Pak. J. Bot., 44: 277-284, Special Issue March 2012.

EVALUATION OF TISSUE CULTURE RESPONSES OF PROMISING WHEAT (*TRITICUM AESTIVUM* L.) CULTIVARS AND DEVELOPMENT OF EFFICIENT REGENERATION SYSTEM

IMRAN MAHMOOD[1], ABDUL RAZZAQ[1*], ZAHEER-UD-DIN KHAN[2], ISHFAQ AHMAD HAFIZ[3] AND SHUAIB KALEEM[1]

[1]*Department of Agronomy, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, Pakistan*
[2]*Department of Botany, GC University, Lahore, Pakistan*
[3]*Department of Horticulture, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, Pakistan*
**Correspondence: arazzaq57@yahoo.co.in*

Abstract

Efficient callusing and regeneration efficiencies and development of reliable regeneration system are prerequisite for improvement of wheat through recombinant DNA technology. Tissue culture responses of wheat are genotypic, media composition and their interaction dependent. Immature embryos of seven elite wheat cultivars were subjected to *in vitro* culture processes to select for most appropriate cultivar for tissue culture responses by using auxins and cytokinins one at a time in solidified MS medium. Callus formation and regeneration varied significantly among varieties and among phytohormone concentrations. The interaction of phytohormones and cultivars for callus induction and regeneration was significant and was found to be genotypic and media dependent. Cultivars AS-2002 (92.75%) and GA-2002 (91.25%) produced maximum number of calli on induction media comprising 4 mg/l 2,4-D; while, cv. Chakwal-50 performed best at 6 mg/l 2,4-D (91.75%). The least callusing was witnessed for cv. Sahar-2006 with 2 mg/l of 2,4-D. Induction media comprising 2 mg/l 2,4-D was found inappropriate for all the cultivars except Inqilab-91. None of the cultivars could produce callus at basal MS medium. Independency of regeneration and callusing was observed with maximum regeneration potential of 41.19% for cv. GA-2002 on regeneration medium comprising 1.0 mg/l kinetin. The least regeneration (12.75%) was recorded for cv. Shafaq-2006 on regeneration medium supplemented with 2 mg/l kinetin. A proficient and reliable *in vitro* regeneration system was successfully established for most tissue culture responsive genotype 'GA-2002' and its regeneration was increased from 38.81% to 63.69% by supplementing 0.2 mg/l IAA, 0.5 mg/l Kn and 0.5 mg/l of BAP in regeneration medium. Regeneration frequency of GA-2002 achieved from control (1.0 mg/l kinetin) was improved up to 64.09%. An efficient protocol was developed for rapid screening of tissue culture responsive genotype; and establishment of high frequency regeneration system of wheat rarely reported earlier.

Abbreviations: 2,4-D (2,4-dichlorophenoxyacetic Acid), BAP (6-benzylaminopurine), IAA (Indole-3-acetic acid), 2iP (6-γ-γ-dimethylallylaminopurine), Kn (Kinetin).

Introduction

Wheat, the second most prominent grain crop of the world is staple food of about 35% of the world masses (Pingali *et al*., 1999). Estimate till 2025 suggest that average yield of about 4 metric tons per year per hectare will be required to feed human population of around 8 billion (Rosegrant *et al*., 1997) and this situation will become more deteriorative in year 2050 with projected world population of 9.5 billion from current population of 6.8 billion (GeoHive, 2009). Achievement of desired goal of yield boost is becoming impracticable due to limiting constraints like drought, salts, diseases and aluminum toxicity scenario. This demands release of high yielding wheat cultivars with improved characteristics particularly resistance to wide range of biotic and abiotic stresses; which seems un-executable with conventional plant breeding strategies being slow, less selective restricted gene pool availability and species barrier in addition to other biological limitations (Razzaq *et al*., 2004; Hamayun *et al*., 2010).

Adoption of novel techniques such as exploitation of *In vitro* tissue culture induced somaclonal variation and recombinant DNA technology involving callus and regeneration phase may facilitate to increase food production and nutritional values of crops. However, lack of regeneration of selected callus lines or transformed cell population is the major limiting factor for producing fertile somaclonal variants or transgenic plants. Whereas, possibility of *in planta* genetic transformation of cereals particularly wheat, without tissue culture based protocol is limited and is reported by few investigators (Razzaq *et al*., 2005; Razzaq *et al*., 2011). The problem is further aggravated by variable tissue culture responses of elite wheat genotypes (Sarker & Biswas, 2002; Satyavathi *et al*., 2004; Ayşe Gul Nasircilar *et al*., 2006; Rahman *et al*., 2008; Hassan *et al*., 2009; Noor *et al*., 2009; Rashid *et al*., 2009; Shah *et al*., 2009; Yasmin *et al*., 2009; Raziuddin *et al*., 2010). It necessitates screening of tissue culture responsive genotypes and establishment of decent plant regeneration system.

Many protocols have been developed for tissue culture based improvement of wheat which are very much genotypic (Shah *et al*., 2009), explant (Sarker & Biswas, 2002; Shah *et al*., 2003) and culture medium (Ekiz *et al*., 1997; Kabir *et al*., 2008; Rahman *et al*., 2008) dependent. Nonetheless, immature embryo explant is always considered good choice for genetic transformation studies due to its highest regeneration potential (Sarker & Biswas, 2002). Excellent callus formation from immature embryo explant of wheat on MS medium supplemented with 3.5 mg/l of 2,4-D was reported by Shah *et al*. (2003). Maximum regeneration was achieved from mature embryo explant of wheat on MS based medium supplemented with 0.5 mg/l kinetin, 0.5 mg/l BAP and 25 mg/l tyrosine (Sarker & Biswas, 2002). Noor *et al*.,

(2009) reported maximum regeneration from mature embryo explant of Pakistani wheat cultivars Kohsar and Khyber-87 on regeneration media comprising 0.5 mg/l BAP, 0.1 mg/l IAA and 0.5 mg/l kinetin. While, Rashid *et al*., (2009) reported highest regeneration from mature seed explants of cultivar Chakwal-97, Inqilab-91 and Manthar on medium having 0.1 mg/l of IAA, 0.4 mg/l kinetin and 0.5 mg/l of 2iP.

At present, it is impossible to predict the exact growth medium and protocol for callusing and regeneration from particular explant of a specific wheat cultivar. A precise tissue culture protocol and growth medium has to be investigated through carefully designed and observed experiments for each explant, for each new plant species or variety of the species using various phytohormones. Present study was therefore, planned to look into most responsive elite genotype of wheat in terms of callusing and regeneration and to further improve regeneration efficiency of most responsive genotype for its genetic improvement with limited resources and time.

Materials and Methods

Experimental site: *In vitro* study pertaining to evaluate wheat cultivar with efficient callusing and regeneration potentials and regeneration improvement of selected one was carried out in Crop Physiology Laboratory of Agronomy Department and Plant Tissue Culture Laboratory, Department of Horticulture, Pir Mehr Ali Shah, Arid Agriculture University, Rawalpindi, Pakistan.

Plant material: Seeds of seven elite commercial cultivars of wheat namely; Inqilab-91, Bhakkar-2002, GA-2002, Chakwal-50, Shafaq-2006, AS-2002 and Sahar-2006, currently under cultivation in the country were collected from National Agricultural Research Center Islamabad as sources of immature embryos for *in vitro* studies.

Tissue culture procedure: The cultivars were grown in the field and in the plant shed for immature embryos. In order to ensure availability of immature embryos through out the study, sowing was done on different dates with 15 days interval. Spikes from plants of all cultivars sown at same date were collected approximately 2 to 3 weeks post-anthesis. Immature caryopses were removed and surface sterilized for 5 min in 90% ethanol and rinsed three times in sterile distilled water. Caryopses were sterilized again for thirty minutes in 6.5% sodium hypochlorite with 0.1% Tween-20 followed by rinsing with four changes of sterile distilled water. One hundred immature embryos of uniform size of each cultivar were removed aseptically using forceps and placed on MS (Murashige & Skoog, 1962) based callus induction medium (MS + 30 g/l sucrose + 0, 2, 4 or 6 mg/l 2, 4-D) in glass jars keeping scutella side upward. Agar (6 g/l) was used for solidification of the medium. Completely Randomized Design (CRD) with factorial arrangement and 4 replications were used for layout of the experiment. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121^{o}C for twenty minutes. Explants were incubated in total darkness at 25±1^{o}C temperature for three weeks. The number of calli induced from embryos was recorded. Then, calli were transferred to fresh maintenance medium (half of the best suited concentration of 2, 4-D for callus induction of respective cultivar) for callus growth and proliferation for a period of three weeks, refreshing callus maintenance medium after every 14-21 days. In the second step of study calli of each cultivar were raised with best suited concentration of 2,4-D and eighty morphogenic calli with green spots were transferred to the regeneration medium (MS medium + different concentrations of kinetin viz., 0, 0.5, 1.0, 1.5 and 2.0 mg/l) and incubated at 26^{o}C temperature with 16 hour light and 8 hour dark photoperiod. Percentage of calli regenerated was calculated after 3-4 weeks of culturing the calli on regeneration medium.

Regeneration improvement: Cultivar with good regeneration potential (GA-2002) was subjected to further *In vitro* experimentations to improve its regeneration efficiency. Immature embryos were collected after one month of previous studies. New fresh calli were initiated and proliferated on best suited concentration of 2, 4-D (4 mg/l) for selected cultivar. The calli were transferred on regeneration medium (MS medium supplemented with various combinations of growth regulators). Following protocols comprising various combinations of phytohormones were tested for regeneration improvement of the selected cultivar along with control treatment (T_1) based on results of preliminary study.

Treatments: Regeneration protocols (Various combinations of phytohormones)

		IAA (mg/l)		Kinetin (mg/l)		BAP (mg/l)
T_1	=	0.0	+	1.0	+	0.0
T_2	=	0.2	+	1.0	+	0.0
T_3	=	0.4	+	1.5	+	0.0
T_4	=	0.6	+	2.0	+	0.0
T_5	=	0.2	+	0.0	+	0.5
T_6	=	0.4	+	0.0	+	1.0
T_7	=	0.6	+	0.0	+	1.5
T_8	=	0.2	+	0.5	+	0.5
T_9	=	0.4	+	1.0	+	1.0
T_{10}	=	0.6	+	1.5	+	1.5

The experiment was laid out following Complete Randomized Design (CRD) with four replications.

Statistical analysis: Data collected were analyzed statistically by using MSTATC software (Freed & Eisensmith, 1986) to find out the variation between and within the treatments, with respect to percentage of callus induction and regeneration at 5% probability level. Differences between means were analyzed by using Least Significance Difference (LSD) test.

Results and Discussions

Visual observations: Callus formation is fundamental to many analytical and applied tissue culture procedures. Callus induction and regeneration potential of seven elite wheat varieties currently under cultivation in the farmers' fields viz; Inqilab-91, Bakhar-2002, GA-2002, Chakwal-

50, Shafaq-2006, AS-2002 and Sahar-2006 were investigated. Swelling of immature embryos was observed within 2 to 3 days after culturing on growth media supplemented with different concentrations of 2,4-D, except on auxin free medium. Initiation of callus started as a white translucent tissue on the surface of scutellar region within 4 to 7 days, depending on the genotype and medium. Almost similar kinds of observations were reported by Satyavathi *et al*., (2004).

Initially, all the cultivars showed frequent callus proliferation which decreased during subsequent time period. Nodular and compact structures were observed on the surface of most of the calli; a characteristic feature of embryogenic callus. These observations are in line with those reported by Rashid *et al*., (2009). Primarily, for all cultivars most of the calli produced were non-embryogenic missing nodular structure. After 20-25 days of culture in the dark, nodular structures emerged on the surface of non-embryogenic calli and the callus turned light yellow to whitish in color. The embryogenic calli continued to proliferate when shifted to fresh medium in subsequent sub culture. These observations are in line with those of Li *et al*., (2009).

Callus formation (%): Data pertaining to callus formation is presented in Table 1. Although uniform *In vitro* cultural conditions were offered to all cultivars but there were marked variations among cultivars for callus induction from immature embryos. Data suggested that all cultivars had potential to produce callus from immature embryos. Mean values for callus induction percentage indicated that cv. Bhakkar-2002 had maximum callusing frequency (63.94%) followed by cv. AS-2002 (62.25%). Cultivar Sahar-2006 proved to be least callogenic with 48.06% callusing potential. Non-significant difference among cv. Inqilab-91 and Chakwal-50 was observed with respective callus formation frequency of 53.19% and 53.31%. Mean values showed that higher concentrations of auxin (2,4-D) inhibited callogensis against occurrence of morphogenesis under lower concentrations (Table 1). Callusing was absent on MS basal medium. However, callus induction percentage varied from 64.36–81.43% with addition of 2,4-D. The optimum concentration of 2,4-D for callus induction was found to be 4.0 mg/l with maximum callus yield of 81.43% against minimum (64.36%) for 2.0 mg/l of 2,4-D. A considerable increment in callus induction percentage was attained with increased concentration of 2,4-D up to 4.0 mg/l but significant decline in callusing frequency was resulted upon exceeding concentration of 2,4-D beyond 4.0 mg/l. However, 2,4-D @ 4.0 mg/l seemed optimum for callus initiation.

Table 1. Callus induction percentage of various wheat cultivars in response to varying concentrations of 2,4-D.

2,4-D	Inqilab-91	Bakhar-2002	GA-2002	Chakwal-50	Shafaq-2006	AS-2002	Sahar-2006	Mean
0.0 mg/l	00.00 n	00.00 n	00.00 n	00.000 n	00.00 n	00.00 n	00.00 n	**00.00d**
2.0 mg/l	89.00 c	79.00 f	56.75 k	52.25 l	51.00 l	76.25 g	46.25 m	**64.36c**
4.0 mg/l	70.75 hi	90.50 bc	91.25 ab	69.25 i	71.75 h	92.75 a	83.75 e	**81.43 a**
6.0 mg/l	53.00 l	86.25 d	74.25 g	91.75 ab	80.50 f	80.00 f	62.25 j	**75.43 b**
Mean	**53.19 d**	**63.94 a**	**55.56 c**	**53.313 d**	**50.81 e**	**62.25 b**	**48.06 f**	

LSD values: **Varieties = 1.048, **2, 4-D = 0.792, **Varieties × 2, 4-D = 2.095

* Non-significant; **Significant

Entries sharing similar letters do not differ significantly at 5% probability level

Interaction of cultivars and concentrations of 2,4-D in induction media was also found significant (Table 1). The auxin induced callus formation was found to be genotype dependent and various genotypes responded differently to various concentrations of 2,4-D (Fig. 1). Optimum concentration of 2,4-D was different for different cultivars viz a viz cv. AS-2002 and GA-2002 produced maximum number of calli on 4 mg/l of 2,4-D with callus yield of 92.75% and 91.25 %, respectively. Moreover, cv. Chakwal-50 performed best (91.75%) on induction media comprising 6 mg/l 2,4-D. Callusing potential of cv. AS-2002 and GA-2002 at 4 mg/l 2,4-D and Chakwal-50 at 6 mg/l was statistically at par. Maximum callus yield was noticed in cv. Inqlab-91 at 2 mg/l, Bhakkar-2002 at 4 mg/l, cv. Shafaq-2006 at 6 mg/l and sahar-2006 at 4 mg/l of 2,4-D (Fig. 1). The least callus yield was observed for Sahar-2006 with 2 mg/l of 2,4-D. MS based medium supplemented with 2 mg/l 2,4-D was found inappropriate for all the cultivars except Inqilab-91. None of the cultivar could produce callus at basal MS medium (Fig. 1) as are reported by Raziuddin *et al*., (2010).

The variability in callus formation frequency in response to various levels of 2,4-D may be due to differences in genes controlling callusing or genes may not express themselves fully in some cultivars contrary to others supplemented with optimum concentration of 2,4-D. Rashid *et al*., (2009) reported variable response of various cultivars to different levels of auxin. Our results suggested varying tissue culture responses of wheat genotypes and it might be due to genotype and media interaction (Yasmin *et al*., 2009). Our finding are in concurrence with other researchers who also suggested genotypic differences of wheat for callus formation and regeneration abilities (Kilinc, 2004; Satyavathi *et al*., 2004; Ayşe Gul Nasircilar *et al*., 2006; Hassan *et al*., 2009; Shah *et al*., 2009).

It was found that increased concentration of 2,4-D above optimum level adversely affected callus induction potential of cv. Inqilab-91, Bhakkar-2002, GA-2002, AS-2002 and Sahar-2006 (Fig. 1). Auxins play a substantial role in activation of genes responsible for cell division and de-differentiation (Dudits *et al*., 1991). Higher concentrations of 2,4-D above optimum had detrimental effect on callusing and callus proliferation (Kabir *et al*., 2008; Rahman *et al*., 2008). Cell division, callus proliferation and other tissue culture responses are mainly

determined by the type and concentration of auxins (Barro *et al*., 1998). Higher concentration of 2,4-D seemed lethal for genes involved in cell division and de-differentiation of cultivars under study. An increase in callus induction percentage with increasing concentration of 2,4-D from 2.5-5.0 mg/l and then subsequent decline in callusing frequency with increasing concentration of 2,4-D beyond 5.0 mg/l had also been reported by Kilinc (2004). Also, maximum callus induction was reported with 6.0 mg/l of 2,4-D on MS based medium, but relatively larger calli were obtained with 4.00 mg/l of 2,4-D (Rahman *et al*., 2008). Li *et al*., (2009) reported that maximum embryogenic callus are production at 1-3 mg/l of 2,4-D.

The mean values for callus induction percentage in wheat cultivars in response to various levels of 2,4-D indicated that 4 mg/l 2,4-D is in general optimum for Pakistani wheat cultivars. However, for maximum callusing the induction media should be standardized. Various workers had standardized optimum concentration of 2,4-D for diverse genotypes of wheat. For example, Sarker & Biswas (2002) obtained maximum callus yield from immature embryos with 5.5 mg/l 2,4-D on MS based medium. Raziuddin *et al*., (2010) obtained maximum callogenesis from immature embryos cultured on MS based medium comprising 2.5 mg/l of 2,4-D. Satyavathi *et al*., (2004) divulged maximum callusing frequency from mature embryos at 2 mg/l 2,4-D. These minor discrepancies in findings as reported earlier and here may be the consequences of diversity of genetic material under investigation and cultural conditions.

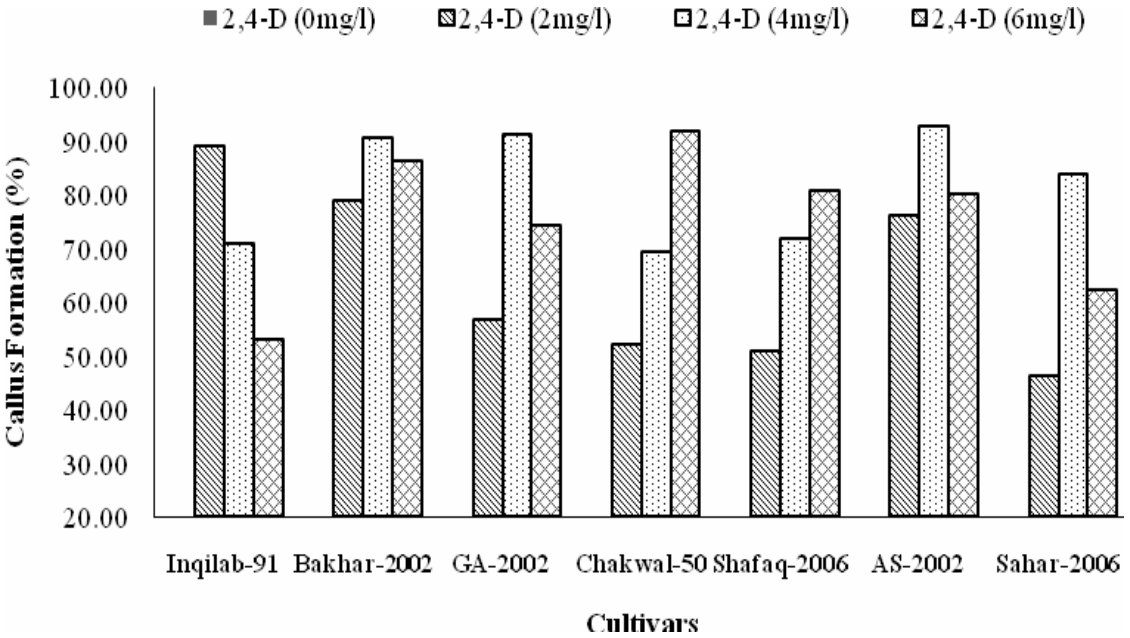

Fig. 1. Callus formation percentage of various wheat cultivars in response to varying concentrations of 2,4-D.

Regeneration (%): Data presented in Table 2 showed that regeneration percentage significantly varied with explant donor genotypes. Mean values indicated that maximum regeneration potential (34.91%) was exhibited by cv. GA-2002 followed by AS-2002 (33.74%), while least regeneration (21.95%) was exhibited by cv. Shafaq-2006. Mean values of various concentrations of kinetin significantly affected regeneration percentage (Table 2) and regeneration percentage ranged from 24.46 to 34.94% for various concentrations of kinetin. Highest regeneration (34.94%) was recorded with MS based medium containing 1.0 mg/l kinetin, followed by 1.5 mg/l kinetin with regeneration frequency of 31.98%. An increasing trend in regeneration from 0-1.0 mg/l kinetin with following slumped was observed.

Significant differences were found between interaction of cultivars and concentrations of kinetin (Table 2). All cultivars performed differently with varying concentrations of Kn (kinetin). Highest regeneration percentage of 41.19% was witnessed for cv. GA-2002 on regeneration medium supplemented with 1.0 mg/l kinetin. The lowest regeneration (12.75%) was recorded for cv. Shafaq-2006 when 2.0 mg/l kinetin was used in regeneration medium. Contradictory with cv. GA-2002, cultivar Bhakkar-2002 showed maximum regeneration (40.12%) on kinetin free medium. Regeneration frequency of cv. GA-2002 at 1.0 mg/l kinetin and Bhakkar-2002 at 0.0 mg/l kinetin was statistically at par. Statistically no differences were found between regeneration frequencies of cv. Bhakkar-2002 (40.12%) at 0.0 mg/l, Chakwal-50 (39.62%) at 1.5 mg/l and AS-2002 (39.19%) at 1.0 mg/l of kinetin (Table 2).

Hormonal balance is critical for regeneration of embryogenic callus. The regeneration was comparatively rapid in calli those were induced on reduced concentration of 2,4-D. Early regeneration happened in Inqilab-91 followed by Sahar-2006 and Bhakkar-2002. Chakwal-50 was last to regenerate; since, higher concentration of auxins in induction medium subsequently adversely affects regeneration efficiency (Fazelienasab *et al*., 2004). However, regeneration happened rapidly when callus are incubated at lower concentration of 2,4-D (Liu *et al*., 1990).

Table 2. Regeneration percentage of various wheat cultivars in response to varying concentration of kinetin.

Kinetin	Inqilab-91	Bakhar-2002	GA-2002	Chakwal-50	Shafaq-2006	AS-2002	Sahar-2006	Mean
0.0mg/l	24.00 q	40.12 ab	27.69 mn	18.25 t	17.25 t	23.75 q	20.12 s	**24.46 e**
0.5mg/l	28.00 m	37.06 fgh	29.49 l	25.88 o	24.50 pq	34.88 ij	25.56 op	**29.33 c**
1.0mg/l	30.75 k	26.50 o	41.19 a	37.00 fgh	31.00 k	39.19 bcd	38.94 cd	**34.94 a**
1.5 mg/l	34.31 j	24.62 pq	38.50 cde	39.62 bc	24.25 q	36.00 hi	26.56 no	**31.99 b**
2.0 mg/l	38.06 def	21.56 r	37.75 efg	36.75 gh	12.75 u	34.88 ij	17.62 t	**28.48 d**
Mean	**31.02 c**	**29.98 d**	**34.91 a**	**31.50 c**	**21.95 f**	**33.74 b**	**25.76 e**	

LSD values : **Varieties = 0.509, **Kinetin = 0.430, **Varieties × kinetin (Interaction) = 1.139

* Non-significant; **Significant

Entries sharing similar letters do not differ significantly at 5% probability level

Bhaskaran & Smith (1990) had reviewed that media supplemented only with cytokinins had the potential to regenerate calli in cereals under certain conditions. Different genotypes of wheat behaved differently to various concentrations of phytohormones and are accredited to genotype and media interaction (Kabir *et al*., 2008). In our study, all the cultivars under investigation showed different regeneration potential in response to various levels of kinetin with highest regeneration of GA-2002 in response to 1.00 mg/l kinetin (Fig. 2). This domino effect are almost analogous to those of Rashid *et al*., (2009) and Yasmin *et al*., (2009) who observed variable response of various wheat genotypes of Pakistan in response to various regeneration protocols. These results are also consistent with findings of Ayşe Gul Nasircilar *et al*., (2006), Mitic *et al*., (2006) and Hassan *et al*., (2009) suggesting existence of genetic variation for regeneration potential.

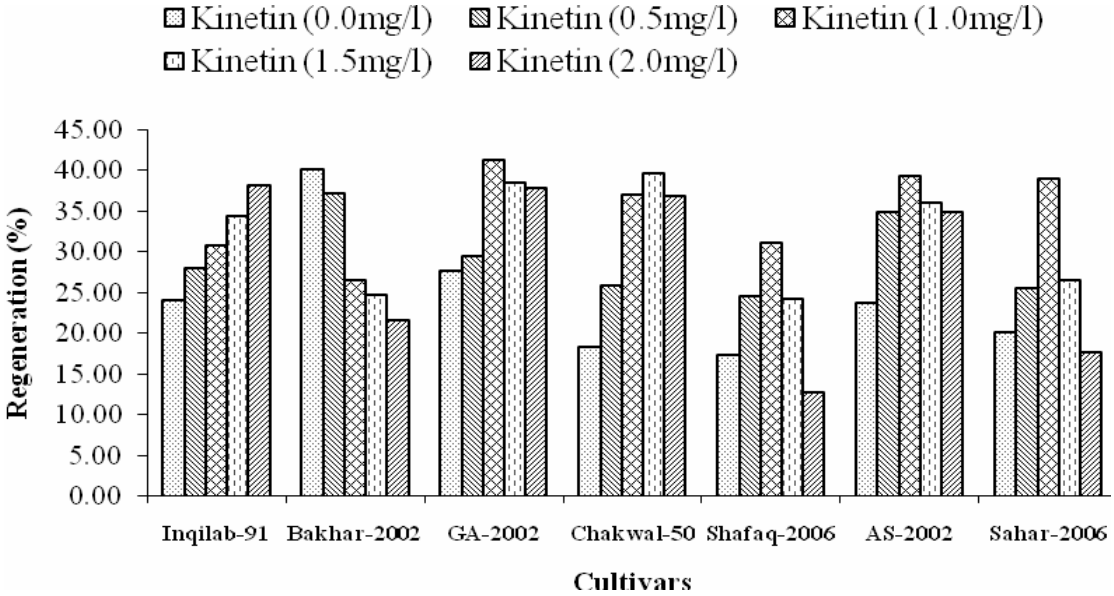

Fig. 2. Regeneration (%) of various wheat cultivars in response to various concentrations of kinetin.

The mean values (Table 2) indicated an increasing trend in regeneration from 0-1.0 mg/l kinetin which declined further with increasing concentration of kinetin. This indicated that high concentration of cytokinins above 1.0 mg/l significantly reduce regeneration efficiency of commercial wheat cultivars of Pakistan origin (Rahman *et al*., 2008; Noor *et al*., 2009). In contrast, Shah *et al*., (2003) observed maximum regeneration on MS based medium comprising as high as 3.0 mg/l Kn. The conflicts might be due to different explant source, genetic material under study, external environmental conditions of explant donor (Mitic *et al*., 2006) and variability of *In vitro* culture conditions. The environment is one of the main causes altering internal hormonal balance of explant and can modify regeneration potential of callus (Biagioli *et al*., 2006).

It was worth accentuated that callusing and regeneration response of all cultivars was independent. Maximum callusing was witnessed for cv. Bhakkar-2002 (mean) (Table 1) while the highest regeneration occurred in cv. GA-2002 (mean) (Table 2). These findings revealed that different genes or combinations of genes may govern the different stages from callus induction to plantlet regeneration (Jia *et al*., 2009).

Variety Inqilab-91 had been extensively studied and reported in literature for its *In vitro* culture responses. In our studies, maximum callus yield of Inqilab-91 (89.00%) was achieved with 2 mg/l of 2, 4-D and the highest regeneration (38.06%) with 2.0 mg/l of kinetin. Earlier, Rashid *et al*. (2009) reported its maximum callus formation (83.25%) with 3.0 mg/l 2,4-D and as high as 87.25% regeneration frequency by supplementing 0.4

mg/l of kinetin, 0.1 mg/l IAA, and 0.5 mg/l of 2iP in MS medium. Similarly, Asma Afzal *et al*., (2010) obtained maximum callus induction percentage (76.04%) from mature embryos of Inqilab-91 by supplementing 3 mg/l of 2, 4-D and 60% callus regeneration from 0.1mg/l of IAA and 0.5mg/l of Kn. They enhance its regeneration up to 65% by supplementing 0.1mg/l of IAA, 0.5mg/l of Kinetin and 1.0mg/l of 2ip. The deviation of our results for callus formation and regeneration of this extensively studied variety might be due to differences of type and concentration of phytohormones, explant and variable environment faced by explant donor and explant source. Since, the choice of genotype and the environment where the explant source is raised is also critical for *in vitro* tissue culture protocols (Biagioli *et al*., 2006).

Regeneration improvement of cv. GA-2002: On the bases of previous results for regeneration potential cv. GA-2002 was found to be the most responsive. Therefore, it was selected and efforts were made to maximize its regeneration efficiency. Various regeneration protocols containing kinetin, IAA and BAP in various proportions were tested for its *in vitro* regeneration response. The immature embryos were collected after four weeks of previous studies and were incubated on MS based medium comprising 4.0 mg/l of 2,4-D based on results of previous study (Fig. 1). After four weeks the calli were shifted onto various regeneration medium (MS based medium with various proportions of phytohormones).

The data pertaining to regeneration (%) of cv. GA-2002 in response to various regeneration protocols is presented in Table 3. Highest regeneration (63.69%) was witnessed with T_8 (0.2 mg/l IAA + 0.5 mg/l kinetin + 0. 5 mg/l BAP) pursued by T_9 (0. 4 mg/l IAA + 1.0 mg/l kinetin + 1.0 mg/l BAP) (Fig. 3). Regeneration acquired from T_8 was 64.09% higher than that accomplished from control (T_1). Least regeneration was observed from media containing T_{10} (0.6 mg/l IAA + 1.5 mg/l Kn + 1.5 mg/l 0f BAP) which was even 2.74% less than control. However, non-significant difference was recorded between regeneration achieved from T_1 (Control) and T_{10}.

Table 3. Regeneration (%) of cv. GA-2002 in response to various combinations of auxins and cytokinins (regeneration protocols).

Treatments (Various combinations of phytohormones)							Regeneration % age	% Increase/ decrease in regeneration over control
		IAA (mg/l)		Kinetin (mg/l)		BAP (mg/l)		
T_1	=	0.0	+	1.0	+	0.0	38.81 gh	control
T_2	=	0.2	+	1.0	+	0.0	46.56 e	19.97
T_3	=	0.4	+	1.5	+	0.0	54.50 c	40.42
T_4	=	0.6	+	2.0	+	0.0	44.81 f	15.46
T_5	=	0.2	+	0.0	+	0.5	50.75 d	30.76
T_6	=	0.4	+	0.0	+	1.0	54.36 c	40.09
T_7	=	0.6	+	0.0	+	1.5	40.19 g	3.54
T_8	=	0.2	+	0.5	+	0.5	63.69 a	64.09
T_9	=	0.4	+	1.0	+	1.0	59.25 b	52.66
T_{10}	=	0.6	+	1.5	+	1.5	37.75 h	-2.74

LSD value = 1.412

Entries sharing similar letters do not differ significantly at 5% probability level

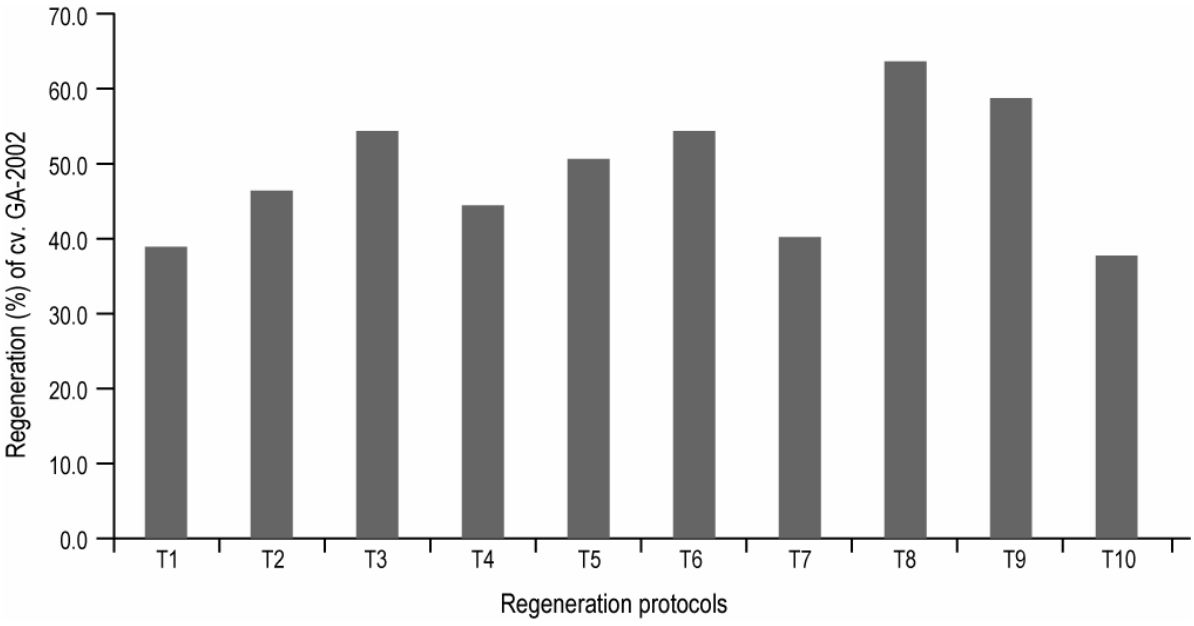

Fig. 3. Regeneration (%) of cv. GA-2002 in response to various combinations of auxins and cytokinins (regeneration protocols).

The ratio of auxins to cytokinins in the culture medium is important; since, their combinations determine the morphogenic responses of calli. Combination and relative concentration of auxins to cytokinins is explant and genotype dependent (Kabir *et al*., 2008). Auxins and cytokinins had major effects on callus induction and regeneration. Varying their concentration in the medium had caused discrepancy in growth rate and growth pattern of calli (Ekiz *et al*., 1997). Cytokinins may express synergistic effects when used in combination with auxins. Mainly individual but also some synergistic effects of NAA and kinetin have been reported in Japanese lily (Takayama & Misawa, 1982). Earlier, kinetin, IAA and BAP or combinations of these were extensively reported for profuse regeneration in cereals (Sarker & Biswas, 2002; Noor *et al*., 2009; Asma Afzal *et al*., 2010).

Our results indicated that excellent regeneration can be achieved on medium comprising a precise combination of auxins and cytokinins. Developed callus can be enforced to form shoots by increasing cytokinins and decreasing auxins concentrations in culture media. Asma Afzal *et al*., (2010) obtained maximum regeneration (60.0%) from mature embryos explant of wheat cv. Inqilab-91 with 0.5 mg/l of Kn and 0.1 mg/l of IAA and managed to enhance its regeneration up to 65% by supplementing IAA @ 0.1 mg/l, Kn @ 0.5 mg/l plus 1.0 mg/l of 2ip in regeneration medium. Excellent regeneration had been reported from various combinations of cytokinins with auxins e.g. media containing IAA @ 5.7 mg/l and kn @ 5 mg/l resulted in excellent regeneration of wheat (Yasmin *et al*., 2009). Sarker and Biswas (2002) verified maximum regeneration from MS based medium having 0.5 mg/l BAP, 25.0 mg/l tyrosine and 0.5 mg/l Kn. Similarly, maximum regeneration of Pakistani wheat cultivars Kohsar and Khyber-87 was achieved by Noor *et al*., (2009) on MS based media comprising 0.5 mg/l BAP, 0.1 mg/l IAA and 0.5 mg/l Kn. While, Rashid *et al*., (2009) reported highest regeneration in cultivar Chakwal-97, Inqilab-91 and Manthar on media containing 0.40 mg/l Kn, 0.10 mg/l IAA and 0.50 mg/l of 2iP.

Conclusion

In conclusion, the study showed that high frequency callus induction and plantlet regeneration can be accomplished for wheat using immature embryos as explants. Callusing and regeneration is genotypic, media and also their interaction dependent. Regeneration frequency of a variety can be enhanced to a considerable extent by combination of auxins and cytokines. Optimization of these growth regulators is fundamental for maximum plantlets regeneration for all kind of tissue culture based studies.

References

Asma Afzal, H. Rashid, M.H. Khan, Z. Chaudhry and S.A. Malik. 2010. High frequency regeneration system optimization for wheat cultivar Inqilab-91. *Pak. J. Bot.,* 42(3): 1857-1862.

Ayşe Gul Nasircilar, K. Turgut and K. Fiskin. 2006. Callus induction and plant regeneration from mature embryos of different wheat genotypes. *Pak. J. Bot.,* 38(2): 637-645.

Barro, F., M.E. Cannell, P.A. Lazzeri and P. Barcelo. 1998. The influence of auxins on transformation of wheat and tritordeum and analysis of transgene integration patterns in transformants. *Transgenic Res.,* 97: 684-695.

Bhaskaran, S. and R.H. Smith. 1990. Regeneration in cereal tissue culture: A review. *Crop Sci.,* 30: 1328-1338.

Biagioli, C., R. Rios, A. Basso, P. Franzone and A. Prina. 2006. *In vitro* culture response of barley (*Hordeum vulgare*) ethylene synthesis mutant MC 169. *Electron. J. Biotechnol.,* 9(3): 271-274. [Online] http://www.ejbiotechnology.info/ content/vol9/issue3/full/7/7.pdf.ISSN:0717-3458.

Dudits, D., L. Bogre and J. Gyogyey. 1991. Molecular and cellular approaches to the analysis of plant embryo development from somatic cells *in vitro*. *J. Cell Sci.,* 99: 475-484.

Ekiz, H. and C.F. Konzak. 1997. Effect of light regime on anther culture response in bread wheat. *Plant Cell Tiss. Organ Cult.,* 50: 7-12.

Fazelienasab, B., M. Omidi and M. Amiritokaldani. 2004. Effects of abscisic acid on callus induction and regeneration of different wheat cultivars to mature embryo culture. *Proc. 4th Int. Crop Sci. Cong. Brisbane, Australia.* 26Sep-1Oct, 2004. | ISBN 1 920842 20 9 | www.cropscience.org.au

Freed, R.D. and S.P. Eisensmith. 1986. MSTATC. Michigan State Uni., Michigan, Lansing, USA.

GeoHive. 2009. Global Statistics [available online] http://www.xist.org/

Hamayun, M., S. A. Khan, Z. K. Shinwari, A. L. Khan, N. Ahmad and I. J. Lee. 2010. Effect of polyethyleneglycol induced drought stress on physio-hormonal attributes of soybean. *Pak. J. Bot.,* 42(2): 977-986.

Hassan, M.U., Z. Ahmed, M. Munir, S.I. Malik and K. Shahzad. 2009. Effect of sorbitol in callus induction and plant regeneration in wheat. *Afr. J. Biotechnol.,* 8(23): 6529-6535.

Jia, H., J. Yu, D. Yi, Y. Cheng, W. Xu, L. Zhang and Z. Ma. 2009. Chromosomal intervals responsible for tissue culture response of wheat immature embryos. *Plant Cell Tiss. Organ Cult.,* 97: 159-165.

Kabir, A.H., I. Mahfuz, M.A. Razvy, M.B. Ahmed and M.F. Alam. 2008. Indirect organogenesis and somaclonal variation in four rice cultivars of Bangladesh. *J. Appl. Sci. Res.,* 4(4): 45-458.

Kilinc, M. 2004. Effects of dicamba concentration on the embryo cultures of some bread wheat (*Triticum aestivum* L.) genotypes. *Biotechnol. Biotechnol. Equip.,* 58-61.

Li, Y., J. Gao and S.Z. Fei. 2009. High frequency *in vitro* embryogenic callus induction and plant regeneration from indiangrass mature caryposis. *Scientia Hortic*., 119: 306-309.

Liu, H.J., S. Misoo, O. Kamijima and M. Sawano. 1990. Effects of plant growth regulators on the response in immature wheat embryo culture. *Plant Tiss. Cult Lett.,* 7(3): 170-176.

Mitic, N., D. Dodig and R. Nikolic. 2006. Variability of *in vitro* culture response in wheat genotypes, genotype and environmental effects. *Genetika*, 38(3): 183-192.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.,* 15: 473-497.

Noor, S., G.M. Ali, U. Rashid, M. Arshad, S. Ali and Y. Zafar. 2009. Optimization of callus induction and regeneration system for Pakistani wheat cultivars Kohsar and Khyber-87. *Afr. J. Biotechnol.,* 8(20): 5565-5569.

Pingali, P.L. 1999. CIMMYT 1998-99. World wheat facts and trends. Global wheat research in a changing world: challenges and achievements. Mexico, D.F., CIMMYT.

Rahman, M.M., A.K.M. Shamsuddin and U. Asad. 2008. *In vitro* regeneration from mature embryos in spring wheat. *Int. J. Sustain Crop Prod*., 3(2): 76-80.

Rashid, U., S. Ali, G.M. Ali, N. Ayub and M.S. Masood. 2009. Establishment of an efficient callus induction and plant regeneration system in Pakistani wheat (*Triticum aestivum*) cultivars. *Electron. J. Biotechnol.,* 12(3): 1-7. [Available on line] http://www.ejbiotechnology.info/content /vol12/ issue3/full/1/].

Raziuddin, J. Bakht, Z.A. Swati, M. Shafi, F. Ullah and M. Akmal. 2010. Effect of cultivars and culture medium on callus formation and plant regeneration from mature embryos of wheat (*Triticum aestivum* L.). *Pak. J. Bot.,* 42(1): 639-652.

Razzaq, A., I.A. Hafiz, I. Mahmood and A. Hussain. 2011. Development of *in planta* transformation protocol for Wheat. *Afr. J. Biotechnol*., 10(5):740-750.

Razzaq, A., M. Zhiying and W. Haibo. 2004. Genetic transformation of wheat (*Triticum aestivum* L): A Review. *Mol. Plant Breed.,* 2(4): 457-464.

Razzaq, A., Z. Yan-min, Y. Fan, Y. Yan-jie, Z. He, M. Zhi-ying and W. Hai-bo. 2005. *In planta* transformation of wheat apical meristem: a preliminary study. *Acta Agric. Boreali. Sinica,* 20(1): 17-22.

Rosegrant, M.W., M.A. Sombilla, R.V. Gerpacio and C. Ringler. 1997. Global food markets and US exports in 21st century. Presented at the Illinois world food and sustainable agriculture program conference, meeting the demand for food in 21st century: challenges and opportunities, 28th May, Univ. Illionois, Urbana-Cahmopaign, USA.

Sarker, R.H. and A. Biswas. 2002. *In vitro* plantlet regeneration and *Agrobacterium*-mediated genetic transformation of wheat (*Triticum aestivum* L.). *Plant Tissue Cult.,* 12: 155-165.

Satyavathi, V.V., P.P. Jauhar, E.M. Elias and M.B. Rao. 2004. Effects of growth regulators on *in vitro* plant regeneration in durum wheat. *Crop Sci.,* 44: 1839-1846.

Shah, M.I., M. Jabeen and I. Ilahi. 2003. *In vitro* callus induction, its proliferation and regeneration in seed explants of wheat (*Triticum aestivum* L.) var.LU-26S. *Pak. J. Bot.,* 35(2): 209-217.

Shah, M.M., Q. Khalid, U.W. Khan, S.A.H. Shah, S.H. Shah, A. Hassan and A. Pervez. 2009. Variation in genotypic responses and biochemical analysis of callus induction in cultivated wheat. *Genet. Mol. Res.,* 8(3): 783-793.

Takayama, S. and M. Misawa. 1982. Regulation of organ formation by cytokinin and auxin in *Lilium bulbscales* grown *In vitro*. *Plant Cell Physiol*., 23(1): 67-74.

Yasmin, S., I.A. Khan, A. Khatri, N. Seema, G.S. Nizamani and M.A. Arain. 2009. *In vitro* plant regeneration in bread wheat (*Triticum aestivum* L.). *Pak. J. Bot.,* 41(6): 2869-2876.

(Received for publication 12 February 2011)