

Plant regeneration through callus initiation from mature and immature embryos of maize (*Zea mays* L.)

M. Guruprasad¹, V. Sridevi², G. Vijayakumar and M. Satish Kumar*³

Department of Crop Physiology,
Acharya N.G. Ranga Agricultural University, Naira-532 185, India.

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ABSTRACT

An efficient regeneration was developed using mature and immature embryos by using Maize (*Zea mays* L.) variety MU 2092. Mature embryos are removed from surface sterilized seeds, slice them into halves and immature embryos are detached from seed endosperm. Both are used as explants to initiate callus on N₆ medium supplemented with 2,4 D @ 4.0 mg.L⁻¹. The induction frequency of primary calli i.e embryogenic callus was 90% in maize. The embryogenic calli on N₆ medium supplemented with 6-benzylaminopurine (BAP) @ 0.5 mg.L⁻¹ and kinetin @ 0.5 mg.L⁻¹ was more effective in producing shoots. The culture expressed maximum plant regeneration potential with eight shoots per embryo on regeneration. Green shoots thus developed were successfully rooted within 20 days on MS media containing IBA (Indole-3-Butyric acid) 1mg L⁻¹. Over 86 % of rooted plants grew well and produced seeds normally when transferred to green house. The important advantage of this improved method is shortening of regeneration time by providing an efficient and rapid regeneration tool for mature and immature embryos.

Key words: Embryogenic callus – Maize (*Zea mays* L), Mature embryo, Immature embryo, Plant regeneration.

INTRODUCTION

Maize (*Zea mays* L.) is a widely grown cereal crop in the world today. Also, maize is the most important fodder crop among cereals in industrialized countries and many developing countries. Under the pressure exerted by limited land and water resources, expanding population and environmental stresses, the great demand for maize of both quality and quantity requires regeneration of maize. However, an efficient plant tissue culture procedure with high regeneration frequency is prerequisite for most of the approaches. The regeneration of plants from tissue culture of maize was first reported by Green and Philips (1975) utilizing immature embryos as explants. In all previous reports only non-competent callus was obtained. It is presented here, regeneration system based on initiation of embryogenic calli from mature and immature embryos of maize are competent. To my knowledge, there are several reports in which differences among genotypes and hormonal regulation during somatic embryogenesis of maize which permit the application for development process to a broad spectrum. Many procedures for procuring embryogenic callus which are capable of plant regeneration, enables cell technologies to be applied, along with conventional methods, for speeding up the selection process and making it easier. Since, biotechnological techniques and culture media required for stimulating the embryogenic response in this plant have to be improving for establishment, growth and

differentiation. So a robust and high frequency regeneration system is needed. In this report, we present a simple and efficient protocol for rapid *in vitro* plant regeneration from mature and immature embryos of maize.

MATERIALS AND METHODS

Seeds of Maize genotype MU 2092 were obtained from Department of Biotechnology, University of Mysore, Mysore. The presented investigations are carried out at the Crop Physiology Lab in Acharya N.G.Ranga Agricultural University, Naira. Seeds are surface sterilized with 70% ethanol for 2-3 min and 0.1% Mercuric Chloride (HgCl₂) for 15-20 min. The sterilized seeds are rinsed 3-4 times in distilled water and soaked in deionized water for 24h. The swollen mature embryos were removed from seeds with a scalpel and longitudinally sliced into halves. The sliced halves of embryos are plated cut-side down on induction medium. The induction medium contains N₆ medium, 3% sucrose, 0.8% agar and supplemented with 2,4 D. A range of concentrations of 2,4-D (1.0 mg/L to 5.0 mg.L⁻¹) are added and the p^H was adjusted to P^H 5.8 before autoclave. The explant (sliced embryo) placed on medium are incubated at 24±2°C in darkness. The immature embryos are excised from seeds under sterilized condition. The seeds are cut without touching the embryo and the endosperm was scooped out of the seed using back of the blade. The embryo was then removed from detached endosperm and placed on N₆

*Corresponding author's e-mail: pesmurari@gmail.com. ¹School of Biotechnology & Genetic Engineering, Bharathiar University, Coimbatore-641 046
²Department of Biotechnology, Sri Padmavati MahilaVisvaVidyalayam, Tirupati – 517 502. ³PET Research Foundation, University of Mysore, Department of Chemistry, PESCE, Mandya – 571 401.

medium same as mature embryos and incubated. After 3 weeks, the embryos producing callus was separated from remnant of the original embryos and classified into embryogenic(E) and non embryogenic(NE) callus under dissecting microscope. The embryogenic callus was transferred to shoot induction medium. The shoot induction medium used was N₆ medium containing 3% sucrose, Casein hydrolysate 1gL⁻¹, proline 550mgL⁻¹, 0.8% agar and combination of different plant growth regulators i.e. BAP and KIN. The plant growth regulator combinations tested are BAP (0.2, 0.4, 0.5 and 0.6 mgL⁻¹) and in combination with KIN 0.5 mgL⁻¹. After 7th week, observation on regeneration frequencies and number of shoots per explant are recorded. Elongated shoots are rooted on MS medium supplemented with 3% Sucrose, 0.8% Agar with ten different concentrations of IBA (0.1 mgL⁻¹ to 1.0 mgL⁻¹) and adjusted to P^H 5.8. Throughout the experiment, cultures are maintained at 24±2°C under a 16h photoperiod with cool white fluorescent light (80 μ mol m⁻¹s⁻¹). After 9th week, the plantlets with healthy roots are transplanted into a mixture of equal parts (v/v) of sterilized soil and vermiculite under humid conditions in a green house for 2 weeks. Then they are transplanted into field and grown to maturity.

RESULTS AND DISCUSSION

Induction of primary callus: Using primary calli, able to induce soft, watery, bruised, brown (even dead) non-embryogenic calli separated from remnants of the original embryo followed by compact, friable, irregularly shaped, light-yellow or creamy embryogenic calli and appear 1-2mm diameter from excised mature embryo and immature embryo within 2-3 weeks on induction medium as shown in fig 1. The induction frequency of primary callus ranged from 28% to 90% depending on the 2,4-D concentrations. In addition, rates of callus induction was relatively low at a low level 2,4-D and it only stimulated the formation of embryogenic callus at each concentration tested. Higher concentration of 2,4-D @5.0 mg. L⁻¹ did not significantly change the callus quantity or quality. In higher concentration of 2,4-D will result in a greater possibility of somatic mutation was proved by Choi *et al.* (2001), So 2,4-D concentration was optimized at 4 mg. L⁻¹. However, Maize (*Zea mays* L) variety MU

2092 showed the highest induction of primary calli in the presence of 2,4-D @4.0 mg. L⁻¹ in induction medium.

Plant regeneration: The concentration of plant growth regulators strongly influenced the embryogenic potential of the mature and immature embryo explant of Maize (*Zea mays* L) variety MU 2092. In response to different combinations of growth hormones, embryo and immature embryo explant responded for shoot initiation was ranging from 70-90%. (Table.1). The frequency of shoot initiation seemed to be more on concentration of BAP and KIN. The shooting frequency was ranged from 2.34% to 87.5%, when BAP and KIN were used. Maximum shoot regeneration frequency (87.5%) was observed on 0.5 mgL⁻¹ BAP and 0.5mgL⁻¹ KIN medium with 8 shoots/explant without any exogenous addition of other growth regulators (Figure). Though BAP@5mgL⁻¹with 0.5mgL⁻¹ IAA responded similar results, but regeneration frequencies was low, proved by Ward and Jordan(2001) on barley. Inclusion of NAA and IAA in shoot induction medium found not useful in improving either shooting frequency or number of shoots/explant in maize regeneration proved by Dahleen and Bregitzer (2002). Benzyl adenine is the most widely used and effective cytokinin for various legumes including maize (*Zea mays* L) (Shahoo *et al* 2002).

Table 1: Effect of various concentrations of growth hormones with shoot regeneration from mature and immature embryos in maize.

Growth regulators (mgL ⁻¹)	Responding explant	Regeneration frequency	Number of shoots per explant
0.2 BAP+0.5 KIN	70±5.77	23.44±5.77	1.3±0.67
0.4 BAP+0.5 KIN	70±5.77	53.3±3.33	1.3±0.33
0.5 BAP+0.5 KIN	90±6.77	87.5±3.33	4.2±0.33
0.6 BAP+0.5 KIN	80±6.77	76.7±3.33	2.7±0.33

Green healthy shoots are transferred on to rooting medium in 9th week by supplementing with MS medium and ten concentrations of IBA (0.1 mgL⁻¹ – 1.0 mgL⁻¹). High concentration IBA (1.0mgL⁻¹) observed highest rooting frequency of 95% in 20 days compared to all other concentrations (Table.2). Similar results have been described by Chang *et al* (2003) for cereals. The elongated shoots of Bermuda grass obtained from callus were rooted on B5 medium with 14.7 mM IBA by Cho *et al* (1998). The IBA was an efficient auxin to produce the roots was proved in maize by Patel *et al* (2006).

Immature embryos have frequently used as an explant source in maize tissue culture (Lu *et al.* 1984), but it is usually difficult to obtain immature embryos throughout the year and their suitable stage for culture is also strictly limited. This is in contrast to the ready availability and abundance of mature embryos from seeds. However, mature embryos are considered more recalcitrant to tissue culture

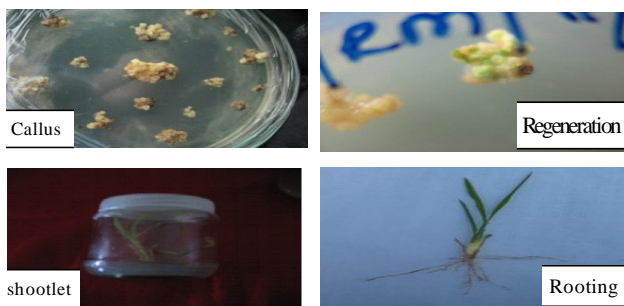
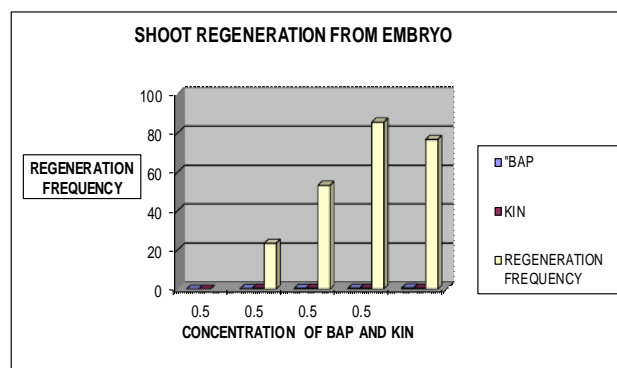


FIG: Effect of growth hormones on shoot regeneration from mature and immature embryos

Graphical representation of effect on various concentrations of growth hormones with shoot regeneration from mature and immature embryos in maize.



than immature embryos. Wang (1987) reported a low frequency and genotype dependent system for maize mature embryo. In the present investigation, high plant regeneration frequency in mature and immature embryos is significantly improved in Maize (*Zea mays* L) variety MU2092. Therefore, mature embryos which are readily available throughout the year can be used as an effective, alternative explant source in maize tissue culture. To obviate the need for isolating immature embryos, Zhong *et al.* (1992) succeeded in developing an *in vitro* method to regenerate clumps of multiple shoots at high frequency from immature embryos of aseptically-grown seedlings of maize. Also, O'Connor-Snchez *et al.* (2002) obtained transgenic maize plants of tropical and sub-tropical genotypes via micro projectile bombardment of callus containing organogenic and embryogenic-like structures derived from shoot tips. Zhang *et al.* (2002) transformed recalcitrant maize elite inbred via microprojectile bombardment of *in vitro* shoot meristematic cultures induced from germinated seedlings in comparison with the high efficient regeneration of multiple shoot clumps from shoot tips of maize. The frequency of plantlet regeneration from embryogenic callus obtained from mature and immature embryos in this study is still high. However, considering the ready availability and abundance of mature embryos from maize seeds, this protocol may be useful for the genetic transformation of maize.

The role of plant growth regulators in cereal tissue culture has been reviewed by Bhaskaran and Smith (1990). In general, auxins, usually 2,4-D in the range of 1–5 mg.L⁻¹ are essential for the establishment of embryogenic callus from

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Table 2: Effect of various concentrations of IBA on rooting of mature and immature embryos in maize

IBA	No of explants	Percent of rooting
0.1	20	Callus
0.2	20	Callus
0.3	20	Callus
0.4	20	20
0.5	20	35
0.6	20	43
0.7	20	50
0.8	20	63
0.9	20	71
1.0	20	80

cereal embryos. Studies have confirmed that the use of 2,4-D to induce callus formation from maize immature embryos was a critical factor (Armstrong and Green 1985; Bohorova *et al.* 1995; Carvalho *et al.* 1997). The results of this paper also showed that the presence of 2,4-D in culture medium was critical for maize callus induction from mature and immature embryo. Use of cytokinin in combination with auxins to induce somatic embryogenesis in callus culture has reported for cereals by Gaspar *et al.* (1996). Chaudhury and Qu (2000), in the turf-type Bermuda grass, reported that inclusion of a low concentration of cytokinin (0.044 mM BAP) in the callus induction medium containing 2,4-D promoted the induction of embryogenic callus. Cho *et al.* (1998) reported that adding 0.1–3 mg. L⁻¹ BAP to the subculture medium is essential for barley embryogenic callus maintenance. These studies indicated that addition of cytokinin into culture medium was important for embryogenic callus formation. In the present investigation, the addition of 2,4-D to callus induction medium, significantly increased the frequency of embryogenic callus formation. The number of plants regenerated was 28% to 87% greater than that produced from callus placed directly on H medium by Duncan and Widholm (1988). This minimizes the amount of time in culture. Further studies to optimize agrobacterium transformation are in progress.

CONCLUSION

In conclusion, we have developed a protocol for maize regeneration from mature and immature embryos. The results of this study suggests that it gives high regeneration frequency by optimizing the composition of shoot induction medium for maize (*Zea mays* L) Variety MU 2092.

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