Tissue culture response of CIMMYT elite bread wheat cultivars and evaluation of regenerated plants

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Summary

Six elite CIMMYT bread wheat genotypes Pavon 76, Seri M82, Opata M85, Mochis T88, Baviacora M93, and the advanced line Attila were evaluated for their response to *in vitro* tissue culture. Donor plants were grown at El Batan and CIANO experiment stations in Mexico in 1992. Immature embryos, about 1.0 mm in length, were excised and placed scutellum side up on callus initiation media. Basal N6 medium supplemented with dicamba (E1), MS medium containing 2,4D (E3), or MS medium containing 2,4D plus different amino acids (E5) was used for callus initiation and maintenance. Plant regeneration and rooting were achieved on basal MS medium with IAA and BAP.

Embryogenic calli with regeneration potentials of 10-92% were obtained on E3 and E5 media; each embryo produced 5-50 plants. A total of 800 regenerated plants were transferred to pots in the greenhouse for evaluation and seed production. Of the regenerated plants, 85% were morphologically normal, reached full maturity, and produced seeds. Seeds (R1) of 360 plants regenerated from Mochis T88, Baviacora M93, and Attila were planted in the field. Field observations indicated that embryos cultured on E3 medium resulted in 29% of the progeny producing deviating plants, compared with 21% of those grown on E5 medium. These results will form the basis for future efforts aimed at transforming CIMMYT bread wheat varieties.

Introduction

In recent years, considerable attention has been focused on improving wheat using genetic manipulation techniques. Production of the first transgenic wheat using microprojectile bombardment of regenerable embrygenic calli was reported by Vasil et al. (1991, 1992, 1993) and Weeks et al. (1993). A fundamental step in the production of transgenic material is establishing stable cultures that have the ability to regenerate phenotypically normal fertile plants and to incorporate introduced DNA into their genome. Callus initiation and shoot regeneration capacities have been studied extensively in wheat. Various nutritional, hormonal, and environmental conditions have been proposed for regenerating plants from immature wheat embryos (Carman et al., 1988; Kaleikau et al.;1989; Maddock et al.; 1983, Ozias-Akins and Vasil, 1982). Mathias et al. (1986) found that the choice of genotype is an important consideration for establishing regenerable cultures. Vasil et al. (1993) cultured immature embryos of highly regenerable wheat cultivars (two spring and one winter), and Weeks et al. (1993) used highly embryogenic cultivar Bobwhite for efficient production of transgenic wheat.

CIMMYT spring bread wheats are grown on more than 70% of the wheat-growing area in developing countries, and thus advances in this germplasm pool could have a major impact on global wheat production. This paper describes a simple procedure for inducing high-frequency somatic embryogenesis in immature embryos of elite CIMMYT genotypes, discusses the subsequent regeneration of complete plants, and reports on field evaluation of R1 progenies for use in future transformation studies.

Materials and methods

Plant material

Six elite CIMMYT bread wheat genotypes - Pavon 76, Seri M82, Mochis T88, Opata M85, Baviacora M93, and the advanced line Attila (CM85836-45Y-0M-0Y-4M-0Y) - were evaluated for *in vitro* culture response Donor plants were grown in experiment stations at El Batan, State of Mexico, in the central highlands of Mexico (19°31'N, 98°50'W, 2249 masl) and CIANO (Centro de Investigaciones Agrícolas del Noroeste), State of Sonora, in northwestern Mexico (27°20'N, 109°54'W, 39 masl) Plant materials were grown in the field in June 1992 and February 1993 Immature embryos were excised from seeds harvested 13-15 days after pollination

Callus initiation and maintenance

Seeds were surface sterilized with 70% ethanol for 1 min, followed by 20% Clorox containing 10 drops Tween-80 for 20 minutes, and then rinsed three times with sterile de-ionized water Immature embryos 1-1 5 mm in size were aseptically removed from the seeds and placed flat-side-down, scutellum up, on the initiation medium Five embryos were placed in each disposable plastic Petri dish (60 x 15 mm), with a minimum of 50 embryos being used per treatment in each experiment The cultures were incubated in the dark at 26°C The embryogenic calli were selectively subcultured by choosing compact nodular tissue and after 21 days were transferred to fresh media

Five media were evaluated for the material from El Batan and three for those from CIANO The basal media used were Chu et al (1975) (N6) and Murashige and Skoog (1962) (MS), and the main components used to supplement the basal media are presented in Table 1 The pH of all of media was adjusted to 5 7, with NaOH and 0 8% agar (Bacto) was added before sterilization

Table1 Components of five culture media for embryogenic callus formation in bread wheat from immature embryos

Culture media				
E 1	**E 2**	**E 3**	**E 4**	**E 5**
Basal N6 medium	Basal N6 medium	Basal MS medium	Basal MS medium	Basal MS medium
2 302 mg/l L-proline	2 302 mg/l L-proline	40 mg/l thiamine	40 mg/l thiamine	100 mg/l meo-inositol
100 mg/l casein hydr	100 mg/l casein hydr	150 mg/l L asparagine	150 mg/l L-asparag ine	75 mg/l glycine
				877 mg/l L-glutamine
				266 mg/l L-aspartic acid
				228 mg/l L-arginine
2 mg/l dicamba	2 mg/l dicamba	2 5 mg/l 2 4 D	2 5 mg/l 2,4 D	2 mg/l 2,4 D
				0 2 mg/l kinetin
				o 1 mg/l GA3
	15 3 mg/l AgNO3		15 3 mg/l Ag NO3	
30 g/l sucrose	30 g/l sucrose	60 mg/l sucrose	60 g/l sucrose	30 g/l sucrose
8 g/l agar	8 g/l agar	8 g/l agar	8 g/l agar	8 g/l agar

Plant regeneration and field evaluation

Plantlets were regenerated by transferring calli to basal MS medium supplemented with 0 5 mg/l Indole-3-acetic acid (IAA), 1 mg/l 6-Benzylamino purine (BAP) and 2% sucrose (MSR) and roots were

formed on half strength of basal MS medium supplemented with 1 mg/l NAA (MSE). Calli were incubated in a culture growth room at 26°C using a 16:8 light:dark photoperiod. When the plantlets reached 10 cm, they were transferred to Jiffy pots and kept in a growth chamber for a week before being transplanted to soil and grown to maturity in the greenhouse.

Plant regeneration frequency (%) was calculated as number of embryos showing plant regeneration out of the total number of embryos plated on the initial medium.

The Waller-Duncan K-ratio T-test (SAS,1994) was used to determine significant differences in regeneration potential between the genotypes tested and the 3 callus formation madia used and two locations (ElBatan and CIANO).

A subset of the varieties and generated progeny - Baviacora M93, Mochis T88, and Attila - was selected for field evaluation. About 10 regenerating embryos were chosen from each of the two media, E3 and E5. Only E3 derived embryos were available for Mochis T88. Approximately 10 plants from each embryo were grown to maturity (R0), and about 40 seeds (R1) from each of those plants were sown in the field at the CIMMYT experiment station in Toluca, located in the central highlands of Mexico (19°N, 2640 masl). The growing cycle was from late May till late October, 1993. The 40 (R1) seeds per regenerated R0 plant were grown in double-row plots, 1.5 m in length. All 360 plots of R1 plants were closely observed throughout the crop cycle.

Results and discussion

Scutellar callus formation

Culture initiation began with enlargement of the scutellar surface of the embryos, which resulted in a dome-shaped scutellum within 7-10 days and by 20 days, the explant produced a callus. Three types of calli were observed: 1) white, friable, 2) pale yellow, compact, nodular, and 3) white, watery. We found that both undifferentiated tissue and compact, nodular calli could be produced in the same explant. Primary calli were obtained in about three weeks from immature embryos of the six genotypes tested.

The embryos formed more friable, white calli on N6 medium supplemented with dicamba (E1).On E3 medium with basal MS components supplemented with 2.5 mg/l 2,4 D, the most commonly used growth regulator in wheat tissue culture (Maddock et al., 1983; Carman et al., 1988; Karadimova, 1989), yellow nodular tissue was produced within the first week; its scutellar surface developed numerous embryoids in the following two weeks. The thiamine and sucrose concentrations were higher in the E3 medium than in the basal MS that induced the high rate (almost 90%) of embryogenic callus formation in all genotypes tested. The E5 medium was supplemented with the same amino acids as reported by Abdullah et al. (1986), which were very efficient for regenerating plants from rice protoplasts through somatic embryogenesis. On this medium, the immature embryos formed irregularly shaped, pale yellow nodular embryogenic calli from the upper part of the scutellum in 7-21 days. Two of the media (E2 and E4) were supplemented with silver nitrate, but this had no beneficial effect on embryogenic callus formation.

Plant regeneration

The yellow, irregularly shaped, nodular embryogenic tissue was transferred to the regeneration medium. After 4-7 days on MSR medium, the calli developed green spots that germinated in 15-20 days and gave rise to normal green plantlets. All six lines tested produced a proliferation of green shoots on the MSR medium, however, growth rates differed among genotypes.

The composition of the medium used for callus formation was found to be important for plant regeneration. Table 2 reports values for regeneration on MSR medium calculated per genotype and per callus induction medium used. The best medium for all genotypes appeared to be E3, followed by E5 and E1. For E3 medium the mean regeneration potential was 62.10 which was not higher than for E5 medium (56.20) and significantly higher than that for E1 (26.20)(Waller-Duncan test). On E3 medium, regeneration

potential ranged from 10 to 92%, and each embryo produced 5-50 plants Of 150 Attila embryos tested on this medium, 138 (92%) produced shoots, and of 150 Mochis T88 embryos, 120 (80%) produced shoots. Four genotypes - Seri M82, Opata, Baviacora M93, and Pavon 76 - showed regeneration frequencies of 10-55%.

High rates of shoot differentiation and plantlet regeneration (29-90%) were also observed for embryogenic calli cultured on E5 medium. Regeneration rates for genotypes Baviacora M93, Pavon 76, and Attila ranged between 68 and 90%. Only one albino was observed in plantlets regenerated from Attila and two from Baviacora M93.

Table 2. Plant regeneration from immature embryos of CIMMYT bread wheats on MSR medium. Plants were grown at CIMMYT's field station in El Batan and CIANO Experiment Station in northwestern Mexico, and embryos were initially plated on one of the three media indicated.

Genotype	Location	Percent regenerated embryos from callus initiation media		
		E1	E3	E5
Sen M82	El Batan	5	55	–
	CIANO	32	50	54
Opata	El Batan	4	10	30
	CIANO	10	42	29
Mochis 88	El Batan	39	80	57
	CIANO	69	75	57
Attila	El Batan	18	92	73
	CIANO	65	88	68
Baviacora M93	El Batan	0	40	73
	CIANO	–	–	90
Pavon 76	El Batan	–	–	–
	CIANO	20	30	90

Compared with the other two media, regeneration rates of embryogenic calli developed on E1 medium were lower for most of the embryos tested. On E1 medium, Baviacora M93 produced watery, crystalline, nonembryogenic calli with roots only. Regeneration rates for Seri M82 and Opata were 4-32%, while the other two genotypes, Mochis M82 and Attila, regenerated plantlets with a frequency of 18-69% The Waller-Duncan test was to compare mean regeneration potentials on the initiation media across all genotypes tested There were significant differences of the regeneration potential among 3 callus formation media ($P<0.0007$) and genotypes ($P<0.0026$).

Differences in regenerable callus production were observed between El Batan and CIANO-grown material; however, there was no significant differences ($P<0.0599$) between the regeneration capacities of immature embryos of the materials grown at these two locations. For example, the regeneration response of Opata embryos harvested at El Batan and cultured on E3 media was 10%, while the response of CIANO-grown embryos was 42% Of genotypes grown at El Batan, Attila achieved the highest frequency of plants regenerated from embryogenic calli - 69 embryos regenerated out of 75 embryos

plated (92%) - while among genotypes from CIANO, Baviacora M93 and Pavon 76 had the highest frequency, with 135 embryos regenerated out of 150 embryos plated (90%).

Plantlets having several well developed green leaves and roots were transplanted to soil in Jiffy pots, acclimatized for a few days in a growth chamber, and then moved to the greenhouse, where 800 plants grew to maturity. A few abnormalities, such as reduced vigor, male sterility, and striped leaves, were observed among 10-15% of the regenerated R0 progeny.

The successful application of immature-embryo-based transformation systems to wheat requires the use of germplasm selected for specific tissue culture response and capacity to regenerate fertile plants. All major crops, including wheat, have been grown in cell culture with various degrees of success. But regenerating large numbers of fertile plants from somatic cells over a prolonged period is still difficult. It has been established that the embryogenic-regeneration character of wheat cultures is genotype-specific. Galiba et al. (1986) found that genes controlling shoot regeneration ability are primarily located on chromosomes 7B, 7D, and 1D, although the possibility that other chromosomes may be involved cannot be discarded. Potrykus and Petruska (1983) indicated that individual lines differed from each other with respect to the extent and type of regeneration.

In this study, elite CIMMYT wheat genotypes showed embryogenic callus formation on at least one of the media tested, and high rates of plant regeneration on MSR medium - at least 50 plants per embryo. Plant regeneration from embryogenic calli of Attila and Baviacora M93 is efficient, and can be used to produce phenotypically normal plants. This embryogenic-regeneration system - E3 or E5 media for callus initiation and MSR medium for plant regeneration - is being used in our transformation experiments.

Field evaluation of R1 progeny

Visual observations suggested that most progenies grown in the field were unaffected by having been tissue cultured and/or regenerated. This is consistent with Maddock and Semple (1986), who found 95% of their tissue-culture-derived lines to be phenotypically normal. However, later in this study, regular observations indicated that some deviating progeny had resulted. Deviating traits included: decrease/increase in stature, lateness/earliness, prostrate growth, decreased tillering, grass-clump growth habit, variations in spike morphology, lax spike, open crown, leaf flecking, leaf curling, and decrease/increase in biomass. Other researchers have observed variation in height, tiller number, spike length, lax spike habit , morphology, heading date, and maturity (Cheng et al., 1992; Larkin et al., 1984; Maddock and Semple, 1986; Sears and Deckard, 1982.). In this study, no variants were noted for fertility, waxiness, awnedness, and glume color.

Table 3 presents the frequency of embryos generating at least one plot containing visually deviating R1 plants. Table 4 lists the actual percentage of embryos that produced deviating R1 progenies.

Table 3. Frequency of embryos generating R0 plants that produced deviating R1 progeny in the field

	Variety			
Medium	Baviacora	Mochis	Attila	Total
E3	3/9 (33%)	1/9 (11%)	3/10 (30%)	7/28 (25%)
E5	2/11 (18%	-	2/9 (22%)	4/20 (20%)

Table 4 Frequency (%) of embryos that produced deviating R1 progeny (mean percentage of the R0 plants per embryo that produced deviating R1 progeny out of all R0 plants tested)

	Variety			
Medium	Baviacora	Mochis	Attila	Total
E3	42	19	20	29
E5	22	–	21	21

Although numbers are low, it appears that E3 medium makes 25% of the embryos subsequently produce some deviating R1 progenies The frequency for E5 medium was slightly lower at 20% Cheng et al (1992) reported a mean frequency of 14 2%, with up to 23 3% for line Jian 78-19, due mainly to physiological disturbances resulting from *in vitro* processes Among those embryos producing some deviating R1 progenies, the actual frequency of actually deviating progeny was also higher for E3 (29%) than for E5 (21%), in particular for the variety Baviacora It appear that E5 may be better suited for use in future transformation studies since it produces fewer variants

Selected entries were promoted to CIMMYT's bread wheat program

Recently 300 elite CIMMYT lines were purified through two generations of controlled selfing, selected lines will again be subjected to tissue culture in order to further determine which culture method produces the lowest frequency of somoclonal variants The selected method will be used to prepare embryogenic culture tissue from genotypes with the highest regeneration rates for the direct introduction of potentially useful agronomic traits using biolistic methodologies

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