IMPROVED SOMATIC EMBRYOGENESIS USING
L-ASPARAGINE IN WHEAT (Triticum aestivum L.)

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SUMMARY

Somatic embryos and plants were produced from mature and immature embryo derived callus of wheat. Immature embryo showed a better capacity for somatic embryogenesis and plant regeneration. Presence of 2, 4-D was shown to be essential for induction and maintenance of somatic embryogenesis. Organic nitrogenous additives (L-proline, L-asparagine and casein hydrolysate) marked an effect on somatic embryo formation when they were used in the callus maintenance medium. Histological analysis confirmed the formation of somatic embryo. The regenerated plants had the same morphology as the original plants.

Key words: Immature embryo, somatic embryo, histology and organic nitrogen, additives.

INTRODUCTION

Modern cereal biotechnology would be impossible without developing a cell culture system characterized by a high embryogenic potential. Fertile plants can be regenerated from zygotic embryo either through organogenesis or somatic embryogenesis via a callus phase in *in vitro*. Immature zygotic embryos often possess a high morphogenic potential and are frequently used for plant regeneration in species where regeneration from other explants is difficult, such as various cereals (Bhaskaran and Smith, 1990). Using such embryos, wheat (Machii et al., 1998; Maddock et al., 1983), barley (Castillo et al., 1998), rice (Alam et al., 2003), and maize (Shohael et al., 2003) cell systems having a high regeneration potential were developed. An embryo-derived wheat embryogenic callus was successfully used for genetic transformation using the biolistic transformation (Takumi and Shimada, 1996), *Agrobacterium tumefaciens* (Cheng et al., 1997) or induction of somaclonal variation (Ahloowalia and Sherington, 1985; Rayan et al., 1987; Mehta and

Angra, 2000). Almost all aspects of plant development are regulated by plant hormones which may act individually or in a concerted fashion. Local concentration of each hormone is important for the cellular response. In tissue cultures, internal hormone concentrations are, in a very complex way, influenced by the plant growth regulators present in the culture medium. The induction of somatic embryos is generally controlled by regulating the composition of phytohormones in the culture. In most cereals including wheat, cells cultured in medium containing an auxin such as 2,4-dichlorophenoxy acetic acid (2,4-D) are transferred to the medium containing no auxin, or only a small amount of auxin to induce regeneration. In addition, supplementation of amino acids in culture media has been reported to enhance somatic embryogenesis in a number of monocots (Claparols et al., 1993; Zhu et al., 1990; Furuhashi and Yatazawa, 1970; Cai et al., 1987; Wang et al., 2002; Kopertekh and Stribnaya, 2003; Abdullah et al., 2005). Amino acids prove an organic form of nitrogen (reduced state), which are readily metabolized by plant cells, stimulating faster cell growth and development (Gamborg 1970; Grimes and Hodges 1990). Therefore, the additional amino acids appear to have the potential to enhance to some extent the roles of suitable nitrogen source. The optimal composition of phytohormones and requirements of other supplements varies with plant species, and massive investigations are required to determine these compositions. Developing methods of supporting the actions of the phytohormones along with other supplements will be useful in the establishment of a reliable operation to induce somatic embryos from various plants. This paper describes a suitable and simple protocol on inducing high frequency somatic embryogenesis using nitrogenous additives and its subsequent regeneration into whole plant of four widely cultivated wheat cultivars of Bangladesh.

MATERIALS AND METHODS

Callus culture was initiated from both mature and immature seeds of four popular wheat Bangladeshi cultivars *viz* Kanchan, Shourav, Gourav and Satabdi. Dehusked immature grains were harvested and immersed in 70% ethanol for 2-4 min and rinsed with sterile distilled water. Then the grains were placed in 30% Clorox solution (1.5% v/v sodium hypochlorite) having a few drops of Tween-80 for 40 min with continuous shaking. After that, grains were rinsed 5-6 times with sterile distilled water. Embryos were then isolated aseptically by cutting from the top of the grain with a sharp scalpel blade and placed in callus induction medium. In case of mature seeds, mature embryos were separated very carefully from the seeds inoculated in germinating media (MS medium without hormones) for 36 hours.

The isolated embryos were cultured in MS (Murashige and Skoog 1962) or N_6 (Chu et al. 1975) basal medium with different concentrations of 2,4-D. For each treatment three replications and five explants per test tube were used. As a source of carbon 3% sucrose was used. Inoculated culture vessels were incubated at 25 ± 1^0C in continuous darkness, until the beginning of the embryogenesis. Induced calli were

subcultured in fresh medium after 21-28 days interval with different additives *viz*. L-asparagine, thiamine, L-proline and casein hydrolysate keeping the concentration of 2,4-D same. Besides these, different concentrations of 2,4-D without additives and MS_0 medium were also used for developing organogenic nature. Watery, spongy, very compact, brown and dead portions of calli were discarded in every subculture. Friable, nodular calli were assumed as potentially organogenic and were selected for maintenance. Subsequently the somatic embryos produced were transferred to regeneration medium.

Selected embryogenic calli were transferred to MS medium supplemented with BAP in combination with IBA for plant regeneration. The cultures were maintained at $25\pm1°C$ under a 16/8-h (light/dark) photoperiod with a light intensity of 28-30 mol $m^{-2}s^{-1}$ provided by cool-white fluorescent lamps. For each treatment three replications were used. Shoots and leaves formed from different globular somatic embryos regenerated into young plantlets on auxin-cytokinin combined medium. In this medium shoot induction frequency and number of shoots per callus were recorded for each treatment.

The shoots having poor roots were removed from the cultures and rooted on MS + NAA medium. For each treatment three replications were used. These plantlets were finally transformed to the soil through a gradual acclimatization process. The rooted plants were potted in washed sand and covered with sealed plastic vinyl bags to keep full humidity at $25\pm1°C$ under 16 h illumination (45 mol m^{-2} s^{-1}) with fluorescent lamps. With the growth of the plants, the bags were poked with chopsticks to allow air enter inside the bags until the plants were self-supported. After 3-5 weeks of acclimatization had been completed, plantlets were transferred to large pots for further growth (Figure G).

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

RESULTS AND DISCUSSION

Callus induction from scutellar tissues of both mature and immature embryos was studied. Generally, callus initiation was observed from both the explants on 10-12th day after inoculation. Use of hormone was found essential for callus induction and 2, 4-D alone was found to be the best to induce callus. Regarding use of 2,4-D (Table 1) 2.0 mg/l was found suitable for producing high amount of callus irrespective of explants and cultivars (51-80%). With the increase of concentrations of 2,4-D, the callus induction efficiency was found reduced in all cases (Table 1). Between two sources of

Table 1. Effect of different concentrations of 2, 4-D in MS and N6 medium on embryogenic callus induction from mature and immature embryos of four wheat cultivars (Data recorded after 28 days of inoculation)

Tablica 1. Djelovanje različitih koncentracija 2,4 – D u MS i N6 mediju na indukciju embriogenetskog kalusa iz dozrelih i nedozrelih embrija četiriju kultivara pšenice. (Podaci dobiveni nakon 28 dana inokulacije)

Treatments - Tretmani		Callus induction frequency (%) Učestalost indukcije kalusa (%)			
2,4-D (mg/l)	Basal medium Mazani medij	Kanchan	Shourav	Gourav	Satabdi
Immature embryo – Nedozreli embrij					
1.0	MS	48.66	45.50	40.62	41.65
	N6	27.36	24.90	20.25	25.56
2.0	MS	80.36	75.40	70.34	72.95
	N6	65.85	60.65	57.82	55.36
2.5	MS	75.45	72.30	65.62	68.18
	N6	58.60	53.25	47.70	50.28
3.0	MS	62.35	57.92	53.20	55.40
	N6	45.90	40.10	36.66	37.25
Mature seeds – Zrelo sjeme					
1.0	MS	30.25	28.60	24.90	25.18
	N6	24.30	20.36	18.65	17.90
2.0	MS	62.90	58.50	51.30	55.46
	N6	42.33	37.90	32.66	35.56
2.5	MS	54.62	51.10	48.15	50.30
	N6	35.75	32.36	26.30	30.80
3.0	MS	47.15	45.20	38.85	41.10
	N6	30.18	26.60	23.90	20.18

explants, immature embryos were found superior for inducing high frequency of callus. In case of mature embryo it was ranged from 17 to 62%, whereas for immature embryo the frequency was ranged from 37 to 80%. Addition of cytokinin (BAP or KIN) in media did not enhance callus induction frequency (data not shown). Comparing MS and N_6 medium, MS was found better for callus induction and further somatic embryo development. Callus induction began with the enlargement of the scutellar tissue. The development of primary callus took three weeks from inoculation of explants. The nature of calli was quite different from each other inducing from mature and immature embryos. Calli induced from mature embryos were whitish in colour, soft and spongy. On the other hand, calli induced from immature embryos were yellowish, more nodular and comparatively hard (Figure 1A).

Calli were further subcultured in the same or reduced level of 2,4-D, MS_0 and 2, 4-D in combination with different additives (L-aspargine, L-proline and casein hydrolysate). The nature of callus after 2nd or 3rd subculture was nodular, hard, creamy

Table 2. Effect of L-aspergin, L-prolin and casein hydrolysate along with 2.0 mg/l of 2,4-D in MS medium on induction of somatic embryos from immature embryo derived callus of four wheat cultivar (MS$_0$ and only 2, 4-D in different concentrations was also used)

Tablica 2. Djelovanje L-aspergina, L-prolina i kazein hidrolizata zajedno s 2.0 mg/l 2,4 – D u MS mediju na indukciju somatskih embrija iz kalusa nedozrelog embrija četiriju kultivara pšenice (Upotrijebljeni su MS$_0$ i samo ",4 – D u raznim koncentracijama

| Treatment Tretman	Callus induction frequency (%) Učestalost indukcije kalusa (%)			
	Kanchan	Shourav	Gourav	Satabdi
L-Proline(mg/l)				
50	48.65	45.34	40.80	41.36
150	54.80	51.33	48.70	43.70
200	43.60	41.50	36.54	39.40
L-asparagine (mg/l)				
100	56.45	54.80	51.46	53.33
150	62.70	60.33	58.65	55.30
200	50.33	46.70	43.67	40.62
CH (mg/l)				
50	35.35	32.38	27.67	30.66
150	42.36	38.25	35.60	32.33
200	39.90	36.55	30.30	33.30
2,4-D only (mg/l)				
2.0	36.33	35.20	31.92	33.45
1.0	33.60	31.45	25.20	26.60
MS$_0$	30.36	26.42	23.60	25.20

and the formation of somatic embryos was observed as well. Amino acids had a marked influence during this phase. Initially, the effects of three different concentrations of three additives (L-asparagine, L-proline and casein hydrolysate) were tested for somatic embryo development (Table 2). The additives used were those which could serve as nitrogen source, known to promote shoot formation. A significant response was found using L-asparagine at 150 mg/l concentration. In cv Kanchan frequency of somatic embryogenesis increased up to 42% using 150 mg/l L-asparagine comparing 2, 4-D only. Using all additives, mature embryo derived calli developed embryogenesis in very low frequency (9-14%, data not shown), so mature embryo was discarded for further experiment. Considering genotype, embryogenic callus production was significantly varied and Kanchan was found best. The effect of L-asparagine on embryogenesis was further studied at varying 2,4-D levels (0.5, 1.0, 2.5, and 3.0 mg/l) and highest embryogenesis was at 2.0 mg/l and lowest at 1.0 mg/l (data not shown). 2, 4-D is widely used to induce callus in cereals (Maddock et al., 1983; Carman et al., 1988). Bohorova et al. (1995) reported three types of calli induced in wheat; white friable, pale yellow compact, nodular and white watery. Our results indicate that use of low concentration of 2, 4-D was found enough for producing high amount of callus and this result confirmed the findings of others (Alam et al., 1994; Rahim et al., 1991 and Abbasi et al., 2000).

Table 3. Effect of different concentrations of IAA and BAP in MS medium on plant regeneration from embryogenic callus (Data recorded after 21 days of culture)
Tablica 3. Djelovanje različitih koncentracija IAA i BAP u MS mediju na indukciju somatskih stanica (Podaci zabilježeni nakon 21 dan uzgoja

PGR	Parameters	Cultivars - Sorte			
IAA+BAP	Parametri	Kanchan	Shourav	Gourav	Satabdi
0.1+0.5	Regeneration frequency (%) Učestalost regeneracije (%)	50.00	47.50	40.33	43.30
	No of shoot/callus ($\overline{X}$±SE) broj izdanaka po kalusu	10.56 ±0.68	9.10 ±0.40	6.65 ±0.25	8.33 ±1.08
0.5+1.0	Regeneration frequency (%) Učestalost regeneracije (%)	65.90	62.19	58.33	60.26
	No of shoot/callus ($\overline{X}$±SE) broj izdanaka po kalusu	15.73 ±0.59	12.69 ±0.79	10.12 ±0.29	12.10 ±0.62
0.5+1.5	Regeneration frequency (%) Učestalost regeneracije (%)	56.35	52.50	49.70	50.65
	No of shoot/callus ($\overline{X}$±SE) broj izdanaka po kalusu	13.69 ±1.12	10.61 ±0.18	7.12 ±0.97	8.50 ±0.33
1.0+2.0	Regeneration frequency (%) Učestalost regeneracije (%)	47.5	42.50	39.60	40.45
	No of shoot/callus ($\overline{X}$±SE) broj izdanaka po kalusu	9.60 ±1.10	8.13 ±0.25	6.56 ±0.78	6.1 ±1.10

However, use of some nitrogenous additives can increase more embryogenic callus and somatic embryo formation. In the present study, superiority of MS basal medium over N_6 proved usefulness of organic nitrogen. Here use of asparagine, CH and proline were found to be effective for producing high amount of somatic embryo. Among the three different additives L-aspergine was found most suitable for induction of somatic embryos from immature embryo derived calli. Requirement of exogenous supply of amino acids for *in vitro* somatic embryogenesis has been reported in a number of plant species, which is also influenced by exogenous supply in culture medium (Basu et al., 1989; Claparols et al., 1993). In the present study too, it was found that exogenous application of a specific amino acid, especially asparagine, promoted the process of somatic embryogenesis and regeneration. Important amino acids were found to be present in regenerated shoots as well as in embryogenic cultures. During embryogenesis (from globular embryo onwards), a few amino acids like Phe, Trp, Lys, Arg, Leu, Tyr, Asp and Ser, present nn higher levels compared to regenerated plantlets (Kamada and Harada, 1984; Murch et al., 1999). In the present study, usefulness of asparagine can be considered to provide organic nitrogen supply associated with *in vitro* culture and embryogenesis. However differential responses of different organic nitrogen sources including amino acids indicate the requirement of specific amino acids for
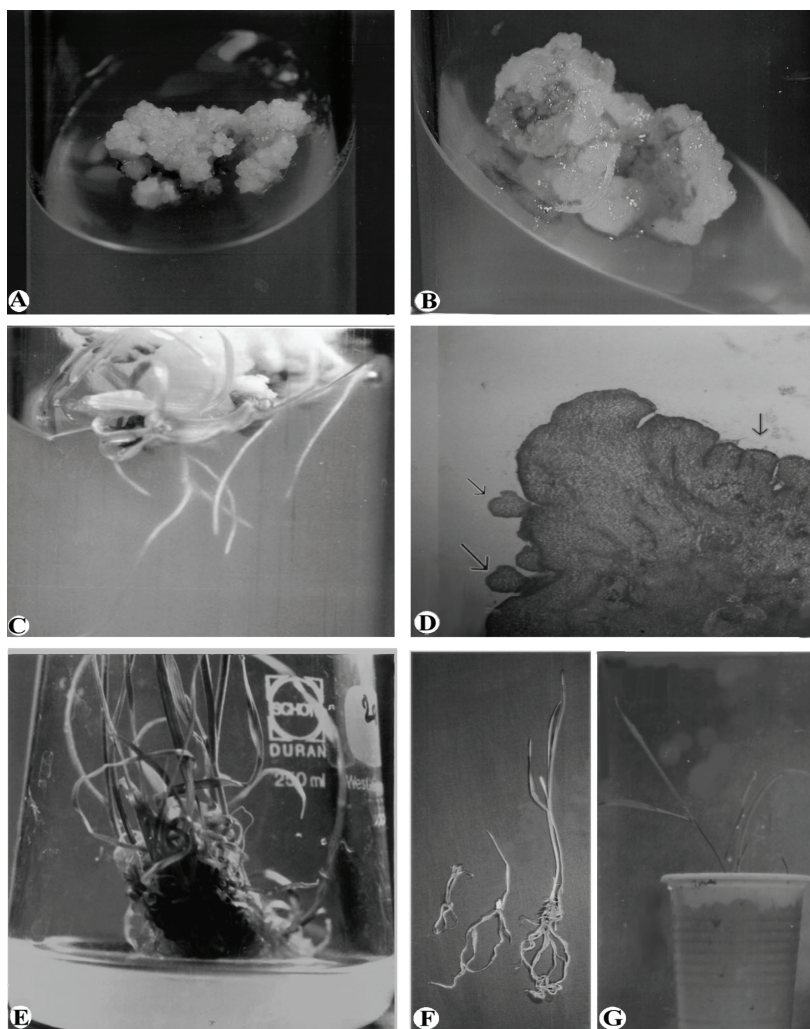
Figure 1. Callus induction and plant regeneration in wheat
Slika 1. Indukcija kalusa i regeneracija biljke pšenice

A. Friable and nodular calli induced from immature embryos
B. Formation of somatic embryo on immature embryo induced callus.
C. Shoots and roots formation from germinating somatic embryos.
D. Histological section showed development of somatic embryo (indicated by arrows)
E. Plantlets regenerated from callus
F. Plantlets showing well developed adventitious roots.
G. Acclimatized plantlets in soil condition.

specific events during morphogenesis. Development of somatic embryo from scutellum of immature embryos via callus was examined histological by (Figure 1D). Small, dense cytoplasmic embryogenic cells with prominent nuclei and starch grain in callus tissues were founded. Here the tissues are dividing periclinally. Somatic embryos appear as short conical structures. This shows the developing shoot and root primordial connected by vascular tissue.

Calli after 3rd subculture were characteristically compact but fragile, nodular and pale yellow in colour. This type of calli was generally considered as highly embryogenic (Figure 1B). These calli were placed on regeneration medium. Results on plant regeneration efficiency are presented in Table 3. After 4-7 days of transfer to regenerating medium immature embryo derived calli developed green spots and total 15-20 days were required to give rise to normal green shoots (Figure 1C, 1E). Although a large number of green spots developed on callus only few of them formed shoots. Roots were developed with shoot development (Fig. 1C). The combination of auxin and cytokinin was found effective for plant regeneration from callus. The combination of BAP (1.0 mg/l) and IAA (0.5 mg/l) was found most effective for plant regeneration from immature embryo derived calli. Shoot formation efficiency of immature embryo derived calli ranged from 40 to 65%. Number of shoot formation per calli of the regenerated shoots was also high in the same medium. Within four weeks of culture on regeneration medium the regenerated shoots attained a height of 5-7 cm (Fig. 1E). The shoots were excised and transferred individually to medium (MS + 0.1mg/l of NAA) for new root induction and proliferation (data not shown). Further root induction was observed within one week of culture and 100% of shoots rooted on this medium with the number of roots ranged form 4-10 (Figure 1F). The levels and combinations of auxin and cytokinin used in regeneration media were variable in previous reports (Chen, 1986; Ouyang, 1986) Indoleacetic acid (IAA) or α- naphthalene acetic acid (NAA) (0.2-2.0 mg/l) such as auxin or kinetin (0.2-2.0 mg/l) as cytokinin is commonly used to initiate plant regeneration. MS medium with 1.0 mg/l each of indole acetic acid and zeatin was reported effective for plant regeneration in wheat (Vasil et al., 1993).

The successful application of embryo based transformation systems in wheat requires the use of popular germplasm selected for specific tissue culture response and capacity to regenerate fertile plants. In this study popular Bangladeshi wheat cultivars showed embryogenic callus formation followed by plant regeneration efficiency. This embryogenic regeneration system for callus initiation and plant regeneration could be used in our wheat transformation experiments.

POBOLJŠANJE SOMATSKE EMBRIOGENEZE PŠENICE (*Triticum aestivml* L.) UPOTREBOM L-ASPARGINA

SAŽETAK

Somatski embriji i biljke proizvedeni su iz dozrelih i nedozrelih kalusa embrija pšenice. Nedozreli embrij pokazao je bolju sposobnost za embriogenezu i regeneraciju

biljke. Prisutnost 2,4 – D pokazala se bitnom za idukciju i održavanje somatske embriogeneze. Organski dušični aditivi (L-prolin, L-aspargin i kazein hidrolizat) djelovali su na stvaranje somatskog embrija kad su upotrijebljeni u mediju za održavanje kalusa. Histološka analiza potvrdila je stvaranje somatskog embrija. Regenerirane biljke imale su istu morfologiju kao i izvorne biljke.

Ključne riječi: Nedozreli embrij, somatski embrij, histologija i organski dušik, aditivi

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