Pak. J. Bot., 48(6): 2499-2504, 2016.

IN VITRO REGENERATION OF FIVE WHEAT GENOTYPES FROM IMMATURE ZYGOTIC EMBRYOS

MUHAMMAD ILYAS KHOKHAR[1*], MUHAMMAD ZAFFAR IQBAL[1] AND JAIME A. TEIXEIRA DA SILVA[2]

[1]*Agricultural Biotechnology Research Institute, Ayub Agricultural Research Institute, Faisalabad, Pakistan*
[2] *P. O. Box 7, Miki-cho post office, Ikenobe 3011-2, Kagawa-ken, 761-0799, Japan*
[*]*Corresponding author's email: khokharab@yahoo.com, ilyasabri@yahoo.com*

Abstract

This study examined the ability to induce callus from immature zygotic embryos of five wheat genotypes (Lu 26, WH 543, Zamindar 80, BT-002 and Seher-06) in response to 2, 4 and 6 mg/L of 2,4-dichlorophenoxy acetic acid (2,4-D). Callus induction was most effective (41% averaged across the 5 genotypes) in the presence of 2 mg/L 2,4-D. Callus induction was highest in Lu 26 (34%) followed by WH 543 (33%). Highest percentage shoot formation (33%) from callus was possible on Murashige and Skoog (1962) medium containing 300 mg casein hydrolysate. BT-002 responded best to shoot formation (26%) followed by WH 543 (24%). Under these optimal conditions, callus could form within 7.4 days and shoots within 20.87 days (fastest growth averaged across the 5 genotypes). Zamindar-80 responded best by taking fewest days to initiate callus formation (7.88 days) while Lu 26 took the least amount of time to form shoots (23.25 days). This study provides a rapid and efficient, as well as cultivar-independent protocol for the indirect formation of shoots from callus, the first such report for WH 543, Zamindar 80, BT-002 and Seher-06. This protocol may be a useful protocol for transgenic wheat plants that are derived from the genetic transformation of callus, either by particle bombardment or *Agrobacterium*-mediated transformation, to produce, for example, insect- or herbicide-resistant plants, since a rapid and effective regeneration protocol is an essential first step for the successful regeneration of transgenic plants.

Key words: Callus induction; Casein hydrolysate; 2, 4-dichlorophenoxy acetic acid (2, 4-D); Shoot formation; Wheat.

Introduction

Wheat (*Triticum* spp.), which has a large genome (~17,000 Mb), is a key staple food crop and source of nutrients and calories (Yasmin *et al.*, 2009). There is thus always the desire to improve current regeneration protocols, or to devise new ones, that would allow for better quality traits, including biotic or abiotic stress resistance, to be improved which is possible through conventional breeding and tissue culture (Schulze, 2007). Such biotechnological approaches would allow the limitations associated with traditional breeding methods – such as a long breeding period or a rather limited gene pool – to be overcome by wheat breeders, for example in programmes designed to induce heat stress (Hossain *et al.*, 2013). Somaclonal variation *in vitro* can provide unique possibilities for the rapid development of new wheat varieties (Malik *et al*., 2004), which is particularly important for the development of heat-resistant cultivars (Farshadfar *et al.*, 2012). Tissue culture has been used to develop useful variation in wheat, including salt tolerance (Zair *et al.*, 2003; Al-Ashkar, 2013), drought tolerance (Gawande *et al.*, 2005), disease resistance (Svabova & Lebeda, 2005), an increase in the protein content in grains (Villareal *et al.*, 1999), or in nutrient biofortification (Velu *et al.*, 2014).

Callus induction and regeneration potential in wheat can be influenced by several factors (Raziuddin *et al*., 2010; Delporte *et al*., 2014; Wang *et al*., 2014). These can be biotic (cultivar, explant, age or degree of differentiation and physiological conditions of the mother plant and explant), or abiotic (carbohydrate, basal medium, concentration of plant growth regulators (PGRs) such as 2,4-dichlorophenoxy acetic acid (2,4-D), and other culture conditions, including light, or temperature). Immature zygotic embryos (ZEs) have been found to be suitable for callus induction (Arzani & Mirodjagh 1999; Hou *et al.*, 1997; Talukder *et al.*, 2004) due to their totipotency (Delporte *et al*., 2014).

Based on these reports from the literature, immature ZEs – which are highly totipotent, i.e., they contain young and highly regenerative tissues that are very receptive to *in vitro* morphogenesis – in the presence of 2,4-D were induced to form callus from five wheat genotypes (Lu 26, WH 543, Zamindar 80, BT-002 and Seher-06), which form part of the germplasm collection of the Agricultural Biotechnology Research Institute, Faisalabad. The characteristics of these cultivars are described in Table 1. Lu 26 was used by (Akhtar *et al.*, 2012) to induce genetic variability and to select for salt tolerance through *in vitro* culture. Callus was first induced because it is not easy to induce shoots directly from ZEs, thus an intermediate callus phase is first required. The ultimate objective of this study was to find *in vitro* variants that could, in a second, subsequent phase, result in greater yield in field trials, or to identify variants with novel traits. This study also quantified the speed of regeneration, which is an important component that is rarely assessed, but is essential for subsequent breeding objectives such as genetic transformation (Wędzony *et al*., 2014).

Materials and Methods

The experiment was performed at the Agricultural Biotechnology Research Institute, Faisalabad during 2012. Immature ZEs were excised from the seeds of all five cultivars with a sharp sterile blade at the milky stage, 75 days after germination. ZEs were surface sterilized with 30% sodium hypochlorite for 45 min on a shaker

(3020 model, GFL, Burgwedel, Germany) at 54 rpm, dipped in 70% (v/v) ethanol for 5 min and washed three times with sterilized (autoclaved) distilled water. The caryopsis was aseptically opened and immature ZEs, including the scutellum, were placed in test tubes holding callus induction medium (CIM) following the procedure of Talukder *et al.* (2004). CIM consisted of Murashige and Skoog (MS; 1962; ID: M519) full micro- and macronutrients, 3% (w/v) sucrose and 2, 4 or 6 mg/L 2,4-D (ID: D299). Medium was solidified with 1.76 g/L Gellan gum (ID: G434). All reagents (product code IDs indicated in parentheses) were purchased from Phyto Technology Laboratories (Shawnee Mission, KS, USA). Sterilized immature ZEs (one/test tube) were incubated in the dark at 25°C for three weeks based on the success of Mahmood *et al*. (2012) with cv. GA-2002. Thereafter, test tubes were transferred to light conditions (incubated at 25 ± 2°C with a 16-h photoperiod and a photosynthetic photon flux density of 83.6 µmol m^{-2} s^{-1} provided by white fluorescent tubes, as suggested by Yasmin *et al.* (2009). Based on Rahman *et al.* (2008), after 28 days of incubation, callus was transferred to shoot induction medium (SIM), which contained MS gelled with 1.76 g/L Gellan gum and supplemented with either: 1) 300 mg/L casein hydrolysate (CH); 2) 400 mg/L CH; 3) 300 mg/L CH and 2 mg/L 6-benzyladenine (BA); 4) MS + 400 mg/L CH and 2 mg/L BA (CIM and SIM factors explained in (Table 2). Days to callus induction and days to indirect shoot formation from callus were assessed for each treatment.

Statistical analysis: The experiment was laid out as a two-factor completely randomized block design (CRBD), replicated three times with 50 samples per replication. Data was analyzed with MSTAT-C software (Russel Freed, Michigan State University, USA) and treatment means were separated by the Least Significant Difference (LSD) test at α=0.05.

Table 1. Unique characteristics of five wheat genotypes studied.

Genotypes name	1000-grain weight (g)	Plant height (cm)	Days to heading	Days to maturity	Number of tillers/plant	Leaf rust*	Yellow rust*
Lu 26 (salt-tolerant)	50.2	118	112	148	8	90S	80S
WH 543	40.8	99	114	154	6.6	5M	10M
Zamindar 80	38.9	121	112	150	7.3	15RMR	10RMR
BT-002	37.2	108	113	148	7	0I	0I
Seher-06	56.1	110	105	148	7.6	10MSS	5MSS

M = moderately resistant, RMS = resistant to moderately resistant, MSS = moderately susceptible to susceptible; I = immune

* Based on Johnson *et al.* (1972)

Table 2. Different combinations of 2,4-D and plant growth regulators on five wheat genotypes, all on MS-based basal medium.

Genotype		CIM	Treatment	Genotype		Treatment	SIM
Lu 26	G1	2 mg/L 2,4-D	T1	Lu 26	G1	T1	300 mg/L CH
		4 mg/L 2,4-D	T2			T2	400 mg/L CH
		6 mg/L 2,4-D	T3			T3	300 mg/L CH + 2 mg/L BA
WH 543	G2	2 mg/L 2,4-D	T1			T4	400 mg/L CH + 2 mg/L BA
		4 mg/L 2,4-D	T2	WH 543	G2	T1	300 mg/L CH
		6 mg/L 2,4-D	T3			T2	400 mg/L CH
Zamindar 80	G3	2 mg/L 2,4-D	T1			T3	300 mg/L CH + 2 mg/L BA
		4 mg/L 2,4-D	T2			T4	400 mg/L CH + 2 mg/L BA
		6 mg/L 2,4-D	T3	Zamindar 80	G3	T1	300 mg/L CH
BT-002	G4	2 mg/L 2,4-D	T1			T2	400 mg/L CH
		4 mg/L 2,4-D	T2			T3	300 mg/L CH + 2 mg/L BA
		6 mg/L 2,4-D	T3			T4	400 mg/L CH + 2 mg/L BA
Seher-06	G5	2 mg/L 2,4-D	T1	BT-002	G4	T1	300 mg/L CH
		4 mg/L 2,4-D	T2			T2	400 mg/L CH
		6 mg/L 2,4-D	T3			T3	300 mg/L CH + 2 mg/L BA
						T4	400 mg/L CH + 2 mg/L BA
				Seher-06	G5	T1	300 mg/L CH
						T2	400 mg/L CH
						T3	300 mg/L CH + 2 mg/L BA
						T4	400 mg/L CH + 2 mg/L BA

2,4-D, 2,4-dichlorophenoxy acetic acid; BA, 6-benzyladenine; CH, casein hydrolysate; CIM, callus induction medium; G, genotype; SIM, shoot induction medium; T, treatment

Fig. 1. Shoot regeneration through an indirect callus route of four-week-old *in vitro* cultures from four wheat genotypes (from left to right: Lu 26, WH 543, Zamindar 80, BT-002).

Results and Discussion

Tissue culture is an indispensable technique for the effective genetic transformation of wheat (Wędzony *et al.*, 2014). Immature ZEs are totipotent and thus an effective and reliable source for *in vitro* regeneration of *Triticum* spp. (Delporte *et al.*, 2014). Thus, in this study, the ability of immature ZEs of five wheat genotypes to induce callus in the presence of multiple 2,4-D concentrations in MS medium was evaluated. The ability of this callus to regenerate shoots and plantlets was also assessed (Fig. 1). A wealth of literature on wheat regeneration *in vitro*, including of Pakistani cultivars, allowed for the development of callus induction and plantlet regeneration protocols for four as yet unexplored Pakistani cultivars (WH 543, Zamindar 80, BT-002 and Seher-06) while Lu 26 served as a model cultivar.

Callus induction: Treatment and genotype strongly influenced the ability to induce callus and shoots (Table 3). Most callus (40.8%) was induced in MS with 2 mg/L 2,4-D (T1), requiring fewest days (7.4) followed by T2 (MS with 4 mg/L 2,4-D), in which 30% of explants formed callus, while least callus was induced (22.80%) in T3 (MS with 6 mg/L 2,4-D) (Table 4). Highest shoot formation (33.4%) was observed on MS medium with 300 mg/L CH, which also took the fewest days (20.87 days) to form shoots (Table 4). Zamindar 80 formed callus most rapidly (7.88 days); callus induction among all genotypes was similar, ranging from 29 to 34% while shoot formation across genotypes was also within a narrow range (19.25 to 25.50) (Table 5). Lu-26 showed the fastest regeneration (23.25 days) (Table 5).

In Pakistan, *in vitro*-based biotechnology has been extensively used to develop resistance to biotic and abiotic stresses (Nasircilar *et al*., 2006; Raziuddin *et al*., 2010; Akhtar *et al*., 2012; Mahmood *et al*., 2012). This study was inspired by the findings of Rahman *et al.* (2008), who indicated that shoots could form early (23 days) from callus when 200 mg/L of CH was added to MS medium. Nasircilar *et al*. (2006) reported immature ZEs of wheat cv. İkizce to be the most efficient explant for the highest frequency (58%) of callus induction, as reported in this study, but the response was genotype-dependent (percentage of explants forming callus): Yakar (53%), Mizrak (45.33%) and Gun 91 (41.67%). Raziuddin *et al*. (2010) reported highest callus induction (67.5%) in an ICARDA (ICP-3) line of wheat from immature ZEs on MS medium with 2 mg/L 2,4-D, or 48% and 100% from mature seeds of cv. Inquilab-91 and cv. Pavon-76 (Malik *et al*., 2004), for cv. Lu 26S (Shah *et al*., 2008), or in eight Chinese cultivars from mature seeds (Yu *et al*., 2008), similar to the findings of this study (Table 4). Our results confirm what Raziuddin *et al*. (2010) discovered, namely that the use of a lower concentration of 2,4-D (2 mg/L) had beneficial effects on callus induction from immature ZEs. Cultivars Inqilab-91, Chakwal-97 and Manthar formed most callus from mature seeds in the presence of 3 mg/L 2,4-D (83.3%, 77.8% and 95.2%, respectively) while 2 mg/L 2,4-D was optimal for cv. Tatara (97.2%). Rashid *et al.* (2009) and Malik *et al*. (2004) found that 4 mg/L 2,4-D inhibited callus proliferation from mature seed while 3 mg/L allowed shoots to form. In this study, 2 mg/L of 2,4-D induced more callus than 4 or 6 mg/L (Table 4). These studies – including ours – all confirm that immature ZEs are an optimal explant for callus induction, and that 2,4-D serves as a strong inducer of callus.

Table 3. Analysis of variance for callus initiation, callus induction %, plant regeneration and days to regeneration % for five wheat genotypes.

SOV	df	Mean square		df	Mean square	
		Days to callus initiation	Callus induction %		Plantlet regeneration %	Days to regeneration
Replications	2	0.156	135.200	2	120.650	0.317
Treatments (A)	2	22.022	50.300	3	1084.950	165.350
Genotypes (B)	4	2.311	1231.200	4	66.525	6.392
A×B	8	1.244	7.700	12	10.825	1.669
Error	28	0.322	16.557	38	16.597	0.703
COV		6.78%	13.04%		17.75%	3.47%

COV = coefficient of variation; SOV = source of variation; DF = degrees of freedom

Table 4. Effect of different concentrations of 2,4-D on days to callus initiation, callus induction %, plant regeneration % and days to regeneration for five wheat genotypes.

Days to callus initiation		Callus induction %		Plant regeneration %		Days to regeneration	
T1	7.400 c	T1	40.80 a	T1	33.40 a	T1	20.87 d
T2	8.000 b	T2	30.00 b	T2	26.20 b	T2	22.27 c
T3	9.733 a	T3	22.80 c	T3	17.40 c	T3	25.27 b
				T4	14.80 c	T4	28.33 a
LSD (α=0.05)	0.949		6.806		6.734		1.386

T = treatment, LSD = least significant difference

Table 5. Response of five wheat genotypes for days to callus initiation, callus induction %, shoot formation % and days to regeneration.

Genotypes	Days to callus initiation	Callus induction %		Shoot formation %		Days to regeneration	
G5	9.000 a	G1	34.00 a	G3	25.50 a	G4	25.17 a
G2	8.778 ab	G2	33.33 a	G2	24.25 a	G2	24.50 ab
G1	8.333 ab	G3	30.67 a	G4	23.25 a	G5	24.25 ab
G4	7.889 b	G4	29.00 a	G1	22.50 a	G3	23.75 b
G3	7.889 b	G5	29.00 a	G5	19.25 a	G1	23.25 b
LSD (α=0.05)	0.949		6.806		6.734		1.386

G = genotype, LSD = least significant difference

Shoot induction and plantlet formation: Shah *et al*. (2008) observed the highest plant regeneration in wheat (Lu-26) on MS medium with 4 mg/L BA alone for shoot formation or 2 mg/L BA in combination with 1 mg/L indole-3-acetic acid (IAA) for root and shoot formation. Sarkar & Biswas (2002) obtained most shoots in wheat on MS medium with 0.5 mg/L of BA and 0.5 mg/L kinetin (Kin). Yu *et al*. (2008) regenerated plants efficiently on MS medium with silver nitrate ($AgNO_3$) and MS with $CuSO_4$ and found that culture efficiency ([number of green plants regenerated/number of explants plated] × 100) ranged from 19 to 35.4 in eight wheat cultivars when mature ZEs were used. Among four Pakistani wheat varieties (Inqilab-91, Chakwal-97, Tatara and Manthar), Rashid *et al*. (2009) found most shoot regeneration in Inqilab-91, Chakwal-97 and Manthar (87.25, 81.75 and 68.75%, respectively) on MS medium with 0.1 mg/L IAA, 0.4 mg/L Kin and 0.5 mg/L 6-γ-γ-dimethylallylaminopurine (2iP) while cv. Tatara formed most shoots (12.25%) in MS medium with 0.1 mg/L IAA and 2 mg/L BA. Yasmin *et al.* (2009) studied three wheat varieties (Sarsabz, Khirman and Soghat-90) in different regeneration media. Most plantlets formed in cv. Sarsabz followed by cv. Soghat-90 on MS medium with 5.7 mg/L IAA and 5 mg/L Kin, and on MS medium with 1.5 mg/L Kin and 2 mg/L NAA for cv. Khirman. Rahman *et al*. (2008) studied *in vitro* regeneration of three wheat genotypes (Sourav, Gourab and Satabdi) and two double haploids (DH-2 and DH-10), most plants forming on MS medium with 1 mg/L Kin while the use of 200 mg/L CH sped up shoot regeneration. Raziuddin *et al.* (2010) studied *in vitro* plant regeneration in two media (MS and Linsmeier & Skoog (1965) (LS) medium) of nine Pakistani wheat cultivars and one ICARDA line. They noted that LS medium was superior to MS medium in terms of compact embryogenic callus formation (non-embryogenic callus was friable and grew slowly) but when PGRs were also considered, plant regeneration was equally good on LS and MS medium containing 0.1 mg/L IAA and 0.5 mg/L BA, with a maximum of 20 and 25.5% plant regeneration, respectively. Akhtar *et al.* (2012) used MS medium without any PGRs to regenerate 20 wheat varieties, but the number of plantlets varied from 1 (cv. SA-42) to 48 (cv. LU-26S) across all varieties in a range of salinity (50-150 mM NaCl). Mahmood *et al*. (2012) obtained most shoots from immature ZEs of wheat cultivar GA-2002 on MS medium with 0.2 mg/L IAA, 0.5 mg/L Kin and 0.5 mg/L of BA. Our study confirms the findings of Mahmood *et al.* (2012) and Yu *et al.* (2008), who used immature ZEs for regeneration (i.e., shoot formation). Other studies also used MS basal medium for effective shoot induction from callus (Sarkar & Biswas, 2002; Shah *et al*., 2008; Yu *et al*., 2008; Yasmin *et al*., 2009) albeit with different PGRs. This study indicates that CH is also an essential component of SIM, having based our levels (300 mg/L) on the levels suggested by Rahman *et al.* (2008), i.e., 200 mg/L. In their study, shoots formed within 23 days

while in our study, shoots developed even more rapidly from callus, only within 21 days, although this response may be strongly dependent on the genotype.

These studies, including ours, indicate that a wide range of protocols can be employed to induce shoots, primarily from callus through an indirect morphogenic route. In all cases, protocols are genotype-dependent, highlighting the need to tweak protocols to suit each genotype. This study not only expands the data-base of information related to wheat *In vitro* regeneration from immature ZEs, but provides a relatively simple, rapid and efficient protocol to regenerate four as-yet unexplored cultivars. Although there was variation in the level of callus that formed or shoots that could be induced among the five genotypes, all could form callus and shoots, which rooted to form plantlets, nonetheless, making this an effective and reliable regeneration protocol.

Conclusion

This study provides the first report for four wheat genotypes of the Agricultural Biotechnology Research Institute: WH 543, Zamindar 80, BT-002 and Seher-06. A low 2,4-D concentration (2 mg/L) was best for rapid callogenesis from immature ZEs while the inclusion of 300 mg/L CH in MS medium benefitted shoot formation. This study's protocol will be used to breed glyphosate (a herbicide)-tolerance plants by *Agrobacterium*-mediated genetic transformation for improving weed control since weeds constitute a major problem in the wheat crop in Pakistan, as they decrease yield. An effective and rapid protocol for shoot induction and plantlet formation, via an indirect callus route, as was achieved in this study, is an indispensable first step to achieving these subsequent transgenic objectives (Wędzony *et al*., 2014).

Authors' contributions: Muhammad Ilyas Khokhar and Muhammad Zaffar Iqbal conceived the experiments. Muhammad Ilyas Khokhar conducted the experiments. Muhammad Ilyas Khokhar and Jaime A. Teixeira da Silva analyzed and interpreted the data, wrote all drafts of the manuscript and approved it for submission to PJB.

References

Akhtar, S., M. Niaz, S. Rahman and M. Asif. 2012. Study of somaclonal variation in wheat for the induction of salinity tolerance. *J. Agric. Res*., 50(2): 165-176.

Al-Ashkar, I.M. 2013. Anther culture response and salt tolerance in some wheat genotypes. *Ann. Agric. Sci*., 58: 139-145.

Arzani, A. and S.S. Mirodjagh. 1999. Response of durum wheat cultivars to immature embryo culture, callus induction and *in vitro* salt stress. *Plant Cell Tissue Organ Cult.,* 58: 67-72.

Delporte, F., A. Pretova, P. du Jardin and B. Watillon. 2014. Morpho-histology and genotype dependence of *in vitro* morphogenesis in mature embryo cultures of wheat. *Protoplasma*, 251: 1455-1470.

Farshadfar, E., B. Jamshidi, K. Cheghamirza and J.A. Teixeira da Silva. 2012. Evaluation of drought tolerance in bread wheat (*Triticum aestivum* L.) using *in vivo* and *in vitro* techniques. *Ann. Biol. Res*., 3(1): 465-476.

Gawande, N.D., D.G. Mahurkar, T.H. Rathod, S.W. Jahagidar and S.M. Shinde. 2005. *In vitro* screening of wheat genotypes for drought tolerance. *Ann. Plant Physiol*., 19: 162-168.

Hossain, A., M.A.Z. Sarker, M. Saifuzzaman, J.A. Teixeira da Silva, M.V. Lozovskaya and M.M. Akhter. 2013. Evaluation of growth, yield, relative performance and heat susceptibility of eight wheat (*Triticum aestivum* L.) genotypes grown under heat stress. *Int. J. Plant Prod*., 7(3): 615-636.

Hou, B., H. Yu and S. Teng. 1997. Effects of low temperature on induction and differentiation of wheat calluses. *Plant Cell Tissue Organ Cult*. 49: 35-38.

Johnson, R., R.W. Stubbs, E. Fuchs and N.H. Chamberlain. 1972. Nomenclature for physiologic races of *Puccinia striiformis* infecting wheat. *Trans. Br. Mycol. Soc*., 58: 475-480.

Linsmeier, E.M. and F. Skoog. 1965. Organic growth factor requirements of tobacco tissue cultures. *Physiol. Plant*., 18: 100-127.

Mahmood, I., A. Razzaq, M. Ashraf, I.A. Hafiz, S. Kaleem, A. Qayyum and M. Ahmad. 2012. *In vitro* selection of tissue culture induced somaclonal variants of wheat for drought tolerance. *J. Agric. Res*., 50(2): 177-188.

Malik, S.I., H. Rashid, T. Yasmin and N.M. Minhas. 2004. Effect of 2,4-dichlorophenoxyacetic acid on callus induction from mature wheat (*Triticum aestivum* L.) seeds. *Int. J. Agri. Biol*., 6(1): 156-159.

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

Nasircilar, A.G., 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.

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. *Electronic J. Biotech*., 12(3): 1-12.

Raziuddin, J.B., 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.

Sarkar, R.H. and A. Biswas. 2002. *In vitro* plantlet regeneration and *Agrobacterium*-mediated genetic transformation of wheat. *Plant Tissue Cult*., 12(2): 155-165.

Schulze, J. 2007. Improvements in cereal tissue culture by thidiazuron: a review. *Fruit Vegetable Cereal Sci. Biotech*. 1: 64-79.

Shah, M.I., J. Mussarat and I. Ihsan. 2008. *In vitro* callus induction, its proliferation and regeneration in seed explants of wheat (*Triticum aestivum* L.). *Pak. J. Bot*., 35(2): 209-217.

Svabova, L. and A. Lebeda. 2005. *In vitro* selection for improved plant *Triticum aestivum* L. *Theor. Appl. Genet*., 79: 609-617.

Talukder, M.Z.I., A.K.M. Shamsuddin, H. Rahman and L. Hassan. 2004. Effect of media components on nature of callus and subsequent regeneration of spring wheat (*Triticum aestivum* L.). *Plant Tissue Cult*., 14(2): 113-121.

Velu, G., I. Ortiz-Monasterio, I. Cakmak, Y. Hao and R.P. Singh. 2014. Biofortification strategies to increase grain zinc and iron concentrations in wheat. *J. Cereal Sci*., 59: 365-372.

Villareal, R.L., K.A. Mujeeb P.J. Peña. 1999. Agronomic performance and quality characteristics of tissue culture derived lines of spring wheat (*Triticum aestivum* L.) cultivar Pavon. *Cereal Res. Commum*., 27: 41-48.

Wang, X.M., X. Ren, G.X. Yin, K. Wang, J.R. Li, L.P. Du, H.J. Xu and X.G. Ye. 2014. Effects of environmental temperature on the regeneration frequency of the immature embryos of wheat (*Triticum aestivum* L.). *J. Integ. Agric*., 13: 722-732.

Wędzony, M., M. Szechyńska-Hebda, I. Żur, E. Dubas and M. Krzewska. 2014. Tissue culture and regeneration: a prerequisite for alien gene transfer. In: (Eds.): Pratap, A. & J. Kumar *Alien Gene Transfer in Crop Plants*. Vol. 1, Springer Science + Business Medium, Berlin, pp. 43-75.

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.

Yu, Y., J. Wang, M.L. Zhu and Z.M. Wei. 2008. Optimization of mature embryo-based high frequency callus induction and plant regeneration from elite wheat cultivars grown in China. *Plant Breed*., 12: 249-255.

Zair, I., A. Chlyah, K. Sabounji, M. Titahsen and H. Chlyah. 2003. Salt tolerance improvement in some wheat cultivars after application of *in vitro* selection pressure. *Plant Cell Tissue Organ Cult*., 73: 237-244.

(Received for publication 22 October 2015)