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Callus Induction and Plant Regeneration from Different *Triticum* Species

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ABSTRACT

Efficient and reliable *in vitro* plant regeneration systems are a prerequisite for *in vitro* propagation of elite genotypes and genetic manipulation aimed at crop improvement. The present study was conducted to study the *in vitro* responses of different *Triticum* species: *Triticum aestivum*, *Triticum dicoccum* and *Triticum durum* using different types of explants for callus induction and plant regeneration. Immature embryos on MS medium supplemented with 18 to 36 µM 2,4-D yielded pale yellow, soft and compact callus and better plant regeneration than callus derived from other explants. Comparison of the callus size in different genotypes indicated that the tall varieties - Unnath C306 and NP200 were numerically better than the semi-dwarfs and significantly better than semi-dwarf variety containing *sphaerococcum* gene used in this study. Callus induction from immature embryos was highly influenced by the developmental stage of the embryo and environmental condition at the time of sampling. The regenerated plants were grown to full maturity and evaluation of agronomic traits showed less variation, suggesting that immature embryo could be used for raising *in vitro* cultures for application in mutation induction or genetic transformation.

Keywords: callus size, genotypic response, tissue culture, wheat

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; **CH**, casein hydrolysate; **Gl**, glumes of immature inflorescence; **Glu**, glutamine; **IE**, embryo from immature seeds; **IS**, immature seed; **Kn**, kinetin; **ME**, embryo from mature seeds; **MG**, mature grain; **MS**, Murashige and Skoog; **Nd**, nodes of one-month-old seedlings; **NAA**, α-naphthaleneacetic acid; **Rc**, rachis of immature inflorescence; **SE**, standard error; **TDZ**, thidiazuron

INTRODUCTION

Wheat is one of the most important cereal crops and is a source of calories and proteins for the world population. A number of environmental factors such as temperature, moisture, soil and light intensity affect the growth and yield of wheat (Keresa *et al.* 2004) and also biotic factors affect plant performance in the field. The developments in plant molecular biology, genetic transformation and molecular breeding have shown great potential (Patnaik and Khurana 2001; Abdul *et al.* 2010) that can be integrated into conventional breeding procedures aimed at crop improvement. Due to low efficiency and genotype dependency, wheat is considered a recalcitrant plant for genetic transformation. Various *in vitro* culture methods have been employed in wheat transformation using different explants such as immature embryos, mature embryos, anther-derived calli, inflorescences, apical meristems, and other floral organs; however, further optimization is needed to establish high frequency regeneration from different *Triticum* species (He *et al.* 2010; Li *et al.* 2012). *In vitro* culture of plant tissue and cells provides opportunities to increase genetic variability besides system for studying physiological and genetic basis of *in vitro* response. In wheat tissue culture, callus induction and regeneration capacity are influenced by the genotype, explant source, physiological status of the mother plant, the culture medium and the interactions between them (Özgen *et al.* 1998; Alok *et al.* 1999; Keresa *et al.* 2004; Dodig *et al.* 2010; Munazir *et al.* 2010; Raziuddin *et al.* 2010). In wheat and other cereals, both mature and immature embryos have been used for obtaining totipotent cultures (Aguado-Santacruz *et al.* 2012). Immature embryos are a better explant source for plant regeneration; however availability of explants due to seasonal growth limits its widespread use. Seasonal time of sampling tissues for explants time has also been shown to influence tissue culture response of immature embryos in barley (Sharma *et al.* 2005; Mitic *et al.* 2009; Fazeli-nasab *et al.* 2012). Mature embryos are available throughout the year, saving on growth facility resources and time required for their collection unlike immature embryos, but recalcitrance is generally observed and hence studies need to be conducted to study which genotypes optimally induce callus and regenerate plants.

Composition of media, plant growth regulators and additives are the main factors influencing *in vitro* culture initiation and plant regeneration (Barro *et al.* 1999; Mendoza and Kaeppler 2002; Raziuddin *et al.* 2010). The auxins, 1-4 mgl^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 2-4 mgl^{-1} dicamba (Dic), 1-2 mgl^{-1} picloram (Pic) alone or in combination with cytokinins, are generally used to improve callus induction and proliferation (Castillo *et al.* 1998; Ren and Wang 2010). The use of 1-3 mgl^{-1} thidiazuron (TDZ) for cell and tissue culture of monocots has been shown to be very effective for plant regeneration from wheat and barley (*Hordeum vulgare* L.) cultures derived from different explants (Shan *et al.* 2000; Ganeshan *et al.* 2003; Li *et al.* 2003; Sharma *et al.* 2004; Aguado-Santacruz *et al.* 2011).

Callus induction and regeneration from immature and mature embryos and immature inflorescence have proved to be genotype dependent and strongly influenced by the composition of the medium used (Carman *et al.* 1988; Zale *et al.* 2004; Islam 2010). A number of workers have reported regeneration of wheat plants from callus culture derived from various plant parts, however, the frequency of green plant regeneration was very low (Ahmad *et al.* 2002; Ayes and Kenanturgut 2006; Miroshnichenko *et al.* 2009). Li *et al.* (2003) also observed that different genotypes gave varied frequency of regeneration suggesting a strong role of geno-

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Table 1 Characteristics of the varieties used in the study.

Variety	Species	Plant height	Dwarfing gene	Remarks
Unnath C306	*T. aestivum*	Tall	nil	Cultivated
HD2189	*T. aestivum*	Semi-dwarf	*RhtB1-b*	Cultivated
Kalyan Sona	*T. aestivum*	Semi-dwarf	*RhtB1-b*	Cultivated
Sphaerococcum line	*T. aestivum*	Semi-dwarf	*RhtB1-b* + *Sph*	Genetic stock
NP200	*T. dicoccum*	Tall	nil	Cultivated
DDK1029	*T. dicoccum*	Semi-dwarf	*RhtB1-b*	Cultivated
MACS2846	*T. durum*	Semi-dwarf	*RhtB1-b*	Cultivated
MACS1967	*T. durum*	Tall	nil	Cultivated
PDW291	*T. durum*	Semi-dwarf	*RhtB1-b*	Cultivated

Table 2 Media tested for plant regeneration in wheat.

Media	Plant growth regulators (mg/L)						Sucrose /	CH	Glut
	TDZ	Pic	Dic	NAA	Kn	Zn	Maltose (%)		
MSDCH	-	-	2		-	-	3S	200	-
MSPTM	1	2	-	-	-	-	3M	-	-
MSNKG	-	-	-	0.5	2.5	-	4S	-	100
TMR2	-	-	-	-	-	2	3S	-	-
TMR3	0.48	-	-	-	-	2	3S	-	-
TMR4	0.99	-	-	-	-	2	3S	-	-
TMR5	0.99	-	-	-	-	1	3S	-	

type which could not be overcome by manipulating the medium compositions or experimental parameters. In wheat, *in vitro* cultures have been useful for the selection of variants for abiotic stress tolerance (Bajji *et al.* 2004; Yadav *et al.* 2004; Gawande *et al*. 2005) and disease resistance (Svabova and Lebeda 2005; Nasir *et al.* 2012).

More often, differences in the production of embryogenic calli and the regenerated plantlets have been observed, depending on the genotype and source of the explants in barley (Luhrs and Lorz 1987; Aguado-Santacruz *et al.* 2011) and wheat (Fennel *et al.* 1996; Bhalla *et al.* 2006; Yin *et al.* 2011). The study of response to *in vitro* conditions and selection of appropriate genotype are a prerequisite for *in vitro* manipulation. In this study, we present a comparative account of three different *Triticum* species and explant sources for callus induction and plant regeneration, and influence of plant growth regulators and seasonal influence.

MATERIALS AND METHODS

All the species of wheat used in this study were spring wheats which are commercially cultivated in India. All plants which were used as source of explants were grown out doors in wheat growing season starting mid October and ending mid April. The varieties included *Triticum aestivum* (HD2189, Kalyan Sona and Unnath C306), *Triticum dicoccum* (NP200 and DDK1029), *Triticum durum* (MACS2846, MACS1967 and PDW291) and a wheat line with sphaerococcum trait developed by backcrossing (BC4) using Kalyan Sona as the recurrent parent (**Table 1**). The line used in the study was true breeding and stable with reduced culm height, condensed spike and round grain which is typical of sphaerococcum trait. All except the sphaerococcum line are cultivated varieties and were chosen on the basis of differences in ploidy and plant height. Different explants viz. immature (IS) and mature grain (MG), embryo from mature (ME) and immature seeds (IE), nodes of one-month-old seedlings (Nd), the rachis (Rc) and glumes (Gl) of immature inflorescence (at boot stage of the tiller) were used for callus induction on MS (Murashige and Skoog 1962) medium: MSCH-2 (MS + 9 µM 2,4-D + 200 mg/L CH + 3% sucrose, pH 5.8), MSCH-4 (MS + 18 µM 2,4-D + 200 mg/L CH + 3% sucrose, pH 5.8) and MSCH-8 (MS + 36 µM 2,4-D + 200 mg/L CH + 3% sucrose, pH 5.8). For immature embryos, developing seeds were collected on 10, 20, 30, 40, 45, 50, 55 and 60 days post anthesis. Mature embryos were derived from seeds which were harvested after full maturity and stored. Mature and immature seeds were surface sterilized with 70% ethanol and 0.1% $HgCl_2$ followed by rinses with sterile distilled water whereas inflorescences and nodes were surface sterilized with 70% ethanol and then used for inoculation.

For callus induction, immature and mature seeds were placed furrow side down on to above MS media while, embryos from mature and immature seeds, nodes of one month old seedlings and the rachis and glumes of immature inflorescence were dissected and placed on the above media. Rachis from immature inflorescence were cut and placed on MS medium (MSNKG) containing 0.5 mg/L α-naphthalene acetic acid (NAA), 2.5 mg/L Kinetin (Kn), 100 mg^{-1} glutamine (Glu) and 4% sucrose. Callus was subcultured every four weeks on to fresh MS medium containing 9 µM 2,4-D, 200 mg/L casein hydrolysate and 3% sucrose (pH 5.8). All the media were adjusted to pH 5.8, and then autoclaved at 121°C for 20 min. Filter-sterilized plant growth regulators were added to the autoclaved media. All the cultures were incubated under cool-white fluorescent light (1000 Lux) under a 16-h photoperiod at 26 ± 1°C. A minimum of 50 explants were used in each replication per treatment. The experiments were performed in at least four replicates in each experiment.

The *in vitro* response was evaluated in terms of callus formation scored four weeks after explant inoculation as: the number of explants with callus formation over the total number of cultured embryos. Callus size as area in mm^2 was measured using the software, 'Image J'. Callus was transferred on to different regeneration media (**Table 2**). Plant regeneration was scored after six weeks based on the percentage of calli regenerating and number of regenerants per 10 calli.

The regenerated plants were separated and allowed to grow on liquid MS medium containing 3% sucrose until roots were fully developed. The fully developed plants were then transferred to paper cups containing soil:Soilrite mixture (in the ratio of 4:1) for acclimatization for 2-3 weeks. The plants were then finally transferred to pots in the field. At maturity, parameters like plant height, spike length, number of spikelets per spike and number of seeds per spike were measured. All the data from different experiments using different parameters were recorded and analyzed statistically. Analysis of variance was carried out and 5% level of significance was considered for differences. For graphical presentation, mean ± 2.96 SE was plotted.

RESULTS

Callus formation was observed within 3-5 days from the time of inoculation on medium supplemented with 2,4-D from MG, ME and IE explants. **Fig. 1A-H** shows callus induction from different explants viz. MG, ME, IE, Rc and Gl. MG upon inoculation showed germination as well as callus formation. The callus formed from MG was white to pale yellow, soft and compact (**Fig. 1A, 1B**). Use of Dic or Pic resulted only in germination without any callus formation. ME showed callus induction within three days without any germination, however, the callus differentiated into roots only, on various regeneration media. In case of IS, low

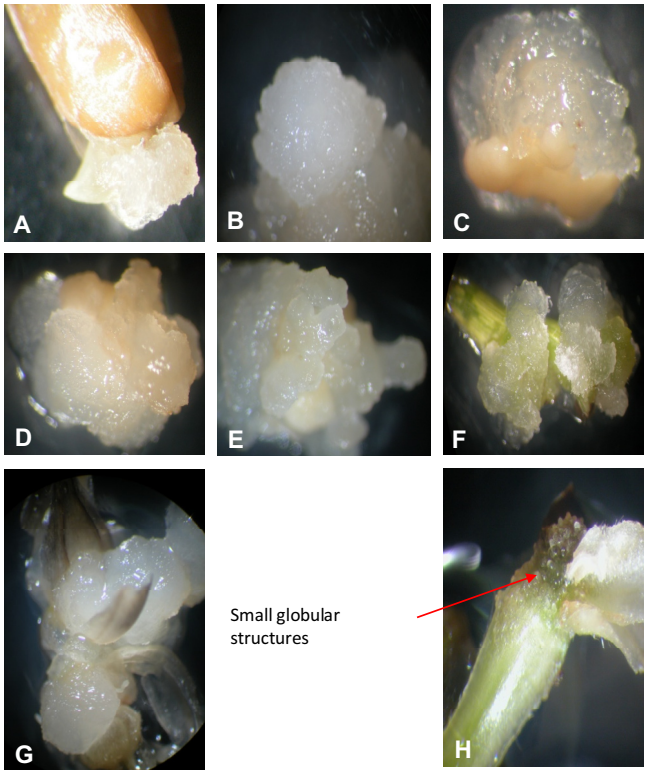

Fig. 1 Callus induction from different explants. (**A**) Callus induced from mature grain (MG) – after 5 days (**B**) callus induced from mature grain (MG) – after 30 days (**C**) callus induced from mature embryo (ME) – after 30 days. (**D, E**) callus induced from immature embryo (IE) – after 30 days (**F**) callus induced from rachis of immature inflorescence (Rc) – after 30 days (**G**) callus induced from glumes of immature inflorescence (Gl) – after 30 days (**H**) rachis of immature inflorescence placed on direct embryogenesis medium. A minimum of 50 explants were used in each replication per treatment.

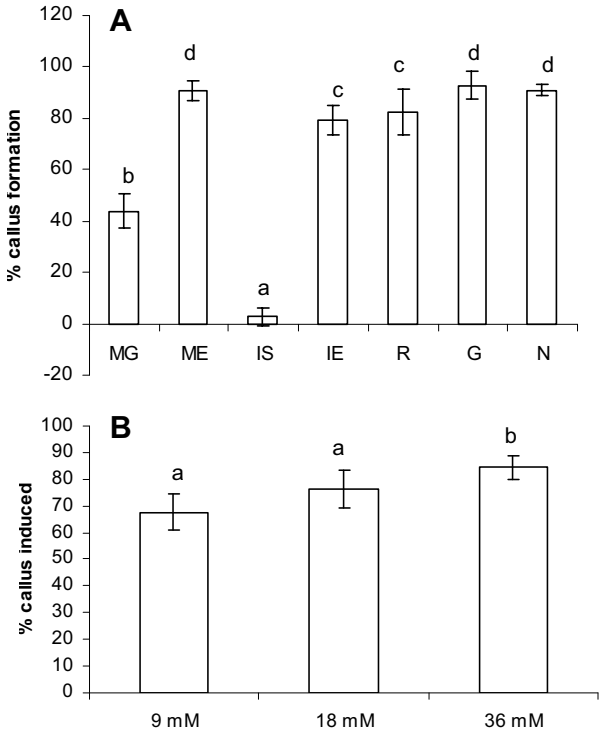

Fig. 2 (**A**) Callus induction percentage from different explants on MS medium containing 18 µM 2,4-D and 200 mg/L CH. MG: mature seeds, ME: dissected scutellum and embryo of mature Seeds, IS: immature seeds, IE: dissected scutellum and embryo of immature seeds, R: rachis from immature inflorescence, G: glumes from immature inflorescence, N: nodes of young plants (30-40 day old plants). (**B**) Callus induction on different concentrations of 2,4-D. Callus was induced from ME of *T. aestivum* Unnath C306 on 9, 18 and 36 µM 2,4-D.

callus formation was observed (**Fig. 1C**) which grew slowly, while with IE, callus formation was rapid and the callus was white to pale yellowish in colour which formed green spots after 3-4 subcultures (**Fig. 1D, 1E**).

In comparison to the above, other explants displayed differential response for callus induction. When rachis of immature inflorescence explants were placed on MS media containing 18 µM 2,4-D, callus formation was observed after a month from the nodal region from where the glumes were excised (**Fig. 1F**). The callus grew rapidly, however differentiation was not observed on any of the regeneration media. Callus formation was also observed at the base of the glumes about 20 days after inoculation on 2,4-D media (**Fig. 1G**). The callus from the nodes of rachis and from the base of the glumes was soft and white in colour and grew very rapidly. This callus also differentiated into only roots on the different regeneration media. Nodal explants showed swelling in the first few days after inoculation, became translucent after two weeks and showed callus growth after four weeks. In order to induce direct somatic embryogenesis, rachis explants were inoculated on medium supplemented with NAA, Kn and glutamine. These responded with the formation of tiny globular structures (**Fig. 1H**) which did not grow even after 30 days of culture and turned brown. In summary, among the different explants, IE was the best responding explant for callus induction with advantages such as short time for callus formation, higher callus growth rate and plant regeneration.

All the explants, except immature seeds, yielded high frequency (45-90%) of callus induction (**Fig. 2A**). High frequency (90-92%) was observed in the case of ME, Gl and Nd while low frequency was observed in IS (2.8%). ANOVA showed that IS and MG were significantly different from each other at 5% level of significance. IE and Rc were comparable and different than ME, Gl and Nd. MEs placed on MSCH medium with 9, 18, or 36 µM 2,4-D resulted in about 68 to 84% with the highest callus induction on 36 µM 2,4-D (**Fig. 2B**). ANOVA showed that 2,4-D treatments (9 and 36 µM) were significantly different from each other and similar to 18 µM.

Callus induction frequencies were 75.5, 79.9 and 83.1% for *T. durum*, *T. aestivum* and *T. dicoccum*, respectively (**Fig. 3A**) and ANOVA showed that the differences were not significant. Comparison of size of IE callus in different species indicated that the tall varieties – Unnath C306 and NP200 showed better callus growth as compared to the semi-dwarfs (containing the *Rht* genes) – HD2189, Kalyan Sona and DDK1029 (**Fig. 3B**), however, this difference was statistically not significant. There was also no significant difference in callus growth between *Triticum aestivum* (Unnath C306, HD2189 and Kalyan Sona) and *Triticum dicoccum* (NP200 and DDK1029) (**Fig. 3**). However, callus growth in IE of the semi-dwarf genotypes of both *T. aestivum* (HD2189 and Kalyan Sona) and *T. dicoccum* (DDK1029) were numerically lower than the respective tall genotypes (Unnath C306 and NP200). ANOVA showed that the sphaerococcum line was significantly different from the rest. HD2189, Kalyan Sona and DDK1029 were similar and different than NP200 and Unnath C306 which were similar to each other.

Isolated embryo explants of Unnath C306 plants that were sown late (mid-December) and explants derived from plants sown in October were used to study if environmental conditions experienced by wheat plants during growth play an important role in callus induction and proliferation. The results showed that low callus induction from explants was observed of plants that were sown late compared to explants derived from plants sown in October (**Fig. 4A**). ANOVA showed that the difference due to time of sowing was significant. In addition, the stage of the immature seed development also showed a significant influence on callus

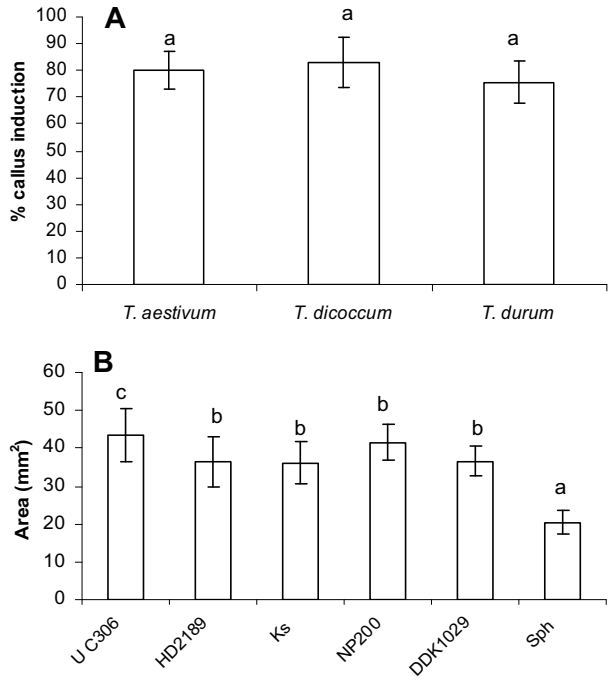

Fig. 3 (**A**) Callus induction in different wheat species. Callus from ME of *T. aestivum* (Unnath C306, HD2189, Kalyan Sona), *T. dicoccum* (NP200, DDK1029), and *T. durum* (MACS2846, MACS1967 and PDW291) on 18 µM 2,4-D. (**B**) Effect of genotype on callus growth. Callus was induced from ME of *T. aestivum* Unnath C306 (tall), *T. aestivum* HD2189 (*Rhtb1-b* semi-dwarf), *T. aestivum* Kalyan Sona (*Rhtb1-b* semi-dwarf), *T. dicoccum* NP200 (tall), *T. dicoccum* DDK1029 (*Rhtb1-b* semi-dwarf), and *Sphaerococcum*- type (cross between *T. sphaerococcum* and Kalyan Sona) on 18 µM 2,4-D and area (mm^2) of each callus was measured using the software image J.

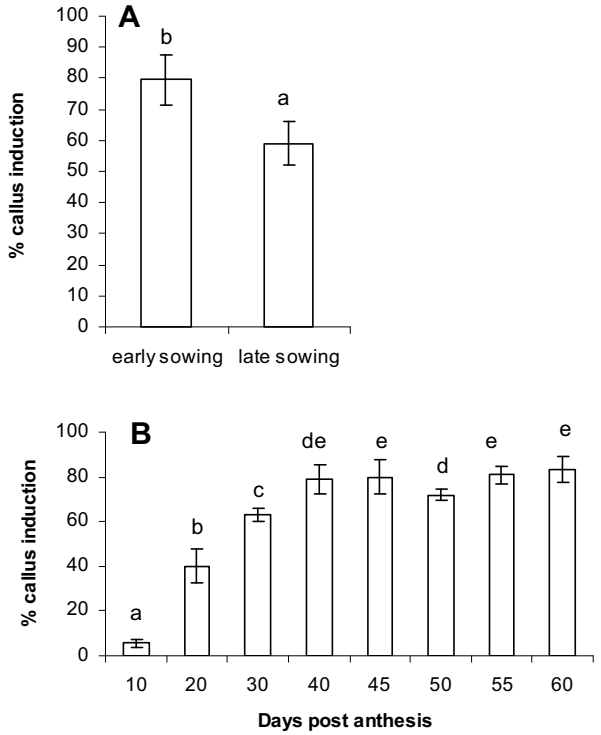

Fig. 4 (**A**) Effect of sowing date on the percentage of callus induction from IE. Callus was induced from IE of *T. aestivum* Unnath C306 on 18 µM 2,4-D. Early sowing: late October, Late sowing: Mid December. (**B**) Effect of the stage of IE on the percentage of callus induction. Callus was induced from IE of *T. aestivum* Unnath C306 on 18 µM 2,4-D. Days post anthesis (DPA) – day 10: milky stage, day 20: dough stage, day 30 onwards hardening and drying of seed.

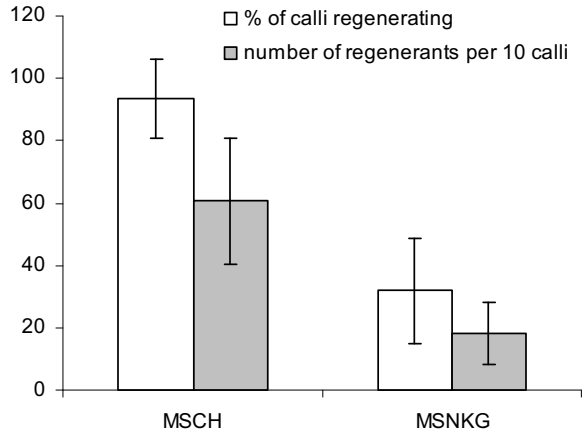

Fig. 5 Comparison of MSCH and MSNKG for plant regeneration from IE-derived callus cultures in *T. aestivum* Unnath C306.

induction. Callus induction at 10 DPA (days post anthesis) was significantly lower than all other stages (**Fig. 4B**). The callus induction percentage increased at 20, 30 and 40 DAP and differences at each stage were significantly different. After 40 DAP (past the dough stage), the callus induction percentage did not change significantly.

Different media were tested to induce plant regeneration (**Table 1**). MSCH media showed significantly higher regeneration percentage (93.4%) and number of shoots (60.7) than MSNKG media (**Fig. 5**). Callus derived from the immature embryos when transferred on to MSCH showed plant regeneration with the appearance of small green spots (shoot primordia) in a week (**Fig. 6A-B**). These greenish regions readily developed further into cluster of shoots and roots (**Fig. 6C-F**). Individual shoots were separated and inoculated in liquid medium containing MS basal medium without any hormones until roots were properly developed (**Fig. 6G**). These plants when transferred to soil showed about 20% survival but, grew better when they were allowed to acclimatize in a mixture of soil to Soilrite in the proportion of 4:1 for about 15 days before transfer to pots in the field (**Fig. 6H, 6I**). The survival rate of regenerated plants increased to ~70% when they were allowed to acclimatize prior to soil transfer (data not shown).

The regenerated plants were grown to full maturity (**Fig. 6J**) and agronomic parameters such as plant height, culm length, spike length, spikelet number per spike and number of seeds per spike were measured. The data on the ten best plants shown as a representative of the population showed that the coefficient of variation for all the above parameters was below 15% (**Table 3**).

DISCUSSION

In recent years, plant tissue culture has emerged as one of the tools which complements other crop breeding methods. It allows propagation and genetic manipulation of crop plants (Datta 2007). The new tools offer the prospect of generating the genetic variability for use in wheat breeding programs and also a resource for genetic manipulation (Li *et al*. 2012).

In wheat, type of explants (Keresa *et al*. 2004), the genotype of cultivar used (Özgen *et al*. 1998; Raziuddin *et al*. 2010), environmental conditions of donor plants (Dodig *et al*. 2010) and tissue culture media composition (Miroshnichenko *et al*. 2009; Ren *et al*. 2010) are important factors affecting *in vitro* tissue culture response. Vasil (1987) suggested that the relationship of genotype to morphogenetic competence *in vitro* is complex. It is suggested that this relationship is influenced by physiological and environmental factors and has a strong effect on the synthesis, transport and the availability of plant growth regulators. It should thus be possible that if appropriate explants are excised from plants and cultured under optimal conditions with

Table 3 Phenotypic parameters of regenerated plants of *T. aestivum* cv. Unnath C306.

	Plant height (cm)	Culm length (cm)	Spike length (cm)	No. of spikelets per spike	No. of seeds per spike
Mean ± SE	82.4	72.9	9.5	18.6	48.9
	0.51	0.52	0.09	0.25	0.86
CoV (%)	4.2	4.9	6.2	8.9	11.8

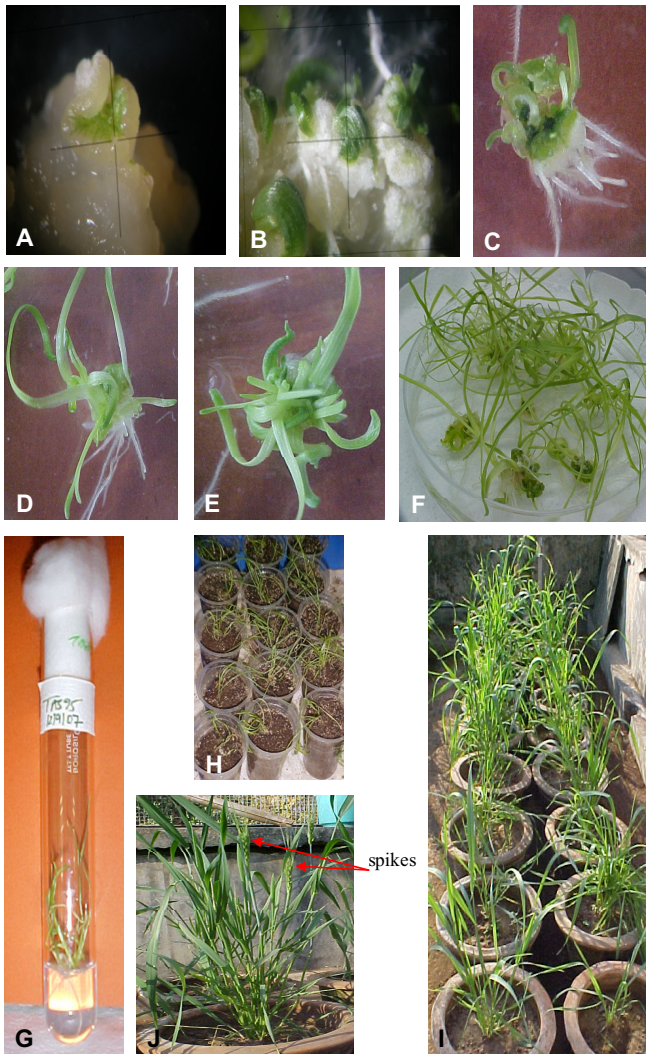

Fig. 6 Steps in plant regeneration from callus cultures obtained from IE explants. (**A**) green spots on callus obtained from IE on MS media containing 18 µM 2,4-D and 200 mg/L casein hydrolysate, (**B-F**) different stages of regeneration from green spot on MS media containing 200 mg/L casein hydrolysate (**G**) regenerated plants transferred to liquid media containing MS and 200 mg/L casein hydrolysate (**H**) regenerated plants transferred to a mixture of soil and 'Soil Rite' in the ratio of 4:1 (**I**) regenerated plants transferred to soil and grown in field (**J**) fully grown, mature regenerated plants with spikes.

appropriate amount of plant growth regulators, plants or genotypes can be induced for morphogenesis (Vasil 1987). A highly efficient and reproducible *in vitro* regeneration system is an absolute prerequisite to produce transgenic plants (Li *et al.* 2012).

Although immature embryos, immature inflorescences and mature embryos are the most widely used explants, immature embryos are the most suitable explants to regenerate wheat plants (Haliloglu *et al.* 2005). Donor plants grown under drought condition, as compared to favourable conditions, resulted in an increased variability and a decreased percentage of callus formation and regeneration frequency and the number of plants regenerated per embryo (Mitić *et al.* 2009). Dodig *et al.* (2008) reported significant association between agronomic traits, and tissue culture response.

In the present study, different wheat species were grown at different times and tested for their ability for callus induction and plant regeneration. Among *T. aestivum* (Unnath C306, HD2189 and Kalyan Sona), *T. dicoccum* (NP200 and DDK1029), *T. durum* and a genotype which was selected for sphaerococcum type, *T. aestivum* genotypes (especially Unnath C306) and durum wheats showed better response. On the other hand, the genotype with sphaerococcum trait showed significantly lower callus size compared to the tall genotypes and also Kalyan Sona which was the recurrent parent. Genotype often plays a significant role, therefore, for a given cultivar, the culture response cannot be assumed and hence, knowledge on environmental influence, tissue culture responses of explants to growth regulators and growth additives needs to be generated. Many factors are known to affect tissue culture responses of wheat which include explant tissue (Vasil 1994), culture medium and its supplements (Mathias and Simpson 1986), and donor plant growth conditions (Hess and Carman 1988). In wheat and other cereals, immature embryos have been shown to be the most responsive explants in tissue culture followed by other explants such as mature embryos, immature inflorescences, anthers and microspores or tissues from seedlings grown *in vitro* (Bhaskaran and Smith 1990; Chauhan *et al.* 2007; Yu *et al.* 2008).

In this study, wheat genotypes, Unnath C306 and NP200 showed better growth of IE derived callus as compared to the semi-dwarfs (containing the *Rht* genes) – HD2189, Kalyan Sona and DDK1029 (**Fig. 3**). The semi-dwarfs contain the dwarfing gene *Rht-B1b* which is known to have altered DELLA proteins involved in gibberellic acid (GA) action (Peng *et al.* 1999). The callus induction and growth could also be influenced by the mutation affecting GA action. The genotype carrying sphaerococcum trait showed significantly lower callus growth as compared to other the genotypes. Since this genotype also carries the *Rht-B1b* and the *Sphaerococcum* gene, the significant reduction could be due to *Sphaerococcum* gene alone or due to the interaction between the two dwarfing genes.

Environmental conditions experienced by wheat plants during growth play an important role in callus induction and proliferation especially when using immature explants. Wheat being a winter crop has less heat tolerance and hence, late sowing (sowing after the recommended date of sowing has passed) could result in increased heat stress on the plants during the grain maturity period. When isolated embryo explants of Unnath C306 plants that were sown late (mid-December) were used, lower callus induction was observed as compared to explants derived from plants sown in October (**Fig. 4A**). The callus induction rate was more sensitive to temperature based factors than the regeneration capacity. Weather conditions between flowering and the medium milk stage were most important for callus formation, while regenerating calli and number of plants per embryo were not particularly related to climatic factors in any period (Dodig *et al.* 2008). In barely, Sharma *et al.* (2005b) observed seasonal influence of harvesting time on tissue culture response of immature embryos. Although callus induction was not much affected, increased frequencies of plant regeneration was observed from January to March, followed by a continuous and strong decrease from May to December. In our study, the stage of the immature seed development also showed a significant influence on callus induction. IE 10 DPA (days post anthesis) showed lower % callus induction as compared to 40 DPA and above (**Fig. 4B**). Callus induction was the highest (80%) when the im-

mature seeds were past the dough stage (40-45 DPA). In *T. aestivum* and *T. durum*, Sharma *et al*. (1995a) observed that immature inflorescences at four different developmental stages (0.5, 1.0, 1.5, 2.0 cm in length) showed a marked variation in callusing response; embryogenic callus formation was observed from entire surface of very young inflorescences (0.5 and 1.0 cm long) while 1.5 and 2.0 cm long inflorescences formed embryogenic callus from the basal spikelets and rachis.

The present study demonstrates that varieties across different *Triticum* species can be induced into callus formation which could be used for plant regeneration. The study of the interaction between different factors like seasonal influence, presence or absence of dwarfing genes(s) will enable the development of genotype-specific culture protocols to exploit regeneration potential of recalcitrant genotypes. Bringing a variety of genotypes under optimized *in vitro* conditions can enable their use in *in vitro* mutagenesis and genetic transformation for crop improvement.

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