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AN EFFICIENT REGENERATION FOR INVITRO MICROPROPAGATION FROM FIRST NODE OF MAIZE

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ABSTRACT

Maize is one of the most important cereal crops around the world. An efficient method for *in vitro* micro propagation and genetic transformation of plants are crucial for both basic and applied research. On the contrary, first node especially tropical maize is recalcitrant toward tissue culture. Here, highly efficient regeneration (90%) system was reported for maize organogenic callus cultures. Seeds are germinated on MS medium supplemented with 3% sucrose with 0.5% agar. Nodal regions of 2 week old seedlings were longitudinally split upon isolation and subsequently placed on callus initiation medium. Maximum frequency of embryogenic callus formation (90%) was obtained on MS medium supplemented with 2,4-D @2 $mg.L^{-1}$ and BAP @1 $mg.L^{-1}$ in the dark conditions for 2weeks. Compact granular organogenic callus (85% frequency) was obtained on MS medium supplemented with 2,4-D @2.5 $mg.L^{-1}$ and BAP @1.5 $mg.L^{-1}$ at light conditions. MS medium supplemented with BAP @3 $mg.L^{-1}$, IAA @0.5 $mg.L^{-1}$ and NAA @0.5 $mg.L^{-1}$ promoted the highest frequency of shoot induction and root formation. The regenerated plants were successfully hardened in earthen pots after adequate acclimatization. The important advantage of this improved method is shortening of regeneration time by providing an efficient and rapid regeneration tool for obtaining more stable transformants from mature seeds of maize.

Keywords: Regeneration *invitro* Micropropagation First Node Maize

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 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. 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. Since then, maize regeneration has been reported from immature embryos, mature embryos, nodal regions, leaf tissues, anthers, tassel and meristems, protoplast and shoot meristems. To the best of our knowledge, almost all the previously reported protocols were developed using the maize, which are characterized by high frequency of embryogenic callus proliferation and plant regeneration. 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 sample amount, round the year and they are responsive to tissue culture. In the present investigation, we report a new plant regeneration method for maize. 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.

MATERIALS AND METHODS

Plant material and seed sterilization

Healthy and mature viable seeds (*Zea mays* L.) 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 for 20 min, next 4% Sodium hypochlorite (NaOCl) for 10 min. To remove the surfactants, sterilized seeds were washed 5 times with sterile deionised-distilled water and blotted on to a sterile Whattman filter paper number 1. All the steps above were performed under the laminar flow.

Seed germination

For seed germination the mature seeds of maize were cultured with the MS medium supplemented with 3% sucrose, 0.5% agar and finally incubated in the light (25 ± 2 °C with 50 μ mol ms^{-1}) at 27°C. After 2 weeks, 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

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^{-1}) + BAP (0.1–2.5 mg.L^{-1}). The plates were transferred to controlled growth chamber at 25 ± 2 °C under continuous dark. Two weeks after incubation embryogenic callus inductions were visible. For better proliferation the callus was removed manually from the internodes, and again sub cultured for another 1 week.

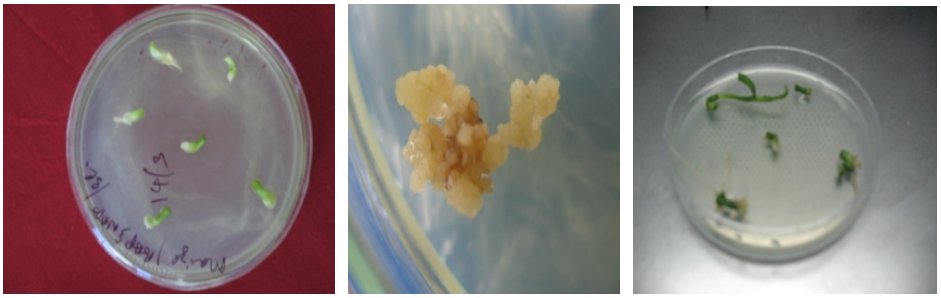
Organogenic callus

The longitudinally split internodes were also transferred to the organogenic callus induction medium (MS + 1.0–3.5 mg.L^{-1} 2,4-D + BAP (0.1–2.5 mg.L^{-1}). The cultures were maintained at 25 ± 2°C with 50 µ mol 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, calli arising from the explants were separated and sub cultured again for 1 week. Three weeks later well-established morphogenetic regenerable organogenic callus were proliferated. To investigate the regeneration potential of internodes derived callus (embryogenic and organogenic) it was transferred to regeneration medium (MS + various concentration of BAP, IAA and NAA). Each flask having media type and their concentration was recorded. The sub cultured flasks were transferred to the light (25 ± 2 °C with 50 µ mol m^{-2} s^{-1}) conditions. The adventitious shoot formation was noticed after one-week. Once the regenerated multiple shoot reached their height 2–3 cm, the shoot bunches were separated and transferred to rooting medium. 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 121^0 C and 15 lbs pressure.

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 maize. Although standard protocols for regeneration of 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, 2) the proliferation of embryogenic callus results only from nodal region. Nodal regions are the most widely used explants for developing maize transgenic. In addition to regeneration response is genotype dependent multiple reports consensus that first node are

relatively more resistant to respond in vitro micro propagation with respect to nodal region. Maize genotypes have variable differences for in vitro culture 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 first node as an explant. Influence of plant growth hormones on callus induction. In vitro micro propagation efficiency differs due to variation in parameters such as concentrations of plant growth regulators and other supplements added to culture media. Mature seeds were germinated in the germination medium which contains in MS and 3% sucrose .The swelling of the cultured internodes was observed in the light conditions within 2 wks and then it longitudinally splitted and finally sub cultured in the dark for induction of embryogenic callus. Splitted internodes on callus induction medium are shown in Figures.



The callus appears from the split portion of the internode.Auxin particularly, 2,4-D (1–3 $mg.L^{-1}$) is essential for induction of embryogenic callus from cereal embryos. In this study, 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. Within 2 wks of incubation, the bulgy internodes form into abnormal callus and then callus were manually removed from the internodes and sub-cultured in the same medium for further proliferation. The yellow friable embryogenic callus was proliferated after 3 wks in dark conditions. Significantly higher (P> 0.05) frequency of well proliferated embryogenic callus (90%) was noticed on the MS medium provided with 2 $mg.L^{-1}$ 2,4-D and 1.0 $mg.L^{-1}$ BAP . The rate of callus formation showed a decreasing tendency at lower concentration (1 $mg.L^{-1}$ 2,4-D, 0.1 $mg.L^{-1}$ BAP). At higher concentrations (2.5–3.5 $mg.L^{-1}$ 2,4-D, 1.5–2.5 $mg.L^{-1}$ 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. To develop organogenic callus, several plant growth regulators (BAP and 2,4-D) with various combinations were studied for sub culture them sequentially under light condition. Two subcultures were found to be necessary for dense, rigid, dry as well as considerably distinguished state of callus and for shoot regeneration in tropical and subtropical

genotypes for production of organogenic callus and no fertility was observed when plants was grown into the greenhouse.

Regeneration

Shoot regeneration is crucial step for cell and tissue culture induction while it played a negative role in plant regeneration. For this auxins are generally reduced or excluded from shoot regeneration media. The plant regeneration efficiency was examined using callus derived from internodes and were sub-cultured in MS medium enriched with varying concentrations and composition of plant growth hormones. Shoot initiation was observed from embryogenic callus within 2 weeks and for organogenic callus it took 1 week after sub culture. No regeneration was found when the calli were sub cultured in the fresh MS medium consisting cytokine (BAP and IAA). To overcome such problem, these calli were further sub cultured in a medium containing cytokine's (BAP,IAA) along with less concentrations of auxin (NAA). Significantly higher shoot induction (90.0%) was achieved (embryogenic and organogenic callus) on MS medium provided with 3.0 $mg.L^{-1}$ BAP + 0.5 $mg.L^{-1}$ IAA . Maximum of 9 shoots were recorded per callus. The regeneration efficiency substantially increased and then decreased for both the callus in presence of growth regulators. The high concentration of auxins was balanced by aggregation of cytokine-like substances in the sub culture media that is needed for shoot regeneration efficiency. The present investigation confirms the significance of the cytokine (BAP) and auxin (IAA&NAA) ratio for the shoot regeneration in maize(Table 1).

Rooting and acclimatization

For root induction, shoots were sub cultured in the MS medium deficient in any growth regulators. One-week after sub culture, rooting was observed and further developed in the same medium (Table 2). After 2 weeks, well established rooted plantlets were obtained 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 peat moss vermiculite and sand in the ratio of 1:1:1 . 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 techniques for plant improvement. Auxins are essential for callus induction from nodal explant of maize (*Zea mays*). The present investigated method takes only 2 month to achieve complete plantlet through embryogenic and organogenic pathway.

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 first node 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 in vitro. As per up-to-date information so far, no such reports have been discussed regarding the efficient regeneration of maize via both embryogenic and organogenic callus from mature seeds.

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Table1.Effect of various concentrations of growth hormones shoot regeneration from first node explant in maize

Growth regulators ($mg.L^{-1}$)	Responding explant	Regeneration frequency	Number of shoots per explant
1.0 BAP	70±5.77	23.44±5.77	1.3±0.67
2.0 BAP	70±5.77	53.3±3.33	1.3±0.33
3.0 BAP	90±6.77	87.5±3.33	4.2±0.33
4.0 BAP	80±6.77	76.7±3.33	2.7±0.33
1.0 BAP+0.5 IAA	60±8.82	23.44±5.77	NS
2.0 BAP+0.5 IAA	70±3.33	33.44±5.77	1.3±0.67
3.0 BAP+0.5 IAA	90±3.33	66.3± 5.77	1.3±0.67
4.0 BAP+0.5 IAA	70±3.33	33.44±5.77	2.3±0.67
1.0 BAP+1.0 NAA	70±11.55	NR	NR
2.0 BAP+1.0 NAA	70±8.82	NR	NR
3.0 BAP+1.0 NAA	80±6.67	NR	NR
4.0 BAP+1.0 NAA	80±5.77	NR	NR

Table2.Effect of various concentrations in root regeneration from first node explant in maize.

Growth regulator IBA $mg.L^{-1}$	Rooting frequency	Days of root
0.1	-	-
0.2	-	-
0.3	-	-
0.4	-	-
0.5	40	17
0.6	50	17
0.7	70	19
0.8	90	21
0.9	NR	-
1.0	95	20