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PLANT REGENERATION THROUGH CALLUS INITIATION FROM MATURE AND IMMATURE EMBRYOS OF MAIZE (*ZEA MAYS* L)

M. GURUPRASAD
Ph.D.Scholar, Dept of Biochemistry, Bharatiyar Univ. Coimbatore, TN.

V. SRIDEVI
Lecturer, Dept. of Biotechnology, Sri Padmavati MahilaVisvaVidyalayam, Tirupati, A.P.

P. UDAYSRI
Asst.Prof, Dept. of Biotechnology, Acharya Nagarjuna University, Guntur, A.P.

M. SATISH KUMAR
Corresponding Author: Assoc.Prof, Dept. of Chemistry, *P.E.S. College of Engineering*, Mandya ***E-mail:*** pesmurari@gmail.com

G.VIJAYA KUMAR
Asst.Prof, Dept. of Crop Physiology, ANGR Agril.University, Ag.College, Naira A.P.

ABSTRACT

An efficient maize regeneration was developed using mature and immature embryos. Embryos were removed from surface sterilized mature and immature embryos, ten slices into halves. They were used as explants to initiate callus on induction medium supplemented with 2,4 Dichlorophenoxyacetic acid (2,4-D) @4.0 mg.L^{-1}. The induction frequency of primary calli was over 90% for all inbred lines tested. After 2 weeks, embryogenic calli were formed. The embryogenic callus readily formed plantlets on regeneration medium supplemented with 6-benzylaminopurine (BAP) @0.5 mg.L^{-1} and kinetin @0.5 mg.L^{-1}. The regenerated plantlets were transferred to half strength Murashige and Skoog medium supplemented with indole-3- butyric acid (IBA) @1 mg.L^{-1} to develop healthy roots. The regenerated plantlets were successful on transfer to soil .The frequency of forming green shoots ranged from 19.8% to 32.4%.

Keywords : Plant regeneration, Callus initiation, embryos of Maize

INTRODUCTION

Maize (*Zea mays* L) is a widely grown cereal crop in the world today. 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. It is presented here another regeneration system based upon initiation of embryogenic calli from mature embryos of maize. The objective of this research was to develop an efficient and less genotype dependent plant regeneration system from mature embryo of maize, for the ultimate utility in genetic improvement of this crop.

MATERIALS AND METHODS

All seeds were surface sterilized with 70% ethanol for 2 min and 0.1% Mercuric Chloride (HgCl2) for 15 min. The sterilized seeds were rinsed six times with sterilized water and soaked in sterilized distilled water containing 2,4 Dichlorophenoxyacetic acid (2,4-D) @4.0 $mg.L^{-1}$ for 24h. The swollen mature embryos were removed from seeds with a scalpel and radicles were then separated from plumules on scutellar nodes. The plumule section (2-5mm) was longitudinally sliced into halves and then plated cut-side down on induction media. A range of concentrations of 2,4-D (0.0, 1.0, 2.0, 3.0, 4.0 and 5.0 $mg.L^{-1}$) were added to the induction medium to test initiation of callus. Mature embryos were incubated for callus induction on the media at 27^0C in darkness. After 3 weeks of culture on induction medium, the percentage of embryos producing primary calli were determined and the primary calli were then transferred to sub culture medium. The basal composition of subculture medium was the same as that of the induction medium except for the plant growth regulators. To test the effect of plant growth regulators on embryogenic callus formation, different concentrations of 2-4 D (0.0, 1.0, 2.0, 3.0 or 4.0 $mg.L^{-1}$) alone were added to the sub culture medium. The cultures were transferred onto fresh subculture medium at 27^0C in darkness, the embryogenic calli were evaluated.

The embryogenic calli were transferred onto regeneration medium supplemented with different concentrations of 6-benzylaminopurine (BAP) and Kinetin (0.0, 0.2, 0.5 and 1.0 $mg.L^{-1}$). The cultures were maintained at 27^0C under a 16h photoperiod with cool white fluorescent lights (40 µmol /m/s). After 2 weeks, the percentage of plant regeneration was calculated based on the number of embryos regenerating plantlets out of the total numbers of embryos plated on the callus induction medium. Regenerated plants were transferred to the rooting medium for root development when their heights reached over 3cm. The medium contained half-stretched

Murashige and Skoog medium supplemented IBA @1mg.L^{-1} was added to the rooting medium. The cultures were maintained at 27 ^{0}C under a 16h photoperiod with cool white fluorescent light (80 µmol /m/s). When plants grown to 10 cm height, the plantlets with healthy roots were transplanted into a mixture of equal parts (v/v) of sterilized soil and vermiculite and grown under humid conditions in a growth room for 2 weeks. Then they were transplanted into green house and grown to maturity.

RESULTS AND DISCUSSION

Induction of primary callus

By careful selection of uniform, healthy and viable seeds, the first two inbred lines tested were able to induce loose, soft and yellowish primary callus from the excised mature embryos within 2–3 weeks on the induction media). The induction frequency of primary callus ranged from 28.5% to 97.6% depending on the 2,4-D concentrations. Swelling of the scutellum region and the appearance of small calli (1–2 mm diameter) from mature embryos were observed 3–5 days and 10–15 days after the mature embryos were plated, respectively. Calli could not be induced in induction medium in the absence of 2,4-D and mature embryos readily germinated within 2–3 days of culture. In addition, rates of callus induction were relatively low at a low level of 2,4-D (1.0 mg.L^{-1}), while shoots and roots appeared at relatively high frequency. However the two inbred lines showed the highest induction of primary calli in the presence of 2,4-D @4.0 mg. L^{-1} in induction medium. Most of the explants exhibited direct callusing without germination. Higher concentration of 2,4-D @5.0 mg. L^{-1} did not significantly change the callus quantity or quality. Because higher concentrations of 2,4-D might result in a greater possibility of somatic mutation 4.0 mg. L^{-1} of 2,4-D may be the optimal concentration (Choi *et al*., 2001).

Formation of embryogenic callus

The primary calli were transferred onto fresh subculture medium containing different concentrations of 2,4-D alone. Following two subcultures, the calli were classified into two types. One type consisted of soft, watery, bruised, brown (even dead) non-embryogenic calli; the other consisted of compact, friable, irregularly shaped, light-yellow or creamy embryogenic calli. Organized somatic embryos in the surface of callus were visible under the stereomicroscope. The frequency of embryogenic callus formation varied depending on the combinations of applied plant growth regulators. On the subculture medium free of plant growth regulator, the primary calli turned brown, watery and died. 2,4-D alone stimulated the formation of embryogenic callus at each concentration tested.

Plant regeneration

The capacity to regenerate plantlets was correlated with the ability to form embryogenic calli. However, not all embryos with embryogenic callus regenerated plantlets. After embryogenic calli were transferred to regeneration medium free of plant growth regulator, green shoots were evident within a week and plantlet regeneration occurred in 20 days. We found that adding BA to the regeneration medium promoted plantlet regeneration. Both the frequency of green shoots and the number of shoots per callus increased. However, it was recorded that BAP and kinetin at 0.5 mg L^{-1} is the optimal level, giving the highest frequency of green shoot (85.2% for 9046 and 82.1% for C8605, respectively) and shoots per callus (1.7 for both inbred lines). When multiple shoots grown on regeneration medium were divided and transferred to rooting medium, thick white roots developed in about 2 weeks. Plantlets with well-developed roots were transferred to soil and transplanted into greenhouse. Nearly all of the established plants were morphologically normal, similar to the original lines, and exhibiting male and female fertility. Using this optimized protocol, plantlets were regenerated from mature embryos of all seven elite inbred lines. The frequency of plant regeneration was ranged from 19.8% to 32.4%.

Immature embryos have been frequently used as an explant source in maize tissue culture but it is usually difficult to obtain immature embryos throughout the year and their suitable stage for culture is also strictly limited (Vasil *et al*., 1984). This is in contrast to the ready availability and abundance of mature embryos from seeds. However, mature embryos are considered more recalcitrant to tissue cultures than immature embryos. Ray and Ghosh (1990) reported a low frequency and genotype-dependent regeneration system for maize mature embryos.

In the present study, using the optimized protocol, the low frequency and genotype-dependent plant regeneration from mature embryos could be significantly improved. The mature embryos of all seven elite inbred lines tested could regenerate plantlets, with a frequency of 19.8% to 32.4%. 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, succeeded in developing an in vitro method to regenerate clumps of multiple shoots at high frequency from shoot tips of aseptically-grown seedlings of maize. Plantlets of 36 genotype tested were regenerated via shoot-tip multiplication, and the regeneration frequency varied from 24% to 97%. Subsequently, the

shoot tip regeneration system was successfully applied to genetic transformation of maize. Gaspar *et al*. (1999) reported the efficient recovery of fertile transgenic maize plants via a shoot-multiplication system after micro projectile bombardment of shoot tips. Also, O'Connor-Snchez *et al.* (2002) obtained transgenic maize plants of tropical and subtropical genotypes via micro projectile bombardment of callus containing organogenic and embryogenic-like structures derived from shoot tips. Transformed recalcitrant maize elite inbred via micro projectile 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 embryos in this study is still low. However, considering the ready availability and abundance of mature embryos from maize seeds, this protocol may be useful for the genetic transformation of maize. Additionally, the study of Agrobacterium-mediated transformation of mature embryo of maize is underway by the authors.

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 1–3mgL^{-1}, are essential for the establishment of embryogenic callus from cereal embryos. Studies have confirmed that the use of 2,4-D to induce callus formation from maize immature embryos was a critical factor (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 and embryogenic callus formation from mature embryo. Use of cytokines in combination with auxins to induce somatic embryogenesis in callus cultures has been reported for cereals in the turf-type Bermuda grass by Chaudhury and Qu (2000), that inclusion of a low concentration of cytokine (0.044 mM BA) in the callus induction medium containing 2,4-D promoted the induction of embryogenic callus. Cho *et al*. (1998) reported that adding 0.1 mg L^{-1} BAP to the subculture medium is essential for barley embryogenic callus maintenance. These studies indicated that addition of cytokines into culture medium containing auxin (2,4-D) was important for embryogenic callus formation. In the present investigation, the addition of BA to sub culture medium containing 2,4-D significantly increased the frequency of embryogenic callus formation. This is consistent with work on corn shoot meristem cultures. The requirement of BA in somatic embryogenesis may be dependent on explant source, as observed by Bhaskaran and Smith (1990).

Usually, somatic embryos of embryogenic callus derived from maize immature embryos germinated into complete plantlets on regeneration medium free of plant growth regulator (Armstrong and Green 1985). The addition of cytokines into regeneration medium had little effect on germination rates. One possible explanation for this is that the somatic embryos capable of germinating to give rise to a new plantlet have already formed and their fate may be predetermined by the initiation media. Other studies, however, have shown that cytokines may promote development and germination of somatic embryos Duncan and Widholm (1988) reported that an effective regeneration protocol for maize callus cultures grown on D medium consisted of sub culturing the callus onto H medium containing 3.5 mg L^{-1} BA for 3–6 days and then transferring the callus to H medium free of plant growth regulator for 15–21 days. The number of plants regenerated was 113% to 148% greater than that produced from callus placed directly on H medium. Chang *et al*. (2003) found that sub cultured embryogenic callus of barley required more BA or kinetin (KT) in the regeneration medium to induce shoot regeneration, and BA was more effective than KT at the same levels. In the present study, we also found that adding 0.5 mg L^{-1} BAP into regeneration medium was more efficient than regeneration medium free of plant growth regulator for plant regeneration from mature embryos.

In comparison with other studies on maize immature embryo culture where multiple shoots per callus were recovered (Duncan and Widholm 1988), the shoots per callus in this study were rather low. This is probably due to the fact that each shoot on embryogenic callus was not removed immediately as it formed. As Ward and Jordan (2001) reported, our study also sought to develop a method where plantlets could be regenerated directly on the callus. This minimizes the amount of time in culture, which can be important for minimizing somaclonal variation. Therefore, it limits the number of plantlets that can be recovered per callus as space and nutrients become limiting as the plantlets grow. Further studies to optimize shoot regeneration are in progress.

CONCLUSION

In conclusion, we have developed a protocol for maize regeneration from mature embryos. The results of this study suggests that it might be possible to improve regeneration from mature embryos by optimizing the compositions of both subculture and regeneration media for specific genotypes. The protocol has four main steps: the induction of primary calli on induction medium supplemented with 4.0 mg L^{-1} 2,4-D for 3 weeks. Plantlet regeneration from the embryogenic callus on regeneration medium

containing 0.5 mg L^{-1} BA and kinetin for 3–4 weeks; and rooting on medium containing 0.6 mg L^{-1} IBA.

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Fig 1 Callus

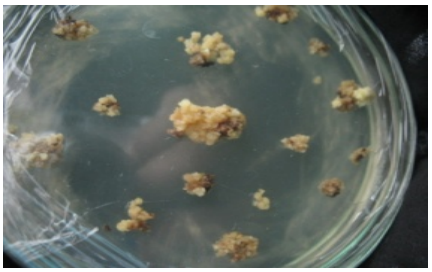

Fig 2 Regeneration

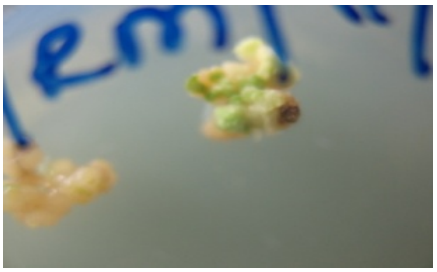

Fig 3 Rooting

Fig 4 Shootlet