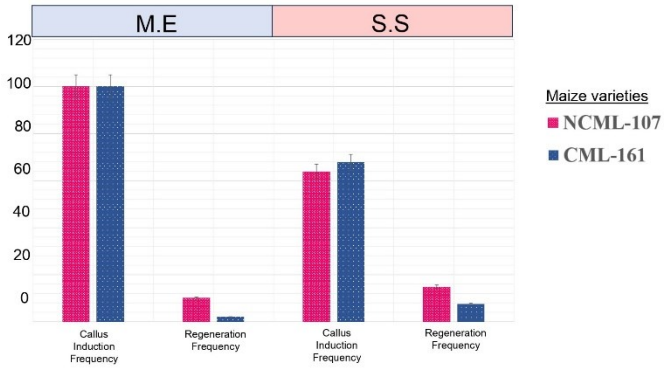

Figure 6. Comparison of Callus Induction & Regeneration frequencies of two elite maize inbred lines using mature embryos (M.E) and split seeds (S.S) as explants. Statistical significance (Tukey's HSD post hoc test) is indicated by asterisks as *p < 0.05, **p < 0.01, or ***p < 0.001

Comparison between mature embryos and split seeds as better explant source

Using the split seed, better regeneration frequencies and successful plant regeneration (**Figure 5**) were observed. However, a huge difference was found in the CIF which were lower as compared to when using mature embryos. Comparison between callus induction and regeneration frequencies using mature embryos and split seeds has been shown in (**Figure-6**). CIF ranged from 63.8-67.8% and regeneration frequencies ranged from 7.5-14.86% using split seeds. Using mature embryos, CIF were 100% for both inbred lines. Regeneration frequency was 10% (NCML 107) and 2% (CML 161). With split seeds, CIF were 63.8% (NCML 107) and 67.8% (CML 161). Regeneration frequencies were 14.86% (NCML 107) and 7.5% (CML 161). In comparison with CML 161, non-significant difference was shown by NCML 107 in callus induction frequency using mature embryos ($p<0.05$). NCML 107 showed a significant difference in regeneration frequency in comparison to CML 161 ($p<0.05$). In comparison with CML 161, NCML 107 showed significant difference in callus induction frequency and regeneration frequency using split seeds ($p<0.05$).

Discussion

Mature embryos are recalcitrant towards tissue culture as compared to immature embryos however, successful regeneration using mature embryos as explants in *Zea mays* has been reported [31, 32]. The response of the explant to the maize transformation procedure is also genotype-dependent [33]. The achievement of high transformation efficiency is contingent upon the selection of the appropriate explant. Moreover, embryogenic calli that are compact, friable, cream, or light yellow in appearance offer competency to the *Agrobacterium* mediated transformation process [34].

The optimum concentration for callus induction in maize tissue culture studies is 1 mg/mL of 2,4-D [35]. Using higher concentrations of 2,4-D causes browning of callus leading to greater possibilities of somatic mutation [36]. CIF using mature maize embryos have been reported upto 90% for inbred lines following two biweekly subcultures at 4 mg/mL 2,4-D and 2 mg/mL 2,4-D, respectively [37]. We report the highest embryogenic CIF (100%) in inbred maize lines (**Table 1**) using 1 mg/L 2,4-D.

Successful transient GUS expression has been reported in monocots such as barley, wheat, and rice [38]. Previously, among 14 maize genotypes, 36-76% transient GUS expression frequencies were observed [39]. The highest transient GUS expression in our study was observed in the inbred line, NCML 107 (93.33%). Transformation frequencies of 3% and 1.1% were reported at different salt concentrations using GUS reporter gene in L3 maize inbred line by *Agrobacterium* mediated transformation [40]. Significant difference was observed in transient GUS expression ($p<0.05$) between SG 2002 and all other maize varieties. Among other five varieties results showed non-significant difference. Acetosyringone is crucial as a virulence inductor in transformation studies of monocots. An ideal co-cultivation time is 3-4 days for maize and plays a vital role in transformation [41, 42]. In the present study, calli were infected with *Agrobacterium* culture having an O.D 600nm of 0.4-0.6. Previously, an O.D 600nm of 0.4-0.5 for Agrobacterium mediated transformation of maize immature embryos has been reported [43].

Highest regeneration frequency has been reported in the inbred line, NCML 107 (43.75%). Plantlet formation was undesirable using mature embryos therefore another explant was used and NCML-107 and CML-161 inbred lines were selected based on the preliminary results. Rooting was observed only in NCML 107. When the longitudinally split seed was used as an explant for maize

transformation and regeneration, good plant development was observed unlike that when using mature embryos. The callus derived directly from mature embryos has a high success rate, but the protocol is laborious. On the other hand, the split-seed technique is a newly developed protocol that can regenerate a higher number of shoots through callus formation in a shorter time. The NCML 107 plant completed its life cycle whilst also producing corn cobs as shown in **Figure 5**. For root development IBA was used in half-strength MS medium. IBA is one of the most efficient auxin to initiate rooting in maize tissue culture [44]. Neelam was the only variety that exhibited 0% regeneration response. Similar results reported regeneration ranging from 2.34% to 87.5% using immature and mature embryos when Kinetin and BAP were used in the regeneration medium [45]. Highest shooting frequency (78%) using mature embryo derived callus has been observed on MS medium (kinetin + BAP) [46]. Difference in varieties could be the reason for relatively lower regeneration frequencies. Kinetin and BAP aided in higher frequency of shoot formation [47]. Better regeneration response in wheat was linked with using endosperm supported mature embryos instead of non-endosperm supported mature embryos [48].

The inadequate regeneration response of various explants poses a significant challenge in tissue culture. Therefore, the selection of an appropriate explant is essential to initiate tissue culture for any crop plant, as it enhances the likelihood of cells acquiring greater regeneration competency. In the past, the production of transgenic maize predominantly relied on immature embryos as regeneration explants. However, the consistent availability of immature embryos throughout the year presents considerable difficulty, necessitating specialized care during winter and access to high-quality greenhouse facilities [49]. The 'split seed technique' has proved successful in the past as high callus induction frequency (82%) and regeneration frequency (65.46%) have been observed [50]. In the present work, callus induction frequency ranged from 63.8-67.8% and regeneration frequencies ranged from 7.5-14.86% using split seeds. Similarly, 13.3-90% callus induction frequency using 2,4-D (3 mg/mL) and 16.5-21.2% regeneration frequencies with slow in vitro shoot development have been reported using split seeds as an explant source and serve as a better alternative to immature embryos [51].

Conclusion

The selection of explant sources has been found to significantly influence callus induction and regeneration responses in maize plants. Our current research advocates for the adoption of the 'Split seed technique' as an essential method for callogenesis and regeneration, owing to its ability to produce superior results within a condensed timeframe. Moreover, certain maize genotypes are recalcitrant towards in vitro plant regeneration therefore it is necessary to screen different genotypes for transgenic maize development. Based on the findings, two inbred varieties (NCML 107 & CML 161) demonstrated a superior overall tissue culture response and hold potential for the future development of transgenic maize lines using cytokinins (BAP + KIN) in the regeneration medium.

Authors' contributions

RA (Conceptualization, Methodology, Visualization, Data Curation, Investigation, Formal analysis, Writing - Original Draft), AM (Conceptualization, Methodology, Writing - Review & Editing, Supervision) , KAM (Conceptualization, Methodology, Writing – review & editing, Supervision, Funding Acquisition), All authors have read and approved the final manuscript.

Competing Interests

All authors declare that there are no competing interests.

Ethical Approval and Consent

Not applicable.

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