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A high-throughput regeneration protocol for recalcitrant tropical Indian maize (*Zea mays* L) inbreds

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Abstract

Immature embryos from five select recalcitrant maize (*Zea mays* L) inbreds used as explants were evaluated for their ability to form callus, somatic embryos and subsequent regeneration into plants. The embryos were placed on N6 basal media with varying levels of 2,4-D (0.5, 1.0, 1.5, 2.0, and 2.5 mg l^{-1}) and were regenerated on MS medium supplemented with BAP (2 - 10 mg l^{-1}), 2,4-D (0.25 mg l^{-1}) and silver nitrate (0.85 mg l^{-1}). Explants cultured on N6 medium supplemented with 2,4-D (2.0 mg l^{-1}) were associated with the highest frequency of embryogenic calli and that of UMI 29 were highly embryogenic (78.67%). When synergism between dicamba and 2,4-D on Type II callus production in UMI 29 was sought to be investigated using 2,4-D (1 or 2 mg l^{-1}) individually and in combination with dicamba (3.7 mg l^{-1}) production of Type II callus with the greatest frequency of 83.33% was observed on N6 medium containing 3.7 mg l^{-1} dicamba + 1 mg l^{-1} 2,4-D. The greatest percentage of shoot induction (82.67%) was observed on MS medium supplemented with BAP (10 mg l^{-1}). Among the five genotypes tested, UMI 29 was associated with the highest percentage of callus initiation, shoot induction and mean number of developed shoots. The protocol described in this study can reliably be used to transform tropical maize inbreds as a routine.

Keywords: callus, immature embryo, maize, regeneration, somatic embryogenesis

Introduction

Maize (*Zea mays* L), dubbed the «Queen of Cereals», ranks the third major crop in global agriculture, and is being grown in diverse environmental conditions i.e. both in tropical and temperate regions. Maize has the highest yield potential among all the cereals. Maize is a principal source of carbohydrates and proteins and it is on par with other cereals in its other nutritional qualities. It has diversified uses as food, feed and as raw material for various industrial products. As special food crop, it is grown as sweet corn and popcorn, although dent, starchy or floury and flint maize are also widely used as food. Due to its high production potential and wider adaptability, maize has wider industrial utilization among all cereals. Maize is a basic raw material for the production of oil, protein, alcoholic beverages and food sweeteners and lately as fuel.

The genetic improvement of maize has been a major focus of plant breeding efforts during the past 50 years, resulting in remarkable improvement in yield and quality of this crop. However, modern plant biotechnology provides novel means for crop improvement through the integration and expression of defined foreign genes into plant cells which further regenerates into whole plants *in vitro* (El-Itriby et al, 2003). Production of genetically transformed plants mainly depends on the ability to integrate foreign genes into target cell and efficient regeneration of such transformed cells into complete plants. Immature embryos are extensively used as primary explants in (Ahmadabadi et al, 2007; Sujay et al, 2010) biolistic method of transformation of maize (Gordon-Kamm et al, 1990; Brettschneider et al, 1997; Frame et al, 2000). Most maize regeneration studies utilized genotypes adapted to temperate zones (Binott et al, 2008) and little attention has been paid for the genotypes of tropical and sub-tropical regions. The objectives of the present study were: i) establishing regeneration protocols for tropical Indian maize inbreds; ii) investigating the effect of different levels of auxin and BAP (6-Benzylaminopurine) on callus induction and regeneration respectively; iii) histological analysis of the maize callus to confirm the regeneration via somatic embryogenesis.

Materials and Methods

Plant material

In the present investigation, five well-adapted tropical Indian maize inbred lines, *viz*, UMI 29, UMI 61, UMI 113, UMI 285 and UMI 1007 were studied. Seeds were sown in greenhouse as well as in field.

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Greenhouse- and field-grown plants were self-pollinated and the whole ears were collected 10 - 12 days after pollination. The husks were removed and the cobs surface sterilized with 70 per cent ethyl alcohol for 1 min followed by 2.5% sodium hypochlorite for 7 min. The cobs were then rinsed 3 - 4 times with sterile distilled water. The immature embryos of appropriate size (usually 1 - 1.5 mm long) were excised and placed on the culture medium with the scutellar side up and the embryo axis side in contact with the media. This embryo orientation permits extensive proliferation in the scutellum and minimizes germination.

Callus induction

For callus induction, N6 medium (Chu et al, 1975) containing N6 salts and N6 vitamins supplemented with 20 g l^{-1} sucrose, 2.8 g l^{-1} proline, 200 mg l^{-1} casein hydrolysate, 10 mg l^{-1} silver nitrate and 2,4-D (2,4-dichlorophenoxyacetic acid) at different levels (0.5, 1.0, 1.5, 2.0, and 2.5 mg l^{-1}) solidified with 4 g l^{-1} Gelrite and adjusted to pH 5.8 with potassium hydroxide was used. Media were autoclaved at 121°C; 1.1 kg cm^{-1} for 20 min, sterile silver nitrate was added to the media after autoclaving. The embryos were cultured at 25 ± 2°C in a culture room in dark for 2 week. The primary calli produced at the end of two week were sub-cultured on to the same media composition periodically and data were recorded. The induction frequencies of primary callus and embryogenic callus were calculated.

To determine the synergistic effect of dicamba and 2,4-D in Type II callus production, UMI 29 was cultured on N6 medium supplemented with various levels of 2, 4-D (1 or 2 mg l^{-1}; alone or a combination of 2, 4-D (1 mg l^{-1}) and dicamba (3.7 mg l^{-1}) at 25 ± 2 °C in a culture room in dark for 2 week. The percentage of Type I and Type II callus induction was determined. Each treatment was replicated thrice and a completely randomized block design was employed in this investigation.

Histological analysis

Microtome sections of callus tissues which were fixed in formalin-acetic-alcohol (FAA) solution (10:7:2:1 parts of 95 % ethanol: distilled water: formalin: acetic acid) were used for histological assays. The stained sections of callus were observed and photographed under 40x magnification of bright field microscope.

Plant regeneration

The embryogenic calli induced from the immature embryos of the target inbred lines were transferred onto regeneration medium containing MS (Murashige and Skoog, 1962) medium supplemented with different levels of BAP (2, 4, 6, 8, and 10 mg l^{-1}), 2,4-D (0.25 mg l^{-1}) and silver nitrate (0.85 mg l^{-1}) for shoot induction. Regenerated shoots were transferred to rooting medium containing MS medium supplemented with silver nitrate (0.85 mg l^{-1}) for root development. The cultures were maintained at 26°C under 16 hour photoperiod with cool white fluorescent lights. With a view to standardizing shoot regeneration and to identifying maize genotypes amenable for regeneration, regeneration frequency was calculated at the expiry of 4 weeks in culture and the experiment was replicated thrice.

Rooted plants were transferred to pots containing sterile soil and vermicompost mixture mixed in a 1:1 ratio. The hardened plants were placed in the culture room under the light at 25 °C for 10 - 15 days. After the plant produced 1 - 2 healthy leaves, they were transferred to larger pots, maintained in greenhouse and grown to maturity.

Statistical analysis

Data analysis was done by AGRES statistical package. Mean values were separated by the Fischer's Least Significant Difference (LSD) at a 5% probability level. All percentage values were subjected to arcsine transformations prior to the statistical analyses.

Results and Discussion

Efficient regeneration of normal and fertile plants is prerequisite to recovery of genetically engineered plants from the transformed cells. Maize regeneration was reported using variety of explants such as leaf bases (Chang, 1983), leaf segments (Ahmadabadi et al, 2007), mesocotyls (Torne et al, 1980), immature embryos (Sujay et al, 2010), anther (Obert et al, 2004), immature inflorescence (Paredy and Petolino, 1990), shoot tips (Zhong et al, 1992), seedling segments (Santos et al, 1984), glumes (Suprassanna et al, 1986), immature tassels (Songstad et al, 1992), ovaries (Tang et al, 2006), mature embryos (Huang and Wei, 2004), and split seeds (Al-Abed et al, 2006).

In the present investigation, we attempted to regenerate tropical Indian maize inbreds using immature embryos as explants. Immature embryos cultured on N6 medium appeared to be more friable and proliferative and their morphogenic and growth responses appeared to be dependent on the individual components added to the N6 basal medium as was reported by Kamo et al (1985). Addition of L-proline in N6 culture medium appeared to be associated with production of a mass of friable and embryogenic calli (Armstrong and Green, 1985). Silver nitrate appeared to encourage embryogenic callus production as reported by Vain et al (1989). Auxins, especially 2,4-D in the range of 1 - 3 mg l^{-1} were essential for the formation of embryogenic callus from cereal embryos (El-Itriby et al, 2003).

Callus induction

Nature of embryogenic calli is highly dependent on choice of tissue, age and size of explant and composition of medium. The frequency of primary callus induction (15 days after culturing) on N6 medium sup-

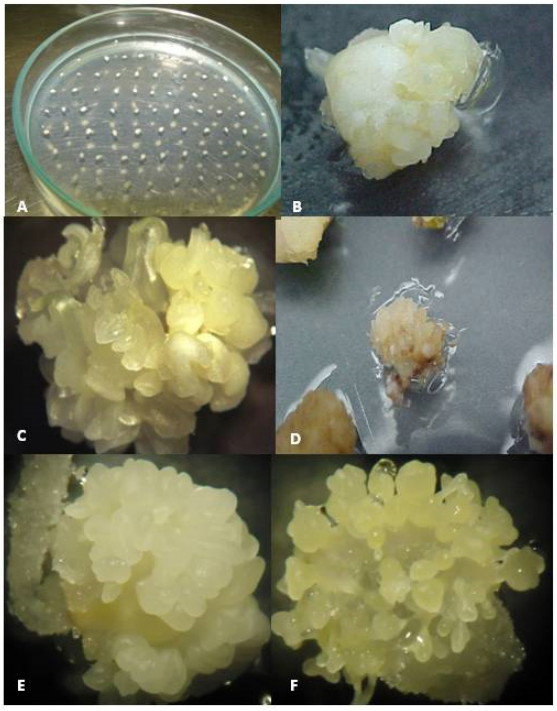

Figure 1 - Callus induction from immature embryos of UMI 29. A) 10 - 12 DAP derived immature embryos on N6 medium; B) primary callus induction; C) fast regenerable embryotic callus; D) watery non-embryogenic calli; E) compact type I embryogenic callus; F) friable type II embryogenic callus.

plemented with 2,4-D ranged from 73.34 to 100%. Culturing immature embryos of 1 - 1.5 mm (Figure 1A) collected 10 - 12 days after pollination resulted in primary callus induction (Figure 1B), which was found to be genotype dependent as reported by Shohael et al (2003). Both UMI 29 and UMI 1007 showed the highest percentage of primary callus induction (100%) from the scutellar region after 15 days of culture. However, differences in primary callus induction frequency was observed with different levels of 2,4-D (Supplementary Table 1).

Both Type I and Type II calli were observed in the cultures as reported by Rasha et al (2008). A closer examination using light microscope could distinguish proliferative, friable, fast growing, regenerable somatic embryos (Figure 1C) from whitish, non-regenerable calli (Figure 1D). Differences in frequency of embryogenic callus formation were observed among different levels of 2,4-D tested (Table 1). Of different levels of 2,4-D tested, N6 medium with 2.0 mg l^{-1} 2,4-D was found to be more effective in embryogenic callus induction with a frequency of 78.67% in UMI 29 (Table 1). The percentage of primary callus induction and embryogenic callus at different levels of 2,4-D was significantly different among the inbreds and media used (Table 1). Similarly difference in embryogenic callus induction with varying levels of 2,4-D in Kenyan genotypes was reported by Binnot et al (2008).

Effect of dicamba on Type II callus production

Though 2,4-D is the most preferred auxin for induction of somatic embryogenesis, the use of dicamba in promoting Type II callus induction was studied in the tissue culture responsive-genotype, UMI 29. The combination of 2,4-D and dicamba was found to be effective in initiating Type II callus. The Type I callus was hard and compact while Type II was friable in nature (Figure 1E and 1F). A similar report on Type I and Type II callus morphology and the ability to maintain Type II callus in culture for longer time with higher regeneration potential by repeated sub-culture was reported by Carvalho et al (1997). Because of this advantage Frame et al (2000) used Type II callus for the generation of large number of transgenic plants successfully. Maximum percentage (83.33%) of Type II callus induction was observed in immature embryos of UMI 29 in N6 media supplemented with 2,4-D (1 mg l^{-1}) and dicamba (3.7 mg l^{-1}; Table 2). A similar observation of enhanced Type II callus induction in Poaceae, *viz*, wheat (Hunsinger and Schauz, 1987), maize (Duncan et al, 1985; Bohorova et al, 1995; Carvalho et al, 1997) and *Dactylis glomerata* (Gray and Conger, 1985) due to the addition of dicamba was reported earlier.

Histological analysis of somatic embryogenesis

The proembryos originated from the scutellar tissues within 6 - 10 days of culture (Supplementary Figure 1A) and were characterized by compact, opaque, globular structures at the early stage of somatic embryo differentiation. The actively dividing meristematic cells with small dense cytoplasm and prominent nuclei were observed in proembryogenic stage in histological analysis (Figure 2A, B). The formation of meristematic zone on the surface of scutellum due to repeated mitotic division formed the proembryonal complexes or the site of somatic embryo origin. A similar observation on the formation of proembryonal complex was reported by Taylor and Vasil (1991)

Table 1 - Effect of different levels of 2,4-D on frequency of embryogenic callus induction in five maize inbreds.

	Treatments	UMI 29	UMI 61	UMI 113	UMI 285	UMI 1007
T1	N6 + 2,4-D - 0.5 mg l^{-1}	37.34±4.81c	25.34±1.33b	20.00±0.00c	25.34±3.53b	29.34±1.33b
T2	N6 + 2,4-D - 1.0 mg l^{-1}	49.34±5.33c	37.34±3.53ab	26.67±2.67b	30.67±2.67ab	34.67±3.53b
T3	N6 + 2,4-D - 1.5 mg l^{-1}	64.00±4.00ab	46.67±3.53a	32.00±2.31ab	38.67±2.67a	48.00±0.00a
T4	N6 + 2,4-D - 2.0 mg l^{-1}	78.67±1.33a	53.34±1.33a	38.67±2.67a	42.67±2.67a	50.68±2.67a
T5	N6 + 2,4-D - 2.5 mg l^{-1}	69.34±3.53a	48.00±4.00a	37.34±1.34a	44.00±2.31a	53.34±1.33a

The values presented are mean % ± SE, followed by alphabets to imply significant difference ($P \leq 0.05$); values with same letters are not significantly different.

Table 2 - Effect of dicamba on frequency of embryogenic type II callus production in maize inbred, UMI29.

Treatments		Callus Induction Percentage	
		Type I	Type II
T1	N6 + 2,4-D - 1.0 mg l^{-1}	75.00±2.50b	14.17±4.16e
T2	N6 + 2,4-D - 2.0 mg l^{-1}	58.33±2.20c	35.00±1.44d
T3	N6 + 2,4-D - 1.0 mg l^{-1} + Dicamba - 3.7 mg l^{-1}	8.33±0.83f	83.33±2.20a

The values presented are mean % ± SE, followed by alphabets to imply significant difference (P ≤ 0.05); values with same letters are not significantly different.

in pearl millet. The periclinal division of superficial callus cells in the proembryonal complex led to the development of unicellular globular somatic embryo attached to a suspensor like organ (Supplementary Figure 1B and Figure 2C). Shohael et al (2003) also observed the development of somatic embryos from the periclinically dividing scutellum derived callus tissue in maize. Later a scutellar notch formed on the globular somatic embryo deepened forming scutellar stage (Supplementary Figure 1C-E). This scutellum later developed into coleoptiar stage (Figure 2D, E) and then to torpedo stage forming shoot apical meristem. (Supplementary Figure 1F and Figure 2F). The matured somatic embryos, which were now capable of germination, produced shoot and root primordia connected by vascular tissue (Supplementary Figure 1G, H and Figure 2G). Freeling et al (1976) also observed a «scutellar-like body» which later produced shoots in maize. A similar observation on the presence of vascular bundle in matured somatic embryos was observed by Shohael et al (2003). Different stages of somatic embryos were observed together and fully developed somatic embryos were clearly distinguishable and produced shoot and root on regeneration media. However, embryos with young coleoptiles became morphologically abnormal and when maintained in the same media for a long time they developed large scutellum and failed to germinate on the regeneration medium.

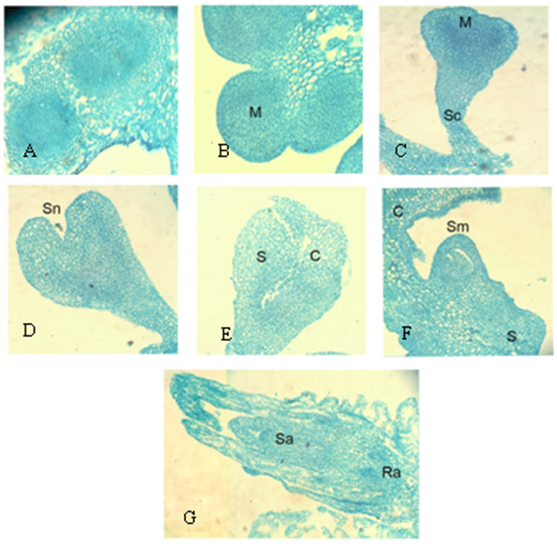

Figure 2 - Histological analysis of maize callus showing different stages of somatic embryogenesis. A) cell division; B) development of proembryos; C) globular; D) scutellar; E) coleoptiar; F) torpedo; G) advanced stage. M - meristematic zone; Sc - suspensor cells; S - scutellum; C- coleoptile; Sa - shoot apex; Ra - root apex.

Plant regeneration

MS medium is considered to be a better medium for plant regeneration (Shohael et al, 2003) and the proper ratio of cytokinin to auxin is reported to stimulate shoot formation (Skoog and Miller, 1957).

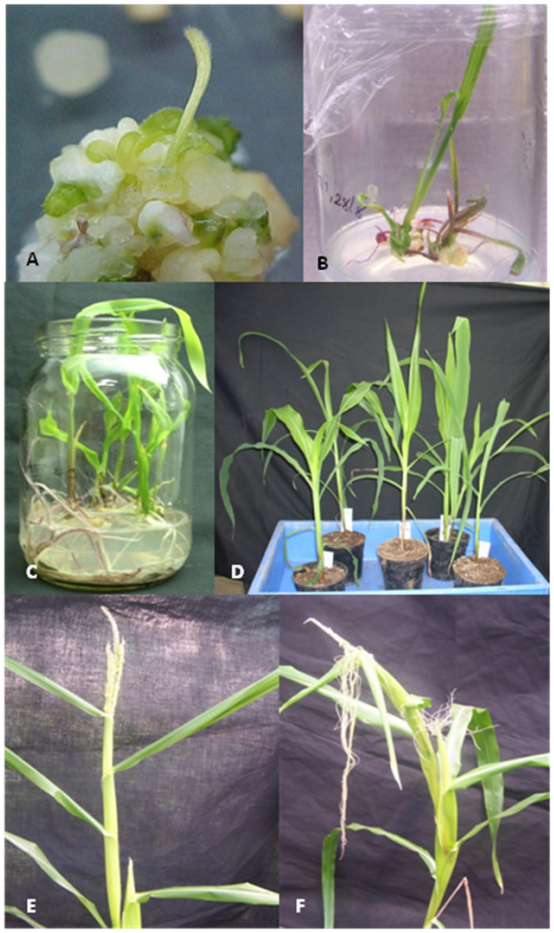

Figure 3 - A) development of green shoots on shooting medium (MS); B) well developed shoots in shooting medium; C) plantlets with healthy roots in rooting medium; D) tissue culture derived plant in green house; E) fully grown fertile tissue cultured plant (UMI 29); F) floral abnormalities of tissue culture derived plant (UMI 29).

Table 3 - Effect of different levels of BAP on frequency of shoot induction in five maize inbreds.

Treatments		UMI 29	UMI 61	UMI 113	UMI 285	UMI 1007
T1	MS + BAP - 2 mg l^{-1}	34.67±3.53d	24.00±0.00d	21.34±1.34b	24.00±0.00c	21.34±1.34b
T2	MS + BAP - 4 mg l^{-1}	53.34±2.67c	30.67±1.34d	28.00±4.00ab	29.34±1.34c	30.67±3.53ab
T3	MS + BAP - 6 mg l^{-1}	66.67±3.53ab	50.67±3.53c	37.34±4.81a	45.34±5.33ab	38.67±2.67a
T4	MS + BAP - 8 mg l^{-1}	72.00±4.00a	60.00±4.00b	42.67±2.67a	54.67±3.53a	45.34±2.67a
T5	MS + BAP - 10 mg l^{-1}	82.67±3.53a	69.34±1.34a	45.34±2.67a	58.67±2.67a	48.00±6.11a

The values presented are mean % ± SE, followed by alphabets to imply significant difference ($P \leq 0.05$); values with same letters are not significantly different.

Although a range of cytokinins are known to affect *in vitro* plant cultures (Bhaskaran and Smith, 1990), addition of BAP (10 mg l^{-1}) to regeneration medium had a significant effect on shoot induction. Experiments conducted to study the effect of different levels of BAP on regeneration indicated that the medium, MS + BAP-10 mg l^{-1} showed the highest percentage of shoot regeneration (Figure 3A) in all genotypes as indicated in Table 3. MS medium with increasing concentration of BAP with 0.25 mg l^{-1} of 2,4-D allowed shoot induction. Besides, this medium produced the highest mean number of shoots irrespective of genotypes (Supplementary Table 2). The genotype, UMI 29 that produced the highest somatic embryogenic calli induction frequency exhibited highest shoot regeneration frequency of 82.67% as well as the highest mean number of shoots per explants. The regeneration frequency at different levels of BAP was significantly different among the inbreds and media used (Supplementary Table 3).

Though shoot induction was observed in all the genotypes studied, frequencies of embryogenic callus and regeneration were higher in UMI 29 and UMI 61. Well developed shoots (Figure 3B) were transferred onto rooting medium for root development (Figure 3C). The plants with 2 - 3 leaves were transferred to greenhouse and grown to maturity (Figure 3D). However, a few abnormal plants were noticed among the regenerants in all genotypes. Among the plants generated, some showed floral or structural abnormalities (Figure 3F) which may be due to stresses induced during various stages of development (Sujay et al, 2010). However, normal regenerated plants flowered (Figure 3E) and cobs were also harvested.

The present tissue culture study sets a starting point for developing more efficient protocol for embryogenic callus production in selected tropical maize genotypes and their future use in transgenic maize development.

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