ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology, Volume 2 Number 2: 154-161, 2003
ISSN 1682-2978

Somatic Embryogenesis and Plant Regeneration from Immature Embryo Derived Callus of Inbred Maize (*Zea mays* L.)

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Abstract: The present study was carried out to develop protocol in which plants could be regenerated with easy and in high numbers from tissue cultures of maize. The tissues of immature embryos were tested in three inbred lines (CML-161, CML-323, CML-327) MS and N_6 were used. In N6 medium, the range of embryogenic calli formation was varied from 56.33-72.0%. The results indicated that the highest amount 72.0% was obtained when N6 was supplemented with L-proline 2.3 gm l^{-1}, Casein Hydrolysate 200 mg l^{-1} and 2,4-D 1.0 mg l^{-1}. Where as in MS medium the embryogenic calli formation was varied from 39.0-68.66%. In this case the highest amount (68.66) was obtained where MS was supplemented with L-Asparagine 150 mg l^{-1}, Thiamin 50 mg l^{-1} and 2,4-D 1 mg l^{-1}. Plants were regenerated successfully from embryogenic callus in hormone free MS medium. The results showed that MS (52.83-61.0%) medium was found better than N6 (35.66-42.49%). The mode of somatic embryogenesis was studied using histological technique.

Key words: Maize inbreeds, immature embryo, embryogenic callus, histology, additives

Introduction

Maize is an amazing crop. In world cereal production it ranks third after wheat and rice. In recent years ***in vitro*** culture technology has received considerable attention for further genetic improvement of maize. Green and Phillips (1975) are creditated to have first reported successful regeneration of the maize plant from tissue culture. Immature embryo has proven to be the best source for the establishment of embryogenic callus and plant regeneration in maize (Green and Phillip, 1975; Freeling ***et al.***, 1976; Armstrong and Green, 1985; Tomes and Smith, 1985; Hodges ***et al.***, 1986; Tuberosa and Lucchese, 1989).

Several factors have been reported to related with the formation of callus as well as plantlet development from immature embryos in maize. The initiation and maintenance of maize cell, tissue culture and successful regeneration of plants depend on the genotype used, choice of tissue, development stage of the plant, culture media and the environment at each stage of the tissue culture process (Phillips ***et al.***, 1988; Armstrong, 1994). The age of embryos, placement of embryos on medium and composition of the culture medium are the major factors (Green and Phillips, 1975; Finar, 1998). Currently hybrid seeds are widely using for commercial maize production. The hybrids are the results of crossing between two genetically different inbred lines. This study report the methods for obtaining embryogenic callus, somatic embryos and plant

regeneration in three maize inbred lines by using immature embryo as explant. The developed information on tissue culture for the studied inbreed lines, eventually would be helpful for improvement of hybrid maize using biotechnological tools.

Materials and Methods

Experimental materials included three maize inbred lines viz., CML-161, CML-323, CML-327. To get genetically uniform explants the purity of inbred lines was maintained through selfing using hand pollination. Initially the dehusked immature cobs were harvested after 10-12 days of post anthesis and the young kernels were used for callus induction. Immature cobs were surface sterilized with 70% ethanol for 2-4 min and rinsed with sterile distilled water. Then the cobs were placed in 30% Clorox bleach solution (1.5% sodium hypochlorite) v/v having a few drops of Tween-80 for 30 min in side the laminar airflow. Embryos were excised aseptically by cutting of the top of the karnel with a sharp scalpel blade. Then the excised embryos were placed as the scutellum up on the medium. About 5-6 explants were inoculated culture-1 bottle containing 20 ml of medium.

Initially different concentrations of plant growth regulators such as 2,4-D (2,4-Dichlorophenoxy acetic acid) single or incombination with BA (6- Benzyl adenine), Kinetin were used for callus induction (Table 1). As basal salts both MS (Murashige and Skoog, 1962) and N6 (Chu ***et al.***, 1975) medium containing 3% (w/v) sucrose as source of carbon and 6 gm l^{-1} agar (w/v) as solidifying agent (pH 5.7) were used. The media were autoclaved at 120°C for 20 min. Each treatment was repeated five times and was incubated in dark for 21 days at 28±1°C. On the basis of initial results on callus induction, different additives (L-Asparagine, Thiamine, L-proline and Casein Hydrolysate) were used. In MS medium, the following supplements Laspergine and thiamin were used. For N6 the supplements were L-proline and Casein Hydrolysate. Callus induction efficiency was measured, as number of explants induced callus^{-1} total no. of explants used × 100. The data were recorded after 21 days of inoculation and the results presented as mean ± standard error.

For histological studies callus was fixed in FAA (5% formaline, 5% acetic acid, 45% ethanol and 45% alcohol) following Johansen (1940). and dehydrated in a absolute alcohol-chloroform series. Paraffin blocks with materials were prepared and sectioned serially at 12 µm thickness using a rotary microtome. Sections were stained with Safranine-Orange-G, Tannic acid (Sharman, 1943) and mounted on glass slides. The picture was taken at 10 to 100X magnification.

To promote regeneration, calli were transferred to either hormone free or with hormones (IAA and BA) in MS and N6 medium. Cultures were incubated under fluorescent light (2000 Lux, 16 hour photoperiod) at 28±2°C. Developed plantlets with primary roots were transferred to ½ strength MS medium for farther root development. After hardening the plantlets with well develop roots were transplanted to small pots containing sterilized soil. The plantlets were developed successfully up to maturity. Like callus induction efficiency plant regeneration efficiency was also measured using similar formula.

The entire experiment was conducted at Plant Breeding and Biotechnology Laboratory in the Department of Botany, Rajshahi University, Bangladesh. During the years 1999-2002.

Results and Discussion

Callus induction

The results on callus induction efficiency are presented in Table 1. When the calli were induced in medium containing only 2,4-D or with either BA or Kinetin were found non-embryogenic type. Because the natures of the calli were white, non-friable, do not subdivide or break up automatically soft and watery and no plants were regenerated from those calli when tested in regeneration medium. This type of non-embryogenic calli production in maize is also reported by others (Lowe ***et al.***, 1985; Tomes and Smith, 1985; Phillips ***et al.***, 1988. Armstrong, 1994). For developing embryogenic callus beside use of 2,4-D other additives *viz*, L-proline,

Table 1: Single use of 2,4-D or in combination with other hormones and additives for inducing embryogenic calli from immature embryos of three maize inbred lines. Data were obtained after 21 days of inoculation

	Callus induction % (±SE)			Embryogenic callus % (±SE)		
	Inbred lines					
Treatment (mg l^{-1})	CML-161	CML-323	CML-327	CML-161	CML-323	CML-327
Ms (Basal medium)						
Control (No hormone used)	NCI	NCI	NCI	NCI	NCI	NCI
2.4-D.0.5	61.33±2.02	56.0±2.30	58.0±1.52	NECD	NECD	NECD
2.4-D.1.0	80.33±1.45	75.66±1.76	77.33±2.02	NECD	NECD	NECD
2.4-D.1.5	74.0±2.08	68.0±1.73	64.33±1.20	NECD	NECD	NECD
2.4-D.1.0+0.1BA	55.33±1.45	54.33±1.20	54.33±1.33	NECD	NECD	NECD
2.4-D.1.0+0.5BA	60.0±0.57	56.66±2.4	48.66±1.20	NECD	NECD	NECD
2.4-D.1.0+0.1KIN	53.33±1.45	51.33±0.33	45.0±1.15	NECD	NECD	NECD
2.4-D.1.0+0.5KIN	50.66±1.20	48.66±0.88	44.33±1.45	NECD	NECD	NECD
Mean	62.14	58.66	55.99	NECD	NECD	NECD
2.4-D. 1.0+L-Asparagine 100+Thiamine 25	84.0±2.08	78.0±1.783	79.33±2.33	58.0±1.15	45.33±1.15	39.0±1.15
2.4-D. 1.0+L-Asparagine 150+Thiamine 50	88.33±0.88	80.66±1.45	82.0±1.52	68.66±2.02	57.66±0.88	60.33±0.88
2.4-D. 1.0+L-Asparagine 200+Thiamine 100	82.0±1.73	76.33±2.18	78.0±1.52	61.0±0.57	53.0±1.73	55.0±1.73
Mean	84.77	78.33	76.77	51.99	51.99	51.44
Grand Mean	73.74	68.49	66.38	51.99	51.99	51.44
Ms (*Basal medium*)						
Control (No hormone used)	NCI	NCI	NCI	NCI	NCI	NCI
2.4-D.0.5	65.0±1.52	56.66±1.20	58.0±1.52	NECD	NECD	NECD
2.4-D.1.0	90.0±1.52	75.66±1.85	77.0±0.57	NECD	NECD	NECD
2.4-D.1.5	78.33±1.76	70.66±1.20	65.0±1.15	NECD	NECD	NECD
2.4-D.1.0+0.1BA	56.0±2.08	49.0±0.57	48.66±0.88	NECD	NECD	NECD
2.4-D.1.0+0.5BA	64.33±1.76	51.66±0.88	52.0±1.73	NECD	NECD	NECD
2.4-D.1.0+0.1KIN	52.33±0.88	66.33±2.33	53.33±2.18	NECD	NECD	NECD
2.4-D.1.0+0.5KIN	57.66±0.88	59.0±0.57	49.66±0.33	NECD	NECD	NECD
Mean	66.23	61.28	58.99	NECD	NECD	NECD
2.4-D.1.0+L-Asparagine 2000+Thiamine 25	89.0±1.15	88.33±1.76	86.0±3.51	65.0±1.15	57.33±1.20	59.0±2.08
2.4-D.1.0+L-Asparagine 2300+Thiamine 50	93.0±1.15	91.66±0.88	89.33±0.88	72.0±1.15	65.66±0.88	53.66±1.76
2.4-D.1.0+L-Asparagine 2500+Thiamine 100	89.33±1.20	85.0±1.15	87.0±2.64	66.33±3.52	56.33±2.08	57.66±1.45
Mean	90.44	88.14	87.44	67.78	59.77	60.10
Grand Mean	78.33	74.86	73.21	67.78	59.77	60.10

Table 2: Effect of IAA and BA in two basal media on plant regeneration of embryogenic calli derived from immature embryos in three maize inbred lines. Data were obtained after 14 days of inoculation

	Embryogenic calli regenerated shoot % (±SE)			Mean number of shoot per calli (±SE)		
	Inbred lines					
Treatment (mg l^{-1})	CML-161	CML-323	CML-327	CML-161	CML-323	CML-327
MS (Basal medium)						
Without hormone	73.0±1.15	64.33±1.76	55.0±1.73	25.66±1.76	20.0±1.52	17.66±1.76
IAA 0.1 + BA 0.5	54.0±4.52	47.66±0.88	51.66±0.33	8.0±1.15	6.66±1.20	7.33±1.45
IAA 0.5 + BA 1.0	62.0±0.57	53.0±1.15	56.66±0.66	12.33±1.45	9.33±1.76	9.0±1.15
IAA 1.0 + BA 2.0	55.0±1.52	50.66±0.88	48.0±1.52	7.33±1.20	5.33±1.20	9.33±1.20
Grand mean	61.0	53.91	52.83	13.33	10.33	10.80±
N_6 (Basal medium)						
Without hormone	45.0±1.52	43.0±1.15	48.0±1.5	6.33±1.20	6.33±1.45	5.66±1.20
IAA 0.1 + BA 0.5	32.66±1.20	33.66±0.66	34.33±2.60	6.33±1.45	5.66±1.76	5.33±0.88
IAA 0.5 + BA 1.0	53.0±1.15	47.66±1.85	32.33±0.88	11.33±1.85	8.66±1.45	8.0±1.73
IAA 1.0 + BA 2.0	39.33±2.33	37.66±1.45	28.0±1.52	6.3±0.88	4.33±0.88	7.33±1.45
Grand mean	42.49	10.49	35.66	7.58	6.24	6.58±

Thiamine, L-Asparagine and Casein hydrolysate were used in both MS and N6 medium. Using those additives enhanced the capacity of immature embryo to produce high amount of callus with excellent embryogenic nature (friable easily sub cultured and regenerated almost exclusively via somatic embryogenesis with light yellow color). The use of additives: L-proline, Thiamine, L-Asparagine and Casein hydrolysate was also reported for developing somatic embryos in maize (Green and Phillips, 1975; Phillips ***et al.***, 1988; Songstad ***et al.***, 1991; Lapitan and Patena, 1993; Tuberosa and Landi, 1991; Carvalho ***et al.***, 1997; Emons and Does, ***et al.***, 1993; Armstrong, 1994; Anonymous, 1999). Regarding use of additives, L-Asparagine (100-200 mg l^{-1}) and thiamine (25-100 mg l^{-1}) were found effective in MS medium, where as in N6 medium L-proline (2.0-2.5 gm l^{-1}) and Casein hydrolysate (150-250 mg l^{-1}) were found effective for inducing somatic embryos (Fig. 1A). The concentration of the additives for producing high amount of embryogenic calli were 2,4-D (1.0 mg l^{-1}) + L-Asparagine (150 mg l^{-1}) + Thiamine (50 mg l^{-1}) in MS medium (68.66% in CML-161, 57.66% in CML-323 and 60.33% in CML-327, respectively) 2,4-D (1 mg l^{-1}) + L-proline (2.3 gm l^{-1}) + CH (200 mg l^{-1}) in N6 medium (72.0% in CML-161, 65.66% in CML-323 and 63.66 in CML-327, respectively).

In comparing the performances of genotypes for callus inducing ability, the inbred line CML-161 was found best among the studied genotypes in both MS and N6 medium. In CML-161 average callus induction and embryogenic callus formation were 73.74 and 62.55, respectively when tested in MS medium. For N6 medium it was 78.33 and 67.78, respectively. The performance of other two inbreeds was moderate.

Histological studies

Development of somatic embryo from scutellum of immature embryos via callus was examined by histological study (Fig 1). Fig 1D shows small, dense cytoplasmic proembryogenic cells with

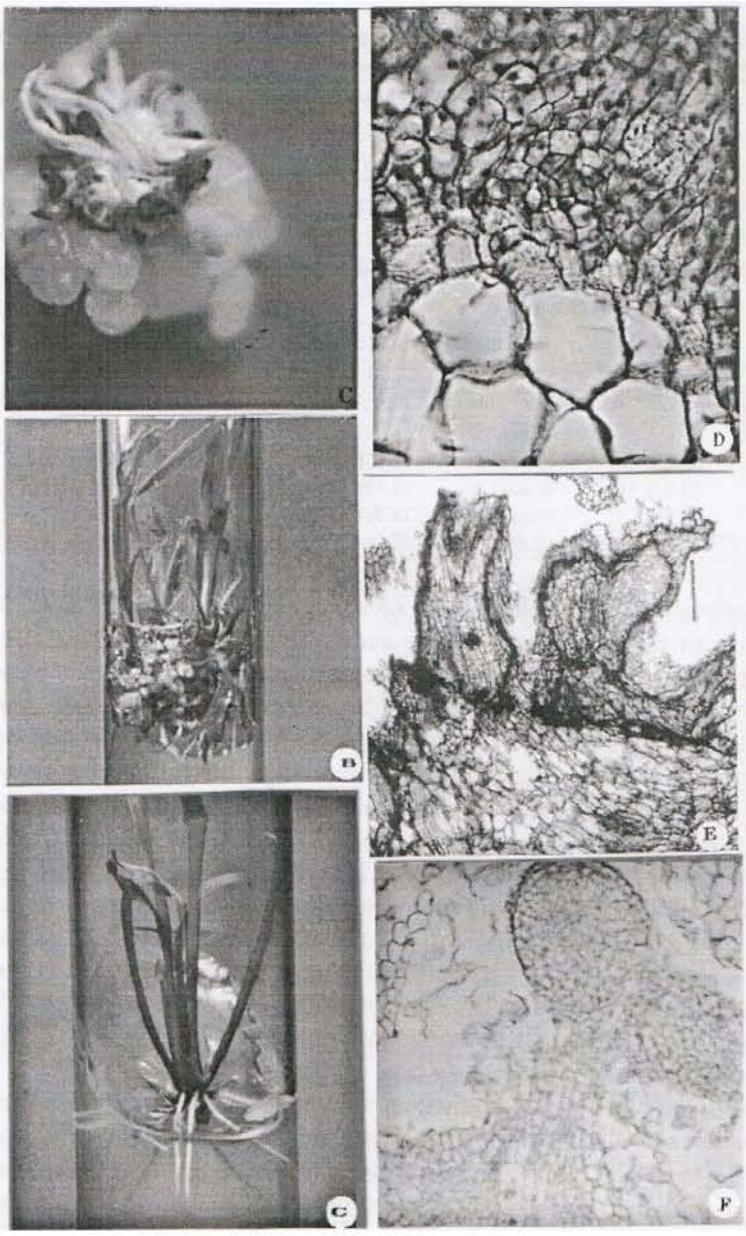

Fig. 1: *In vitro* culture and regeneration from immature embryo in *Zea may* L.

A) Somatic embryo formation from callus cultures
B) Root and shoot formation embryonic cali after transfer to MS medium without growth regulation and incubation in light
C) Single platelet with roots prior to transfer in soil; Histology
D) Embryogenic callus showing small, dense cytoplasmic proembryogenic cells with Very prominents nuclei and starch grain. Section through callus piece showing formation of shoot apices
E) and shoot primordial

very prominent nuclei and starch grain in callus tissues. Here the tissues are dividing periclinally. Fig 1E, further proved the development of somatic embryo from scutellum derived callus tissues. This shows the developing shoot and root primordial connected by vascular tissue. The development of lateral root from outer portion of vascular bundle in callus tissues is shown in Fig. 1E.

Plant regeneration

Primary callus (21 days old) was tested for regeneration efficiency. Three media composition including control (without hormone) were used in both MS and N6 medium (Table 2). The results revealed that use of hormone (s) is not essential for plant regeneration from callus derived from immature embryos. In medium without plant hormones the percentage of average number of regenerated shoot was ranged from 43.0-73.0% and the average number of shoot callus-1 was ranged from 5.66-25.66. When hormone was used in regeneration medium, the average number of regenerated shoot was ranged from 28.0-62.0% and the average number of shoot callus-1 was ranged from 4.33-12.33%. This indicates that the primary callus derived from immature embryo has contained sufficient amount of endogenous hormones for totipotency.

In comparing between MS and N6 as basal media, MS was found to be better than N6 as plant regeneration medium (Fig. 1B). Similar observation was also supported in earlier worker (Armstrong and Green, 1985; Anonymous , 1999). High frequency of regenerated shoot per calli was also reported in maize (Vasil ***et al.***, 1985; Emons and Does, 1993; Rosati ***et al.***, 1994; Carvalho ***et al.***, 1997; Bohorova ***et al.***, 1995; Das ***et al.***, 2001).

Regarding genotype used, CML-161 was identified favorable for ***in vitro*** culture. In both MS and N6 medium CML-161 was found better than other inbred lines for plant regeneration and shoot formation per callus. In MS medium the grand mean for plant regeneration was 61.0, 53.91 and 52.83 for inbred lines CML-161, CML-323 and CML-327, respectively. The no. of developed shoots was 13.33,10.33 and 10.83 for inbred lines CML-161, CML-323 and CML-327, respectively. Similar trend was noticed in N6 medium. Half strength of MS salt without any growth hormone was found effective for vigorous root development in regenerated plantlets (Fig.1C). The plantlets were successfully established in soil.

This work sets a starting point for developing more efficient protocol for embryogenic callusproduction on the treated three maize inbred lines and their further use in biotechnological research for hybrid maize development. Of special interest will be determining the amenability of the faster growing embryogenic callus generated in the study of the ***Agrobacterium*** mediated gene transfer.

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