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In vitro Plantlet Regeneration and *Agrobacterium*-mediated Genetic Transformation of Wheat (*Triticum aestivum* L.)

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Abstract

Different explants such as mature and immature embryos, seeds, endosperms, leaves, shoot bases and root tips of four local wheat (*Triticum aestivum* L.) varieties, namely Sourav, Gourav, Kanchan and Protiva were evaluated for their *in vitro* callus induction and regeneration of plantlets as well as *Agrobacterium*-mediated genetic transformation. Among the explants immature embryo was found to be the best for callus induction and shoot regeneration. Profuse callus developed when MS was supplemented with 5.5 mg/l 2,4-D. Maximum shoot regeneration was obtained on MS medium containing 0.5 mg/l BAP, 0.5 mg/l Kn and 25 mg/l tyrosine. Majority of regenerated shoots (90%) developed roots on half strength of MS without any hormonal supplement. Plantlets were success-fully established in soil. No remarkable variation was observed among the wheat varieties regarding their ability for callus induction and shoot regeneration. Two strains of *Agrobacterium*, namely LBA4404 containing pBI121 and EHA105 having plasmid pCAMBIA1301 were used in transformation experiments. Among the two strains EHA105 was found to be compatible to the aforesaid four local varieties of wheat. Maximum transformation efficiency, monitored by transient GUS histochemical assay, was obtained in callus derived from immature embryos with a bacterial suspension of optical density 0.75 at 600 nm.

Introduction

In Bangladesh wheat currently ranks next to rice in terms of production and consumption (Rahman et al. 1986). Wheat has a comparative advantage over other rainfed crops in Bangladesh. For example the amount of irrigation required for wheat is 15 times less than boro rice. Not only that, wheat fits well into a rice-wheat rotation system. Wheat continues to fill the food gap caused during failure of rice cultivation which makes this crop even more important. However, the

productivity of wheat is being hampered due to the incidence of various fungal, viral, bacterial and more so diseases caused by nematodes (Ahmed and Meisner 1996). Among these diseases, leaf rust, stem rust, loose smut, foot- and root-rot, leaf blight and losses caused by different fungi during grain storage are mainly responsible for major yield losses.

In recent years genetic transformaton techniques are used to improve different crop plants. It has been possible to transform many agronomically important crops, namely rice, (Hiei et al. 1997; Rasul et al. 1997; Chen et al. 1998; Mohanty et al. 1999) and maize (Thompson et al. 1995; Wang et al. 2000). There are also some reports on *Agrobacterium*-mediated transformation in wheat (Hess et al. 1990; Deng et al. 1990; Mooney et al. 1991; Peters et al. 1999). In the light of these reports the present investigation was undertaken with a view to developing *Agrobacterium*-mediated transformation protocols for the said wheat varieties cultivated in Bangladesh.

Success of plant transformation largely depends on the efficient regeneration protocol for the particular plant and therefore before embarking upon such transformation experiments the regeneration protocols for the selected wheat varieties were developed. In this paper results of *in vitro* plant regeneration as well as the preliminary findings of *Agrobactrium*-mediated transformation are presented.

Materials and Methods

Seeds of four local wheat (*Triticum aestivum* L.) varieties, namely Sourav, Gourav, Kanchan and Protiva were collected from the Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur. These varieties were maintained in the Plant Breeding and Tissue Culture Laboratory and in the Botanical garden, Department of Botany, University of Dhaka.

Different explants such as mature and immature embryos, seed, endosperm, leaf, shoot base and root tip were used for the induction of callus and subsequent development of shoots. Explants like root tip, shoot base and leaf were collected from the aseptically grown two - three-day-old seedlings. Immature embryos were collected from the spike after 14 - 18 days of anthesis.

Both mature and immature seeds were washed thoroughly under running tap water and then rinsed several times in distilled water. Then they were dipped in 70% ethanol (v/v) for two - three min followed by washing with distilled water. Finally surface disinfection was done with 0.1% $HgCl_2$ solution (w/v) for 5 min followed by several washes with sterilized distilled water. Then the seeds were dissected. Excised embryos were cultured in tubes.

Various explants were cultured in MS medium supplemented with various hormonal combinations and were incubated under 16/8 hrs light/dark cycle at 25 ± 2_C. After 17 to 25 days the calli were transferred to either fresh or regeneration medium. For the regeneration of shoots MS supplemented with various combinations and concentrations of BAP, Kn, IAA with or without tyrosine were used. All media contained 3 % sucrose and 0.8 % agar with pH 5.8 which was adjusted before autoclaving.

For the induction of roots, regenerated shoots (3 - 5 cm long) were excised and transferred on half strength of MS. The shoots which did not produce roots were later transferred to rooting medium consisting of half strength of MS with 0.2 mg/l IBA. Plantlets (6 - 8 cm) were transferred into sterile soil after they produced sufficient roots. They were first adapted at room temperature and then transplanted to the field.

In the transformation experiments *Agrobacterium tumefaciens* strain LBA4404 (Ooms et al. 1982) harboring the binary plasmid pBI121 and strain EHA105 (Hood et al. 1993) containing the plasmid pCAMBIA1301 were used. Both plasmids contain the GUS reporter gene as well as the *npt*II gene conferring resistance to kanamycin.

Besides immature and mature embryos embryonic calli were also used in the genetic transformation. The *Agrobacterium* suspension was centrifuged at 10000 rpm for 10 min and the precipitate resuspended in appropriate volume of MS medium to allow the bacterial suspension to reach an OD of 0.6 - 1.0 (at 600 nm). The explants were then immersed in the resuspended *Agrobacterium* suspension for different periods and then blotted dry and co-cultured for three to four days in the dark in MS with 0.5 mg/l BAP + 0.5 mg/l Kn + 25 mg/l tyrosine. After co-culture the explants were washed in 500 mg/l cefotaxime to kill the *Agrobacteria* and cultured in regeneration medium.

Following cocultivation the explants were subjected to transient GUS histochemical assay according to Jefferson (1987) to monitor the efficiency of infection. The cocultivated explants were incubated for 24 - 48 hrs at 37_C in the substrate X-gluc (5-bromo, 4-chloro, 3-indolyl-glucuronide) and subsequently bleached with 70% ethanol before scoring for transient GUS expression.

Results and Discussion

For callus induction MS medium supplemented with differnt concentrations of 2,4-D (1.5 - 13 mg/l) was used. Among the concentrations 5.5 mg/l was found to be the best for callus induction from immature embryo, shoot base and root tip. On the other hand, 6.0 mg/l 2,4-D showed the best response for callus induction

from the seeds and embryos (Table 1). All explants except leaf and endosperm produced callus of various amounts. Among these explants immature embryos were found to be the best for callus formation (Table 1). Calli developed from immature embryos were compact, nodular, whitish to creamy in colour and larger than those obtained from of other explants (Fig. 1). Calli obtained from immature embryo showed the best potential for shoot regeneration. The above results are in agreement with those reported by Eapen and Rao (1982), Sears and Deckard (1982), Reddy and Reddy (1983), Heyser et al. (1985), Lazar et al. (1988) and Galiba and Sutka (1989) in a number of varieties of wheat.

Table 1. Responses of different explants towards callus formation in the MS containing 2,4-D (Only optimum concentrations are shown).

Name of the	Type of	Supplements	% of responsive	Days required for
	A	5.5	95.0	17
	B	5.5	93.3	20
Sourav	C	5.5	93.3	20
	D	6.0	65.0	31
	E	6.0	83.3	21
	A	5.5	93.7	17
	B	5.5	93.3	20
Gourav	C	5.5	86.6	20
	D	6.0	66.6	31
	E	6.0	66.6	21
	A	5.5	93.3	17
	B	5.5	85.0	20
Kanchan	C	5.5	86.6	20
	D	6.0	70.0	32
	E	6.0	80.0	21
	A	5.5	92.0	17
	B	5.5	88.8	20
Protiva	C	5.5	80.0	20
	D	6.0	80.0	32
	E	6.0	84.0	21

A = immature embryos, B = shoot bases, C = root tips, D = seeds, E = embryos.

Unlike the results reported by Vasil and Vasil (1981), Zamora and Scott (1983), Zimny and Lorz (1986), Ahloowalia (1986) and Vazquez et al. (1991), in the present investigation leaf explant did not produce any callus. However, the present findings are in agreement with those of Bhaskaran and Smith (1990) who reported that leaf tissue from most cereals (unlike dicots) is not a satisfactory explant for callus induction.

Six different combinations of BAP, Kn, IAA and tyrosine were used for shoot regeneration from immature embryo derived callus and three combinations of BAP, IAA and tyrosine were used for mature embryo derived callus (Table 2). Among these combinations 0.5 mg/l BAP, 0.5 mg/l Kn and 25 mg/l tyrosine showed the best response for shoot regeneration from immature embryonic calli (Table 2). In this combination about 65% calli formed shoots. In case of mature embryonic callus 0.5 mg/l BAP, 0.5 mg/l IAA and 40 mg/l tyrosine was found to be the best for shoot regeneration (Table 2). Initially four to five shoots were found to develop from each callus within 5 to 12 days (Fig. 2). The number of shoots were found to increase following subculture (Fig. 3). It was noted that from a piece of callus of Sourav, the number of multiple shoots increased up to 18. The remaining calli which did not differentiate into plantlets produced only roots in the regeneration media.

Immature embryonic calli were found to be the most efficient tissue source for regeneration of whole plantlets in large numbers. This is in agreement with the findings of Shimada (1978), Shimada and Yamada (1979), Gosh-Wakerle et al. (1979); Sears and Deckard (1982); Ozias-Akins and Vasil (1982, 1983).

Callus derived from root tip and shoot base did not differentiate into shoots. Regenerants were obtained only from the calli of mature and immature embryos. Similar findings were reported by Heyser et al. (1985), Eapen and Rao (1982), Huno Singer and Schauz (1987), Ozias-Akins and Vasil (1982), Fennell et al. (1996). Contrary to these findings, Hossain (1993) did not find any plantlets from mature embryo derived callus.

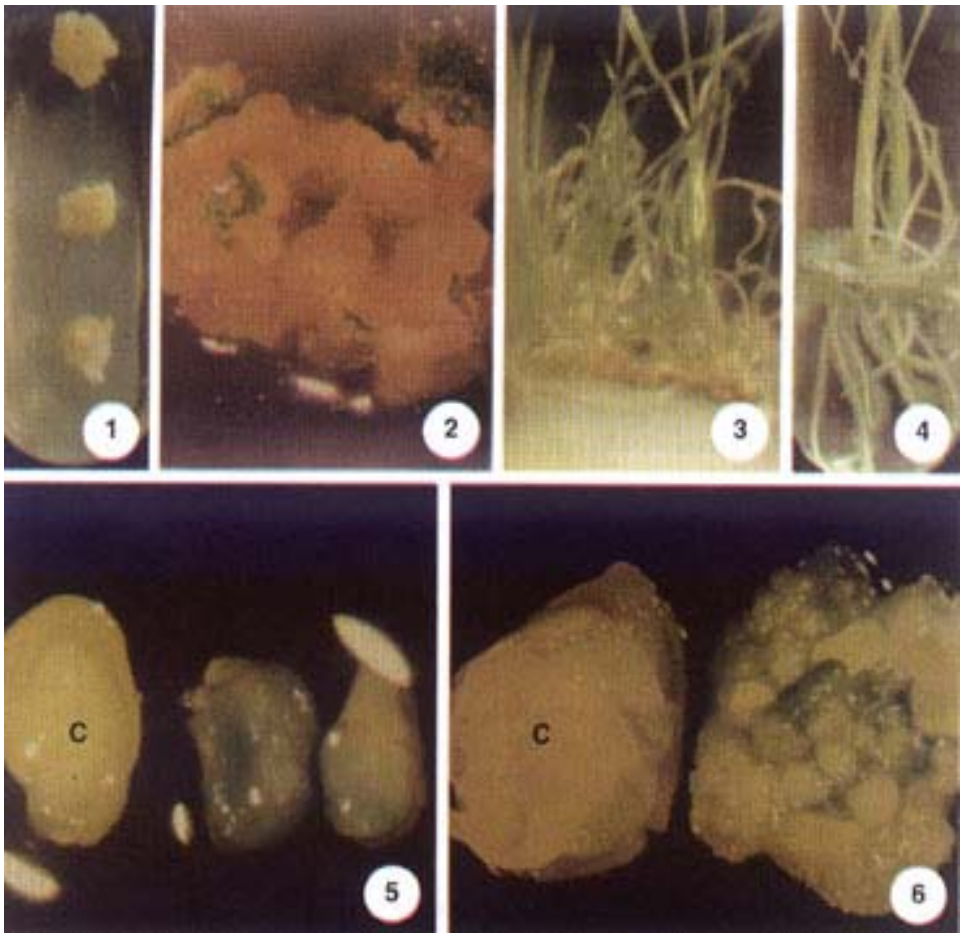
For root induction, shoots of 3 - 5 cm in length were excised and cultured on half strength of MS. In this medium more than 90% of the shoots of all varieties produced roots without the addition of any hormonal supplements (Fig. 4). The shoots that failed to grow roots were transferred to the rooting medium i.e. half strength of MS with 0.2 mg/l IBA successfully produced roots. The results are in agreement with those of Hossain (1993). Eapen and Rao (1982) reported that plantlets rooted when they were transferred to MS supplemented with 1.0 mg/l NAA. On the other hand, Fennell et al. (1996) reported that plantlets rooted when they were cultured on half strength of MS supplemented with 1.0 mg/l NAA. The fully developed plantlets were then transferred to small pots for further development. Gradually the plantlets were adapted to the soil.

Four different explants, namely mature and immature embryo and the calli derived from them were tested for their ability for transformation. For this purpose two strains of *Agrobacterium* (LBA4404 containing pBI121 and EHA105 containing pCAMBIA1301) were used. Between these two strains only EHA105

Table 2. The effect of different hormonal combinations in MS for shoot regeneration from immature and mature embryo derived callus.

Name of the variety	Type of callus	Supplements (mg/l)				No. of callus ino-culated	Per cent calli forming shoot	Days to shoot formation
		BAP	IAA	Kn	Tyro-sine			
Sourav	A	0.5	0.5	0	25	75	44.0	25
		0.5	0.5	0	0	40	20.0	27
		1.0	0.5	0	12.5	40	15.0	27
		0.5	0	0.5	25	80	68.7	22
		1.5	0	0.5	25	50	52.0	25
		1.0	0.5	0	40	50	10.0	28
	B	0.5	0.5	0	25	30	16.6	30
		0.5	0.5	0	40	44	31.8	29
		0.5	0.5	0	45	35	28.5	29
Gourav	A	0.5	0.5	0	25	75	42.6	25
		0.5	0.5	0	0	40	20.0	27
		1.0	0.5	0	12.5	40	12.5	27
		0.5	0	0.5	25	80	63.7	25
		1.5	0	0.5	25	48	50.0	27
		1.0	0.5	0	40	48	12.5	28
	B	0.5	0.5	0	25	40	17.5	30
		0.5	0.5	0	40	49	30.6	29
		0.5	0.5	0	45	50	28.0	29
Kanchan	A	0.5	0.5	0	25	70	42.8	27
		0.5	0.5	0	0	38	21.0	28
		1.0	0.5	0	12.5	35	14.2	28
		0.5	0	0.5	25	75	65.3	23
		1.5	0	0.5	25	50	50.0	25
		1.0	0.5	0	40	50	10.0	28
	B	0.5	0.5	0	25	35	17.1	30
		0.5	0.5	0	40	40	25.0	30
		0.5	0.5	0	45	40	27.5	30
Protiva	A	0.5	0.5	0	25	70	40.0	27
		0.5	0.5	0	0	35	20.0	27
		1.0	0.5	0	12.5	35	11.4	27
		0.5	0	0.5	25	75	62.6	25
		1.5	0	0.5	25	50	56.0	25
		1.0	0.5	0	40	50	14.0	28
	B	0.5	0.5	0	25	35	17.1	31
		0.5	0.5	0	40	45	26.6	31
		0.5	0.5	0	45	45	26.6	31

A = immature embryonic calli, B = mature embryonic calli.



Figs. 1 - 6 : 1. Callus formation from immature embryos of Kanchan on MS with 5.5 mg/l 2,4-D. 2. Stereomicroscopic view of shoot initiation from immature embryo derived callus of Kanchan on MS + 0.5 mg/l BAP + 0.5 mg/l Kn + 25 mg/l tyrosine. 3. Multiple shoots regenerated from immature embryo-derived callus of Kanchan on the same medium. 4. Development of roots at the base of regenerated shoot of Sourav on half strength of MS. 5. Histochemical localization of GUS activity in immature embryo of Sourav with control (c). 6. Same as 5 but showing of immature embryo derived callus of Kanchan.

showed a positive response, LBA4404 was found to be incompatible with all the explants. Factors influencing successful transformation, namely optical density of the bacterial suspension, incubation and infection period were optimized. It was found that EHA105 having an OD 0.75 at 600 nm when used for infection under 5 min vacuum showed response. For good transformation efficiencies the bacteria had to be subsequently shaken with explant in infection media for 50 min followed by a cocultivation of 96 hours. Transformed cells were obtained

from all the explants. Immature embryos of all the four wheat varieties showed the best transformation ability (Table 3). Histochemical GUS assay of immature embryo of Sourav and callus of immature embryo of Kanchan are shown in Figs. 5 and 6.

Table 3. Response of various explants of four wheat varieties towards GUS histochemical assay for *Agrobacterium* strain EHA105 containing binary plasmid pCAMBIA1301.

Name of the explants	Name of the variety	No. of explants infected	No. of explants assayed for GUS	No. of explants +ve for GUS	% of GUS +ve explants
Immature embryo	Sourav	80	20	17	85
	Gourav	80	20	15	75
	Kanchan	80	20	16	80
	Protiva	80	20	15	75
Mature embryo	Sourav	80	20	15	75
	Gourav	80	20	13	65
	Kanchan	80	20	15	75
	Protiva	80	20	12	60
Immature embryo derived callus	Sourav	70	15	12	80
	Gourav	70	15	12	80
	Kanchan	70	15	13	87
	Protiva	70	15	12	80
Mature embryo derived callus	Sourav	50	15	10	67
	Gourav	50	15	11	73
	Kanchan	50	15	10	67
	Protiva	50	15	10	67

The *in vitro* regeneration procedure described here provides a rapid and reproducible protocol for four locally grown wheat varieties. The protocol for successful transformation established in the present study can now be used for developing transgenic wheat plants for incorporation of disease resistance and other agronomically important traits.

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