An efficient and rapid regeneration via multiple shoot induction from mature seed derived embryogenic and organogenic callus of Indian maize (Zea mays L.)

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Abbreviations: BAP, 6-Benzylaminopurine; KIN, kinetin; 2,4-D, 2,4-Dichlorophenoxyacetic acid; MS, Murashige and Skoog (1962) medium; NAA, 1-naphthaleneacetic

Introduction

Maize (Zea mays) is a major cereal crop and ideal model monocot plant for studying genetics, genomics, and molecular biology. Millions of people living in the tropical and subtropical zones of the world are largely dependent on maize for their subsistence. It is a C₄ crop with outstanding ability to maintain high rates of photosynthetic activity that is important for grain yield and biomass. Being a cross-pollinating species, it maintains broad morphological features, genetic variability and geographical adaptability. Among the cereals, maize is the most important crop in the world in terms of productivity, industrial products (fermentation and pharmaceuticals), animal feed and fodder. There is a continued increase in the demand for maize across the world, and more predominantly in Asia.¹ Maize yield is largely affected by various biotic and abiotic stresses. Several factors such as lack of useful variation and the long time duration required in conventional breeding affect the development of plants resistant to biotic and abiotic stresses through conventional breeding. To overcome such problems effective and reliable genetic transformation techniques are required.² ³ The insertion of desired trait into the maize genome will allow improvement in the productivity, nutritional quality and development of parental stock which in turn will lead to the development of new resistant varieties against environmental factors. Reliable in vitro regeneration techniques permitting the production of complete plants from single cells are of central importance to both clonal propagation and successful genetic
Engineering of plants. Green and Philips have first reported regeneration from immature embryos of maize. Since then, maize regeneration has been reported from immature embryos, mature embryos, nodal regions, leaf tissues, anthers, tassel and ear meristems, protoplast, and shoot meristems. To the best of our knowledge, almost all the previously reported protocols were developed using the maize model genotype A188 and their hybrid Hi-II (A188 · B73), which are characterized by high frequency of embryogenic callus proliferation and plant regeneration. These genotypes have poor agronomical value; therefore it would be a time-consuming and costly procedure to introduce transgenes into local maize varieties by backcrossing. Although the protocol of Agrobacterium-mediated transformation using explants of immature zygotic embryos is reproducible, one of the limitations is unavailability of the explants to perform transformation frequently. The production of immature embryos of maize is difficult and time consuming and it requires a well-equipped greenhouse and laborious artificial pollination system. Furthermore, the efficiency of artificial pollination varies among seasons and in the winter, the efficiency is usually much lower as compared with other seasons. Moreover, dry mature seeds are available in ample amount, round the year and they are responsive to tissue culture.

In the present investigation, we report a new plant regeneration method for Indian maize cultivar (HQPM-1). This method is efficient, rapid, simple, genotype independent for obtaining shoots from mature seed-derived callus with successful rooting. This efficient regeneration system facilitates the application of plant tissue culture and genetic engineering approach in maize.

**Results and Discussion**

An effective regeneration protocol has become paramount importance in transgenic research for efficient genetic transformation in any crop plant. The present work focuses to develop a standard regeneration protocol for tropical Indian maize inbreds. Although standard protocols for regeneration of

**Table 1. Effects of plant growth regulators on callus induction rate**

| Plant growth regulators (mg/l) | Embryogenic callus | Organogenic callus |
|-------------------------------|--------------------|--------------------|
| 2,4-D | BAP | Callus induction frequency (%) | Callus formation (mean ± SE) | Callus induction frequency (%) | Callus formation (mean ± SE) |
| 1.0 | 0.1 | 36.23 ± 1.3 | 37.45 ± 1.5 | 32.11 ± 1.4 | 31.75 ± 1.4 |
| 1.5 | 0.5 | 48.56 ± 0.96 | 55.32 ± 0.6 | 39.05 ± 1.7 | 45.63 ± 1.7 |
| 2.0 | 1.0 | 90.34 ± 2.02 | 95.73 ± 1.8 | 52.22 ± 0.74 | 58.79 ± 1.9 |
| 2.5 | 1.5 | 62.75 ± 1.01 | 75.31 ± 2.1 | 85.67 ± 1.03 | 89.92 ± 2.8 |
| 3.0 | 2.0 | 55.35 ± 0.98 | 63.32 ± 1.6 | 57.58 ± 1.6 | 67.38 ± 3.1 |
| 3.5 | 2.5 | 38.42 ± 1.2 | 42.65 ± 1.1 | 40.34 ± 1.5 | 47.76 ± 2.1 |

LSD 0.05 | 1.033 | 0.228 | 1.918 | 0.396

Least Significant Difference (LSD) among the means at P > 0.05 level of probability was considered as significant.
temperate maize are established worldwide but little information is available for tropical maize. Callus induced transformation for maize is restricted because of following reasons: A) the regeneration of plants from non embryogenic callus (Type 1) is very less, 2) the proliferation of embryogenic callus results only from immature embryos.\textsuperscript{23-25} Immature embryos are the most widely used explants for developing maize transgenics.\textsuperscript{26} The difficulties of using immature embryos are unavailability of explants round the year, seasonal variation for pollination and also the requirement of high quality greenhouse facility. In addition to regeneration response is genotype dependent multiple reports consensus that mature embryos are relatively more resistant to respond in vitro micropropagation with respect to immature embryos.\textsuperscript{9,27} Maize genotypes have variable differences for in vitro culture\textsuperscript{23} and only a few maize genotypes possess regeneration capacity. Hence, it becomes important to specify growth condition for specific genotypes under in vitro culture namely; doubled haploid, somaclonal variation, genetic transformation and somatic hybridization. Therefore we developed a new regeneration method for maize by using mature seed as an explant.

**Influence of plant growth hormones on callus induction**

In vitro micropropagation efficiency differs due to variation in parameters such as concentrations of plant growth regulators and other supplements added to culture media.\textsuperscript{9} Mature seeds were germinated in the seed germination medium which contains 5 mg/l 2,4-D and 3 mg/l BAP in MS medium (Fig. 1A). The swelling of the cultured internodes was observed in the light conditions within 2 wks (Fig. 1B) and then it longitudinally splitted and (Fig. 1C) finally subcultured in the dark for induction of embryogenic callus. Splitted internodes on callus induction medium are shown in Figure 1D. The callus appears from the split portion of the internode. In dicots, including *Gossypium arboreum* L., the callus initiated from the longitudinally cut portion of the hypocotyls sections were reported.\textsuperscript{28} Auxin particularly, 2,4-D (1–3 mg/l) is essential for induction of embryogenic callus from cereal embryos.\textsuperscript{28} In this study we didn’t get any embryogenic callus by using only 2,4-D (data not shown) while various composition of 2,4-D and BAP showed embryogenic response. Media enrich with 2,4-D and BAP is a common phenomenon for induction of embryogenic callus.\textsuperscript{29} Within 2 wks of incubation, the bulgy internodes form into abnormal callus and then callus were manually removed from the internodes and subcultured in the same medium for further proliferation. The yellow friable embryogenic callus was proliferated after 3 wks in dark conditions (Fig. 1E). Vladimir\textsuperscript{11} reported callus formation from internodes. Significantly higher ($P > 0.05$) frequency of well proliferated embryogenic callus (90%) was noticed on the MS medium provided with 2 mg/l 2,4-D and 1.0 mg/l BAP (Table 1). The rate of callus formation showed a decreasing tendency at lower concentration (1 mg/l 2,4-D, 0.1 mg/l BAP). At higher concentrations (2.5–3.5 mg/l 2,4-D, 1.5–2.5 mg/l BAP), the maximum number of callus formation is observed (38%). So, excessive concentrations of 2,4-D and BAP negatively affected the callus formation rate (Table 1).

To develop organogenic callus, several plant growth regulators (BAP and 2,4-D) with various combinations were studied for subculture them sequentially under light condition. Two subcultures were found to be necessary for dense, rigid, dry as well as considerably distinguished state of callus (Fig. 1H) and for shoot regeneration. Among the six tested plant growth hormone combinations in MS medium, 2.5 mg/l 2,4-D and 1.5 mg/l BAP were significantly produced higher organogenic callus ($P > 0.05$). The maximum callus proliferation frequency recorded was 85% (Table 1). Similar results have been reported in tropical and subtropical genotypes for production of organogenic callus and no fertility was observed when transgenic plant was grown into the greenhouse.\textsuperscript{30}

**Regeneration**

Shoot regeneration is crucial step for cell and tissue culture techniques for plant improvement. Auxins are essential for callus
The present investigation confirms the significance of the cytokinin (BAP, KIN) and auxin (NAA) ratio for the shoot regeneration in maize. This is consistent with our previous report in tobacco. After 2 wks, well established rooted plantlets were obtained (Fig. 1G and J) and individual rooted plantlets 7–10 cm in length, were transferred directly from the culture room to a glasshouse, small pots composed a mixture of peatmoss vermiculite and sand in the ratio of 1:1:1 (Fig. 1F and J). The regeneration efficiency was examined using calluses derived from internodes and were subcultured in MS medium enriched with varying concentrations and composition of plant growth hormones (Table 2). Shoot initiation was observed from embryogenic calli (Fig. 1F), within 2 wks and for organogenic callus (Fig. 1I) it took 1 wk after subculture. No regeneration was found when the calli were subcultured in the fresh MS medium consisting cytokinin (BAP and KIN). To overcome such problem, these calli were further subcultured in a medium containing cytokinins (BAP, KIN) along with less concentrations of auxin (NAA). Significantly higher shoot induction (90.0%) was achieved (embryogenic and organogenic callus) on MS medium provided with 2.0 mg/l BAP + 1 mg/l KIN + 0.5 mg/l NAA (Fig. 1G and J and Table 2) at P > 0.05. Maximum of 9 shoots were recorded per callus (Table 2). The regeneration efficiency substantially increased and then decreased for both the callus in presence of growth regulators (Table 2). The high concentration of auxins was balanced by aggregation of cytokinin-like substances in the subculture media that is needed for shoot regeneration efficiency. The present investigation confirms the significance of the cytokinin (BAP, KIN) and auxin (NAA) ratio for the shoot regeneration in maize.

**Rooting and acclimatization**

For shoot regeneration, shoots were subcultured in the MS medium deficient in any growth regulators. One-week after subculture rooting was observed and further developed in the same medium. This is consistent with our previous report in tobacco. After 2 wks, well established rooted plantlets were obtained (Fig. 1G and J) and individual rooted plantlets 7–10 cm in length, were transferred directly from the culture room to a glasshouse, small pots composed a mixture of peatmoss vermiculite and sand in the ratio of 1:1:1 (Fig. 1G2 and J2). The humidity was maintained by covering them with the polythene bags and subsequently it was decreased by making small hole to the polythene covers. After proper acclimatization, the plantlets were transferred to natural condition with 90% survival rate. In this investigation, a reproducible protocol for plant regeneration was established through callus induction from nodal explants of *Zea mays*. The present investigated method takes only 2 mo to achieve complete plantlet through embryogenic and organogenic pathway as compare with 9 mo for the existing method of O’Connor-Sánchez et al. 30

**Conclusion**

The regeneration method standardized in the present investigation relies on the fact that it is efficient, quick and highly reproducible method which might be useful for genetic transformation studies. In this study we have used mature seed as a novel explant and the regeneration was achieved through callogenesis (embryogenic and organogenic). The regenerated callus yielded higher number of shoots (nine) in both organogenic and embryogenic callus within a short duration of time (50–56 d). The development of multiple self-growing shoot buds indicates several independent transgenic events which can be potentially useful to screen out the performance of transgenic events in vitro. As per our knowledge, no such reports have been discussed regarding the efficient regeneration of Indian maize inbred line (HQPM-I) via both embryogenic and organogenic callus from mature seeds.

### Materials and Methods

**Plant material and seed sterilization**

Healthy and mature viable seeds (*Zea mays* L.) local ecotypes (HQPM-I) were collected from National Seed Corporation (NSC), Indian Agricultural Research Institute (IARI) Pusa New Delhi, India. Approximately 2 g of mature seeds were initially surface sterilized with 70% ethanol for 2 min and rinsed with distilled water. Following sterilization, the seeds were treated with 4% Bavistin Carbendazim 50% (Benofit, Coromandel International Limited, Andhra Pradesh, India.) for 20 min, next 4% sodium hypochlorite (NaOCl) for 5 min and finally treated with 0.1% mercuric chloride (HgCl₂) for 10 min. To remove the surfactants, sterilized seeds were washed 5 times with sterile deionised-distilled water and blotted on to a sterile Whatman filter paper number 1. All the steps above were performed under laminar flow. All the plant growth hormones which used in this study were purchased from Duchefa Biochemie.

**Seed germination**

For seed germination the mature embryos were cultured with orientation having the embryo-axis side in contact (scutellum side up) with the MS medium supplemented with 3% sucrose, growth regulators (5 mg/l 2,4-D and 3 mg/l BAP) and finally incubated in the light (25 ± 2 °C with 50 μmol m⁻² s⁻¹) at 27 °C. After 2 wks, the bulged internodes were longitudinally split with a sharp scalpel to expose shoot meristem and simultaneously cultured on callus induction media with the split side facing the media.

### Table 2. Effects of plant growth regulators on regeneration of maize

| Plant growth regulators (mg/l) | Regeneration (%) (mean ± SE) | No of shoots per callus (mean ± SE) |
|-------------------------------|-------------------------------|-------------------------------------|
| BAP  | Kin  | NAA  |                     |                               |
| 0.5  | 0.25 | 0.0  | NR                  | NS                            |
| 1.0  | 0.5  | 0.1  | 41.65 ± 1.6         | 4 ± 1.1                       |
| 1.5  | 0.75 | 0.25 | 78.74 ± 0.63        | 7 ± 0.52                      |
| 2.0  | 1.0  | 0.5  | 90.23 ± 1.4         | 9 ± 0.41                      |
| 2.5  | 1.25 | 0.75 | 69.56 ± 0.95        | 6 ± 1.2                       |
| 3.0  | 1.5  | 1.0  | 35.14 ± 1.1         | 5 ± 0.85                      |
| LSD  | 0.05 |      | 1.789               | 0.198                         |

Least Significant Difference (LSD) among the means at p > 0.05 level of probability was considered as significant. NR, no regeneration; NS, no shoots.
Embryogenic callus

To induce calluses from split internode it was transferred to callus induction medium containing MS + 2,4-D (1.0–3.5 mg/l) + BAP (0.1–2.5 mg/l). The plates were transferred to controlled growth chamber at 25 ± 2 °C under continuous dark. Two wks after incubation embryogenic callus inductions were visible. For better proliferation the callus was removed manually from the internodes, and again subcultured for another 1 week.

Organogenic callus

The longitudinally split internodes were also transferred to the organogenic callus induction medium (MS + 1.0–3.5 mg/l 2,4-D + BAP (0.1–2.5 mg/l)). The cultures were maintained at 25 ± 2°C with 50 μmol m⁻² s⁻¹ irradiance produced by cool fluorescent lamps (Philips) and were exposed to a photoperiod of 16 h light, 8 h dark and 55% relative humidity. Two weeks later, calluses arising from the explants were separated and subcultured again for 1 wk. Three weeks later well-established morphogenetic regenerable organogenic calluses were proliferated.

Plant regeneration, rooting and acclimatization

A schematic representation of complete protocol for high frequency callus induction and plant regeneration of Indian maize (HQPM-1) is shown in Figure 2. To investigate the regeneration potential of internodes derived callus (embryogenic and organogenic) it was transferred to regeneration medium (MS + various concentration of BAP, KIN and NAA). The regeneration potential of internodes derived callus (embryogenic and organogenic) it was transferred to regeneration medium again for 1 wk. Each flask having media type and their concentration was recorded. Once the regenerated multiple shoot formation was noticed after one-week. Each flask having media type and their concentration was recorded. Once the regenerated multiple shoot reached their height 2–3 cm, the shoot bunches were separated into individual shoots and transferred to rooting medium. The initiation and establishment of the roots were continued in the MS medium. Plantlets with the hardened roots were transferred to small pots containing a mixture of vermiculite, sand and peat moss in 1:1:1 ratio. Each pot was covered with a polythene bag to maintain high humidity initially for the few days. Subsequently, the humidity was reduced by making holes in the polythene bags to harden the plants. All the media above mentioned were adjusted to pH 5.8, solidified with 0.8% agar. PGRs (Plant growth regulators) were added to the medium after autoclaving. The media were autoclaved for 20 min at 121 °C and 1.1 kg/cm² pressure.

Data analysis

In the present study, 50 explants were used for each combination of different hormones supplemented with MS medium under similar conditions. Data represent the mean ± SE of 3 independent explants (n = 3), calculated by using MSTAT computer program. Least Significant Difference (LSD) among the means at P > 0.05 level of probability was considered as significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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