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ORIGINAL PAPER

Comparative analysis of the differential regeneration response of various genotypes of *Triticum aestivum*, *Triticum durum* and *Triticum dicoccum*

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Abstract An efficient genotype independent, in vitro regeneration system was developed for nine popular Indian wheat cultivars, three each of *Triticum aestivum* L. viz., CPAN1676, HD2329 and PBW343, *Triticum durum* Desf. viz., PDW215, PDW233 and WH896, and *Triticum dicoccum* Schrank. Schubl. viz., DDK1001, DDK1025 and DDK1029, by manipulating the concentration and time of exposure to the growth regulator, thidiazuron (TDZ). A total of 18 (for immature inflorescence and embryo explant) and six (for mature embryo explant) different combinations of growth regulators were tried for callusing and regeneration, respectively. Media combination with low concentration of TDZ (2.2 μM) in combination to auxin and/or cytokinin (depending upon culture stage), was found to be effective for immature and mature explants. Compact, nodular and highly embryogenic calli were obtained by using immature embryo, immature inflorescence and mature embryo explants, and regeneration frequency up to 25 shoots/explant with an overall 80% regeneration was achieved. Comparable regeneration frequency was achieved for mature embryo explants. No separate hormone combination for rooting was required and plantlets ready to transfer to soil could be obtained in a short period of 8–10 weeks. This protocol can be used for raising transgenic plants for functional genomics analysis of agronomically important traits in the three species of wheat.

Keywords Callus · Genotype · Immature inflorescence · Immature embryo · Mature embryo · Regeneration · Somatic embryogenesis · Thidiazuron

Abbreviations

2,4-D	(2,4-Dichlorophenoxy) acetic acid
TDZ	1-Phenyl-3-(1,2,3-Thia-Diazol-5-YL) urea
DAP	Days after pollination
CM	Callus media
RM	Regeneration media
Inf	Inflorescence

Introduction

Wheat, a major food crop has remained a challenge for biotechnologists due to its recalcitrant for in vitro propagation. Many attempts have been made to establish reproducible regeneration systems for wheat, but these have either genotypic dependency, poor or no regeneration, or prolonged and tedious culture conditions. Moreover, most protocols have been developed with spring wheat 'Bob White' as a model system, which has limited agronomic qualities (Vasil et al. 1993; Weeks et al. 1993; Bhalla 2006).

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Suitable explants are another major constraint for routine regeneration of large number of fertile plants (Ganeshan et al. 2006; Khurana et al. 2007). In the present investigation, we present a highly efficient and reproducible regeneration protocol through indirect somatic embryogenesis, involving three popular wheat cultivars each of bread, pasta and emmer wheat, by using mature embryos, immature inflorescence and embryo as explants. Traditionally, immature embryo derived-calli have been found efficient for in vitro regeneration in wheat, but more recently, mature embryo derived tissues such as callus and leaf bases have also shown promise for in vitro propagation and transformation studies (Ahmed et al. 2002; Mendoza and Kaeppler 2002; Chugh and Khurana 2003; Zale et al. 2004; Patnaik et al. 2006). Initial attempts to culture mature wheat embryos failed to regenerate as only callusing was observed (McHughen 1983; Heyser et al. 1985; Bartok and Sagi 1990; Ozgen et al. 1996). Delporte et al. (2001) using mature embryo fragments of wheat induced embryogenesis and though achieved embryogenic callus, the efficacy of regeneration was not appreciable. Genotype dependent regeneration efficiency was reported by Zale et al. (2004). An alternative approach by culturing immature inflorescence of Canadian wheat was adopted by Caswell et al. (2000) but regeneration frequency in terms of shoots per explants was low (maximum 15 shoots per 10 explants). Chugh and Khurana (2003) successfully raised bread and emmer wheat transgenics by particle bombardment through callus induction from leaf basal segments. Regeneration frequency in emmer wheat basal segments (80%) was higher than bread wheat (68%), however, transformation frequency of bread wheat was far better (8.6 and 4.9% for bread and emmer wheat respectively). To keep pace with functional genomics analysis involving transgenic approaches, it is imperative to have an efficient and robust regeneration protocol. This lack of reproducible and dependable regeneration protocols suitable for different type of explants and genotype is a major hurdle towards an effective wheat functional genomics programme (Bhalla 2006; Ganeshan et al. 2006, Vasil 2007).

Thidiazuron, a substituted phenyl urea, originally developed by A.G. Scherning as a cotton defoliant (Arndt et al. 1976), has been shown to be an efficacious regulator of in vitro morphogenesis of many dicot plants, influencing callusing, shoot regeneration, somatic embryogenesis, and protoplast division (Khurana et al. 2005). Biologically, TDZ converts cytokinin nucleotides to more active nucleosides (Capelle et al. 1983) and stimulates accumulation of endogenous cytokinins (Thomas and Katterman 1986). While application of TDZ in dicot plants during in vitro propagation is very well documented (see Khurana et al. 2005), its application to cereal tissue culture have come only recently (Shan et al. 2000; Ganeshan et al. 2006; Gairi and Rashid 2004; Sharma et al. 2004, 2005).

In India, bread wheat and pasta wheat are cultivated in northern parts of India while, in peninsular India emmer wheat is commercially cultivated along with bread and pasta wheat. In the present article we present a highly reproducible, genotype independent and efficient regeneration protocol for nine agronomically important and popular bread, pasta and emmer wheat varieties using immature inflorescence, immature embryo and mature embryo as explants by optimizing both the amount and time of application of TDZ.

Material and methods

Plant material

Seeds of *Triticum aestivum* L. (bread wheat) varieties viz., CPAN1676, HD2329 and PBW 343, *T. durum* Desf., (pasta wheat) PDW215, PDW233 and WH896 were obtained from Directorate of Wheat Research ICAR, Karnal, India, while seeds of *T. dicoccum* Schrank. Schubl. (emmer wheat), DDK1001, DDK1025 and DDK1029, were obtained from University of Agricultural Sciences, Dharwad, Karnataka, India. Plants were raised in the departmental garden during the crop season. For immature inflorescence, the tillers were disinfected in 4% sodium hypochlorite (Qualigens, India) for 8–10 min, and than rinsed 3–5 times in sterile water under a laminar airflow transfer hood, and the enclosed young spikes in the tillers excised with forceps to yield immature spikes of around 1–1.5 cm in length. For immature embryos, green caryopsis were harvested from 12–14 DAP, and surface sterilized by 4% sodium hypochlorite (Qualigens, India) for 20 min in a laminar flow hood after rinsing briefly for five times with sterile RO water. Immature inflorescence were cut in to small pieces (2–5 mm), and then transferred to

Table 1 Composition of different media

Medium	Composition
CM1	MS + 2,4-D (9 μM)
CM2	MS + 2,4-D (9 μM) + TDZ (2.2 μM)
CM3	MS + 2,4-D (9 μM) + TDZ (4.5 μM)
RM1	MS + TDZ (4.5 μM)
RM2	MS + TDZ (9 μM)
RM3	MS + Zeatin (9 μM)
RM4	MS + Zeatin (9 μM) + TDZ (2.2 μM)
RM5	MS + Zeatin (9 μM) + TDZ (4.5 μM)
RM6	MS

Petri dishes containing different callus media combinations while, the immature embryos were dissected from the caryopsis and placed scutellum side up on different callus induction media in 90 mm Petri plates (Tarsons India Ltd.). For mature embryo culture, dry seeds were first washed in running tap water with Teepol (Reckit and Colman India Ltd.) and then surface sterilized with 4% sodium hypochlorite for 30 min in a laminar flow hood followed by five washing with sterile RO water. Mature embryos were excised with a fine scalpel and placed scutellum side up on callus induction media.

Table 2 Percentage callusing response of different wheat genotypes

Genotype	Immature inflorescence			Immature embryo			Mature embryo
	CM1	CM2	CM3	CM1	CM2	CM3	CM1
T. aestivum							
CPAN1676	85 ± 2.6	80 ± 2.6	77 ± 1.7	88 ± 2.5	80 ± 1.1	75 ± 0.5	85 ± 2.0
HD2329	83 ± 1.7	78 ± 3.5	75 ± 2.5	85 ± 0.5	85 ± 0.5	76 ± 1.0	82 ± 1.5
PBW-343	85 ± 2.0	82 ± 2.6	75 ± 0.5	89 ± 2.0	81 ± 2.6	75 ± 1.5	80 ± 1.0
T. durum							
PDW-215	82 ± 2.6	79 ± 1.5	78 ± 2.0	85 ± 2.0	80 ± 0.5	76 ± 1.0	77 ± 1.6
PDW 233	79 ± 1.5	76 ± 2.6	76 ± 2.8	84 ± 2.5	74 ± 0.5	72 ± 0.5	78 ± 0.5
WH 896	82 ± 2.6	80 ± 3.0	77 ± 0.5	87 ± 1.5	79 ± 1.0	74 ± 2.0	79 ± 2.5
T. dicoccum							
DDK-1009	88 ± 2.6	78 ± 1.5	75 ± 0.5	82 ± 1.5	76 ± 0.5	71 ± 0.5	71 ± 0.6
DDK-1025	87 ± 3.6	72 ± 1.5	70 ± 1.5	75 ± 0.5	70 ± 0.5	69 ± 1.0	75 ± 2.0
DDK-1029	86 ± 1.7	79 ± 3.0	76 ± 1.1	80 ± 0.5	75 ± 1.5	70 ± 1.5	78 ± 2.5

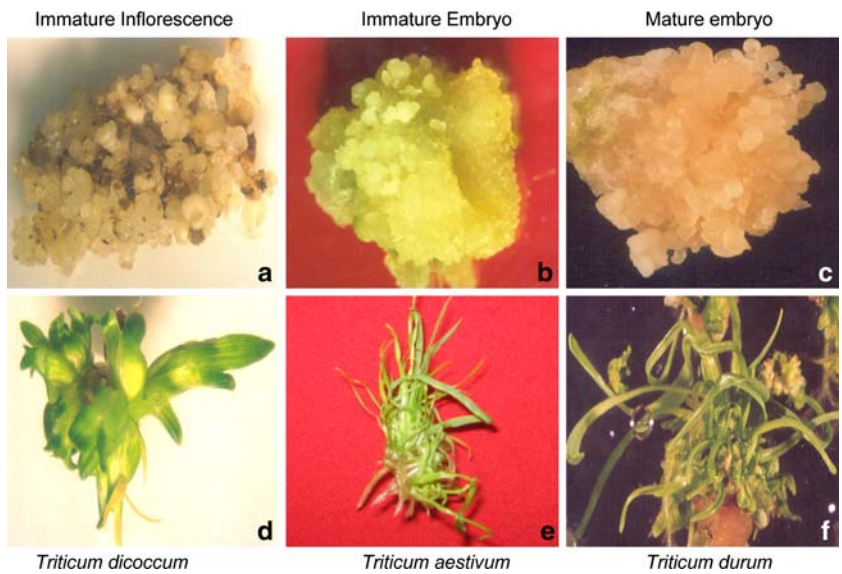

Fig. 1 Representative callus response (a–c) and in vitro regeneration (d–f) of different wheat genotypes on different CM and RM, respectively

Culture conditions and media

Callus induction medium comprised of MS (Murashige and Skoog 1962) supplemented with 30 g l^{-1} sucrose, 1 g l^{-1} casein hydrolysate (Pronadisa, Spain), 100 mg l^{-1} myo-inositol (Sigma, USA), 0.7 g l^{-1} L-proline (Sigma, USA) and solidified with 0.4% Phytagel (Sigma, USA). For immature embryo and inflorescence culture, three and six different hormone combinations were used for callusing and regeneration, respectively (Table 1). For mature embryo culture, callus media CM1, was used. Explants were cultured for 4 weeks in the dark in a culture room maintained at 25 ± 2°C. A total of 3 and 4 subcultures were done for immature and mature explants, respectively before transfer to various regeneration media (RM). After 4 weeks of callusing in dark, the explants were transferred to different regeneration media in light, with a day night photoperiod of 16:8 h and light intensity of 90 µmol m^{-2} s^{-1}. Explants were subcultured every 10 days on RM and after 3 weeks explants with actively growing leaves and shoots were transferred to magenta boxes-containing MS basal medium for rooting, followed by transfer of plantlets to potted soil. Each experiment contained a minimum of 25 explants per culture plate, five replicates were maintained for each treatment and data analysis was done by using three individual experiments.

Data analysis

For statistically analyzing the results, observations on callusing and regeneration were taken after 4 weeks of incubation in dark and light, respectively. Analysis of variance (ANOVA) was calculated for each set of experiment and calculations done by using the MSTATC package (Russell 1989). Means were compared using Least Significant Difference (LSD) test.

Results and discussion

The responses of cereals to tissue culture have been studied extensively over the years, except for popular agronomically important varieties, thereby limiting the role of biotechnological advances to breeding

Table 3 Regeneration response in terms of shoots/explants. (The figure followed by same letters are not significantly different at 5% level of probability)

	Triticum aestivum				*Triticum durum*				*Triticum dicoccum*		
Medium/genotype	Imm. infl.	Imm. emb.	Mat. emb.	Medium/genotype	Imm. infl.	Imm. emb.	Mat. emb.	Medium/genotype	Imm. infl.	Imm. emb.	Mat. emb.
CM1	8.14c	13.06b		CM1	6.82c	11.27a		CM1	8.97a	7.08a	
CM2	12.07a	14.36a		CM2	10.86a	11.44a		CM2	9.22a	6.54b	
CM3	9.18b	10.42c		CM3	7.98b	8.60b		CM3	6.25b	6.22c	
RM1	6.44d	8.91d	7.00c	RM1	5.48c	6.95e	5.80c	RM1	5.04e	5.36c	2.86b
RM2	5.20e	7.20e	5.40d	RM2	4.44d	5.97f	3.80d	RM2	4.26f	4.58d	2.33b
RM3	10.55c	13.02b	10.06b	RM3	9.64b	11.02c	9.06b	RM3	7.80d	5.75b	2.93b
RM4	12.66a	17.53a	13.00a	RM4	10.82a	13.80b	11.06a	RM4	10.8b	9.04a	4.40a
RM5	11.57b	11.49c	7.46c	RM5	10.02b	10.17d	6.06c	RM5	9.13c	5.78b	2.40b
RM6	12.37a	17.56a	9.13b	RM6	10.93a	14.71a	8.13b	RM6	11.8a	9.18a	2.26b
CPAN1676	10.43a	13.32a	9.06a	PDW215	9.15a	11.34a	8.03a	DDK1009	9.13a	7.30a	3.20a
HD2329	9.27c	11.90c	7.90b	PDW233	7.72c	9.60c	6.53b	DDK1025	7.27c	5.92c	2.43b
PBW343	9.70b	12.63b	9.06a	WH 896	8.80a	10.37b	7.40a	DDK1029	8.04b	6.62b	2.96a

programmes in wheat. The aim of present investigation was to develop a rapid and efficient regeneration protocol for popular Indian wheat varieties with different explants source and to study the effect of TDZ on wheat regeneration through indirect somatic embryogenesis. Analysis of variance revealed significant differences in callus media, regeneration media and the varieties/genotypes used. All the varieties produced regenerable calli on the various callus induction media (Table 2) and showed good callusing on induction medium CM1, CM2 and CM3 for immature inflorescence and embryo explants (Fig. 1a–c). The maximum callusing was observed in CM1 containing 9 μM of 2,4-D (Table 2) for all the genotypes and various explants. Up to 90% callusing was achieved for immature embryo explant of bread wheat genotype CPAN1676 (Table 2). Initially CM2 and CM3 media were also employed for mature embryo culture, but it was seen that TDZ containing medium inhibited callus formation in all the genotypes. Additionally TDZ promoted shoot elongation as evident from the ever growing main shoot axis; there was practically no callusing even after several subcultures and eventually some rudimentary callus appeared which turned brown leading to death of explant, as reported earlier also by Shan et al. (2000). For CM1 also, weekly subcultures were required in case of mature embryo to remove the growing original embrynal axis to facilitate proper callusing. Inclusion of TDZ in CM2 yielded the highest number of shoots per explants on RM6 in bread wheat followed by pasta and emmer wheat. All the cultivars produced highly regenerable, well formed embryoids at the surface, on the callus induction media (Fig. 1a–c). For the various genotypes, CM2 gave maximum regeneration frequency except for immature embryo explants of emmer wheat where CM1 is best (Table 3).

With respect to regeneration, all the varieties tested differed significantly in terms of shoots/explant except for mature embryo explants (Table 3). Analysis of variance also revealed significant differences in callus media, varieties and regeneration media and significant differences were observed in their interactions (Table 4). The highest regeneration was observed in RM6 for bread and pasta wheat varieties followed by RM4. Table 3 shows comparison and effect of different factors on shoot regeneration per explants. It was noted that callus induced on 2,4-D media (CM1) from mature embryo resulted in good regeneration even if media was growth hormone free (RM6). However, addition of growth hormones

Table 4 Analysis of variance

		T. aestivum				*T. durum*				*T. dicoccum*			
		Imm. Infl.		Imm. emb.		Imm. Infl.		Imm. emb.		Imm. Infl.		Imm. emb.ryo	
Source	df	MS	Pr > F	MS	Pr > F	MS	Pr > F	MS	Pr > F	MS	Pr > F	MS	Pr > F
Callus media (CM)	2	373.62	0.00	363.62	0.00	390.00	0.00	229.33	0.00	244.07	0.00	16.80	0.00
Regeneration media (RM)	5	459.12	0.00	837.66	0.00	363.90	0.00	557.93	0.00	419.85	0.00	176.81	0.00
CM * RM	10	55.90	0.00	59.90	0.00	60.28	0.00	24.68	0.00	36.52	0.00	38.47	0.00
variety	2	30.77	0.00	45.52	0.00	50.13	0.00	68.73	0.00	78.24	0.00	42.71	0.00
CM * variety	4	2.95	0.13	3.52	0.31	1.95	0.30	0.24	NS	1.90	0.00	1.69	0.03
RM * variety	10	9.92	0.00	3.40	0.32	2.38	0.14	0.82	NS	0.44	0.28	1.98	0.00
CM * RM * variety	20	5.60	0.00	4.93	0.04	0.73	NS	0.57	NS	0.89	NS	1.10	0.03
Error	216	1.62		2.95		1.59		1.58		1.48		0.64	
Mature embryo		*T. aestivum*		*T. durum*		*T. dicoccum*							
Source	df	MS	Pr > F	MS	Pr > F	MS	Pr > F						
Variety	2	13.61	0.00	17.01	0.00	4.63	0.00						
Regeneration media (RM)	5	107.53	0.00	102.06	0.00	9.65	0.01						
RM * variety	10	1.59	NS	0.89	NS	0.52	NS						
Error	72	1.70		2.01		0.96							

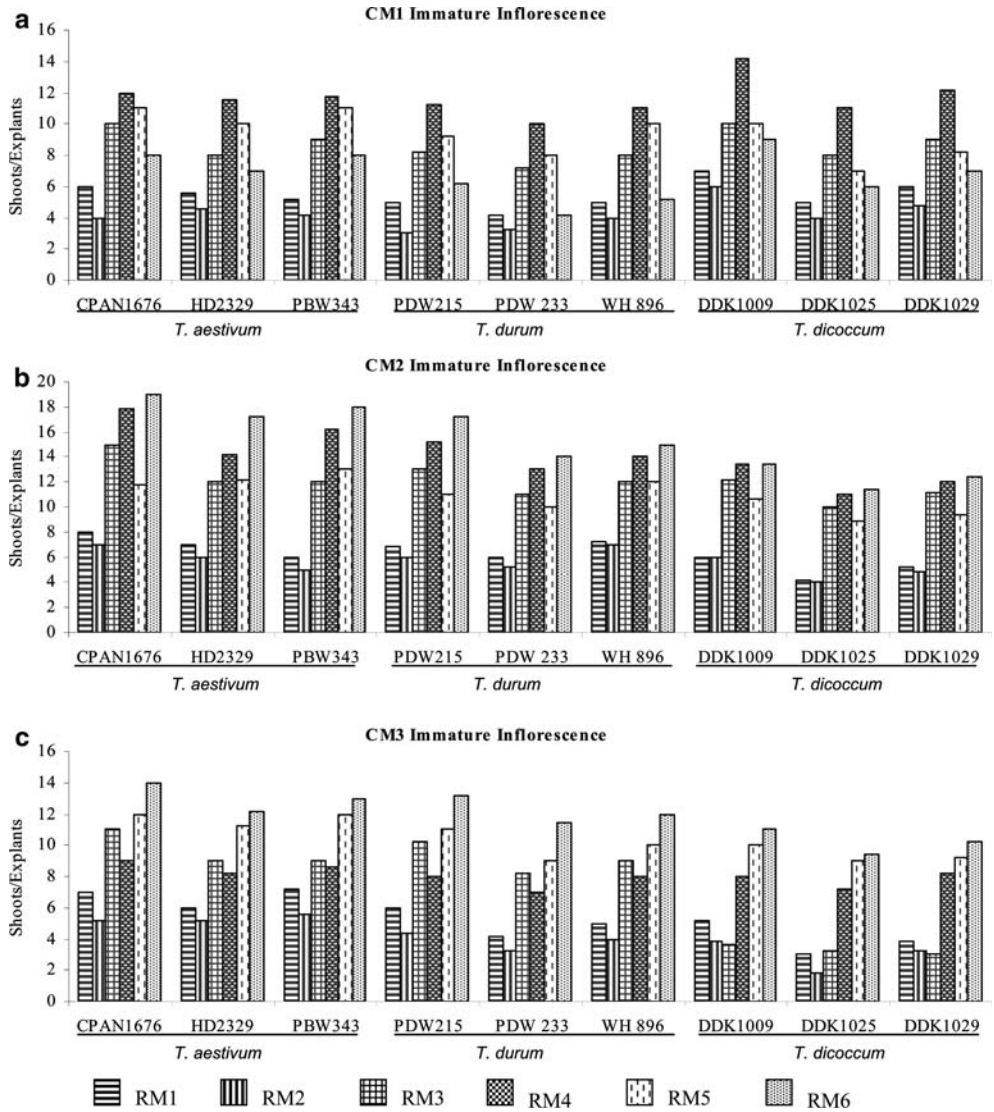

Fig. 2 In vitro culture response of immature inflorescence of different wheat genotypes. (**a**) Cultures raised on CM1 for callusing & than transferred on different RMs, (**b**) Cultures raised on CM2 for callusing & than transferred on different RMs, and (**c**) Cultures raised on CM3 for callusing & than transferred on different RMs

(RM4) increased the regeneration frequency. RM1 and RM5 did not differ significantly in both bread and pasta varieties in case of mature embryo explant, indicating that TDZ at higher concentration is not suitable for enhanced regeneration. In contrast, emmer wheat displayed good regeneration on RM4, while other regeneration media did not differ significantly for mature embryo explants (Table 3). Among various genotypes tested, CPAN1676 displayed highest callusing (Table 2) and regeneration (Table 3). While for bread and durum wheat immature embryo and mature embryo gave maximum regeneration, immature inflorescence performs better for emmer wheat genotypes, where the maximum shoots per explants were observed in DDK1009 followed by bread and pasta wheat on CM1 and RM4 combination (Figs. 2, 3). TDZ was found to be highly beneficial during regeneration in mature embryo explant and enhanced regeneration frequency comparable to immature embryo derived calli were observed (Fig. 4). Nonetheless higher concentration of TDZ was found inhibitory for regeneration (Figs. 2–4).

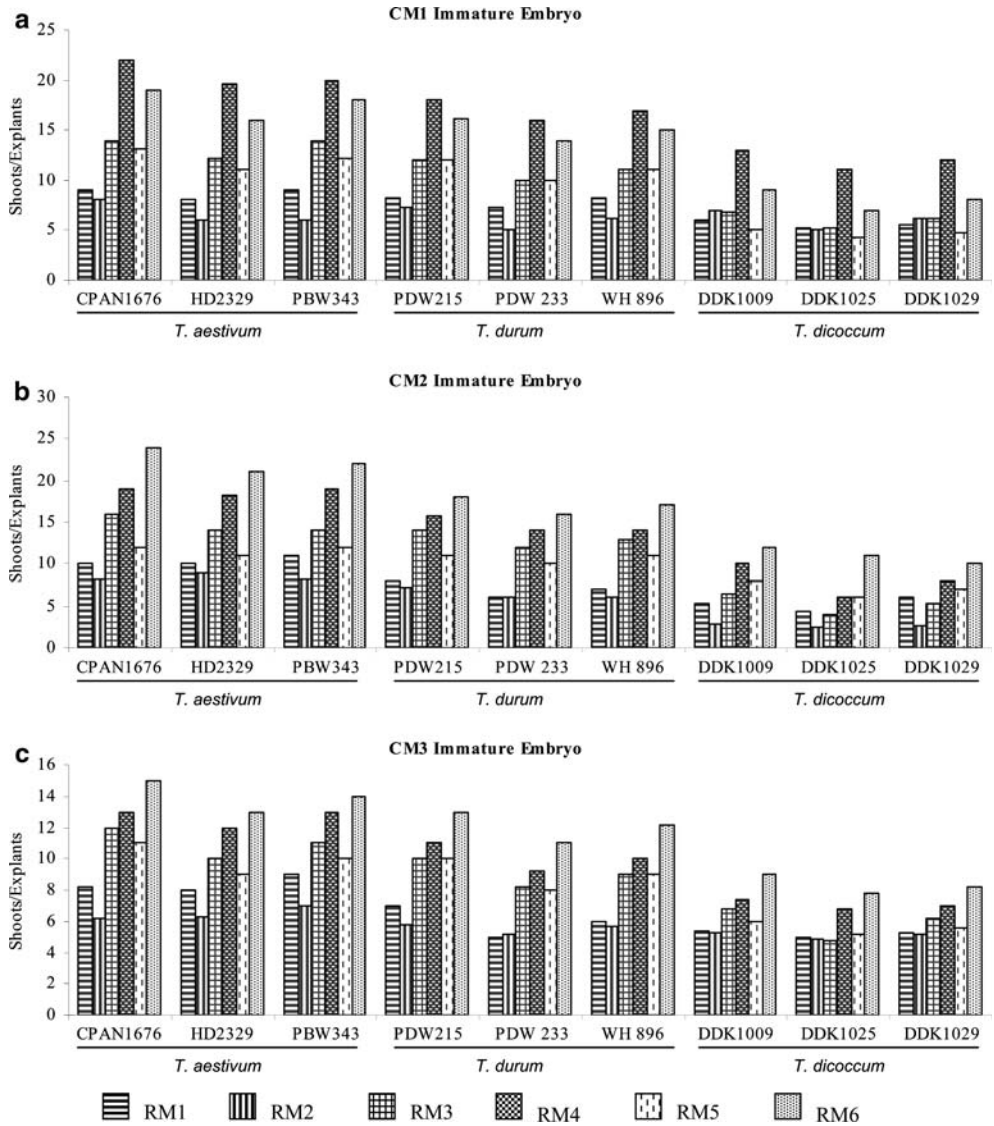

Fig. 3 In vitro culture response of immature embryo of different wheat genotypes. (**a**) Cultures raised on CM1 for callusing & than transferred on different RMs, (**b**) Cultures raised on CM2 for callusing & than transferred on different RMs, and (**c**) Cultures raised on CM3 for callusing & than transferred on different RMs

In a recent study, Fillipov et al. (2006) studied effect of different auxins on embryogenic callus response of six Russian winter and spring wheat cultivars using mature embryo explant. They found Dicamba was more effective at higher concentration (12 mg/l) for embryogenic callus induction. Moreover, addition of other auxins along with Dicamba significantly increased the embryogenic callus frequency. They also reported that this has little effect on plantlet regeneration as maximum frequency of plant regeneration was 8.8 and 8.6 for 2,4-D and Dicamba raised callus, respectively. In the present study, we found that though 2,4-D alone can generate a good amount of embryogenic callus, addition of TDZ during callusing from immature embryo (24 shoots/explants) and immature inflorescence (19 shoots/explants) increased regeneration frequency significantly (Figs. 2, 3).

In contrast to the in vitro culture of dicot plants, where TDZ alone is sufficient to enhance

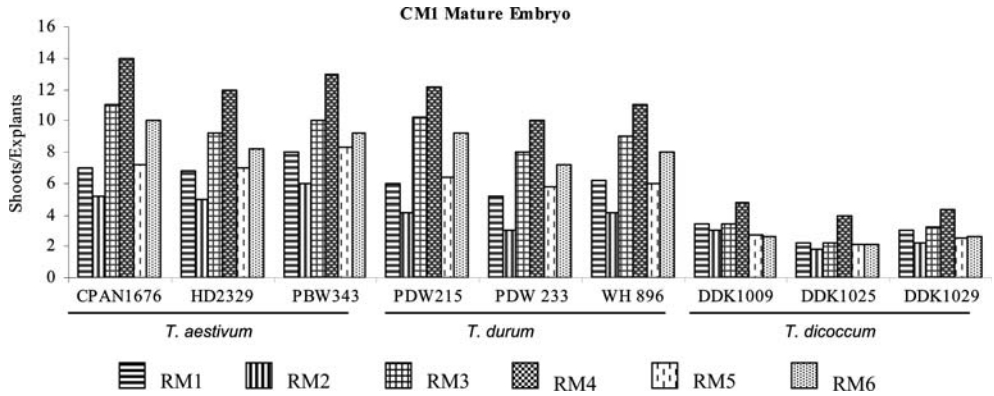

Fig. 4 In vitro culture response of mature embryos of different wheat genotypes raised on CM1

regeneration, in monocots, TDZ works well when used in combination with other growth regulators (Shan et al. 2000; Vikrant and Rashid 2002; Gairi and Rashid 2004; Sharma et al. 2004, 2005). In barley in vitro culture, Sharma et al. (2004) found that the combination of Picloram (2 mg/l) and TDZ (3 mg/l i.e. 13.5 μM) were suitable for direct multiple shoot regeneration, but not for callusing. They also found this combination to be more suitable for direct shoot regeneration in two winter wheat genotypes (Sharma et al. 2005). The present study also included TDZ at a lower concentration with different auxin and cytokinin combinations depending upon culture stage. The regeneration was not optimal when TDZ was used alone (RM1and RM2), however, cytokinin (zeatin) when used alone (RM3) produced better and significant regeneration over RM1 and RM2 in all the varieties and explants tested. Similar results were seen in wheat mature embryo culture where no callusing was observed when TDZ was included in the callusing media even at a low (2.2 μM) concentration. Moreover, during regeneration also media containing higher concentration of TDZ (RM1 and RM2) was found to have less regeneration potential (Figs. 1–3), and lower concentration of TDZ proved more potent.

It is clear from the above discussion that in monocots TDZ has more often been used for direct shoot regeneration, while in the present study the possibility of TDZ in induction of somatic embryogenesis regeneration is demonstrated. We found that both the concentration and time of TDZ application are critical for efficient wheat regeneration and a balanced exposure is needed when working with mature embryos, immature inflorescence and embryo explants. While it was beneficial to provide TDZ in lower concentration (2.2 μM) during callusing in combination with auxin, for immature inflorescence and embryo culture, TDZ should be provided in combination with cytokinin during the regeneration phase in mature embryo culture. More over, for bread and pasta wheat all three explants can gave good regeneration on TDZ containing media, but for emmer wheat mature embryo explant more work needs to be done as it is the only instance where all RM do not differ significantly among the three genotypes tested.

The key factor in the successful generation of transgenic plants is the optimization of conditions for regeneration of plantlets after several rounds of selection (Patnaik et al. 2006). Plantlets regenerated through this protocol were tested for morphological abnormalities after transfer to soil. We found no visible morphological abnormality and seed setting was almost 100% and seed germination was not compromised in the progeny. This protocol is being used for producing transgenic wheat lines with genes of interest for popular Indian wheat cultivars. This report of high frequency regeneration for agronomically important Indian wheat cultivars with use of TDZ for indirect somatic embryogenesis and regeneration can be of immense use for functional genomic analysis through transgenic approaches.

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