Pak. J. Bot., 45(SI): 545-549, January 2013.

TISSUE CULTURE RESPONSES OF SOME WHEAT (*TRITICUM AESTIVUM* L.) CULTIVARS GROWN IN PAKISTAN

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Abstract

Good tissue culture response for callus induction and regeneration is prerequisite for improvement of wheat through genetic transformation. Tissue culture response of 6 wheat cultivars was studied using MS and N6 medium supplemented with different concentration of 2, 4-D (2,4-Dichlorophenoxyacetic acid) and BAP (6-benzylaminopurine) for callus induction and regeneration, respectively. Mature seeds were used as explants. All cultivars exhibited best response for callus induction and regeneration on MS medium as compared to N6 medium. However, significant differences among cultivars were observed. Each cultivar responded differently at different levels of growth regulator for callus induction. Inqalab-91 and Lasani-08 showed maximum callus induction (90%) and (78.78%), respectively at 3mg/l of 2, 4-D. Tatara showed 84.43% callus at 2.0mg/l, chakwal-97 77.08% at 2.5mg/l while GA-02 and Khyber showed 74.30% and 65.97% callus induction response, respectively, at 3.5mg/l of 2, 4-D. As regards regeneration, direct shoots and roots development were observed by using different concentration of BAP. Significantly higher regeneration (59.33%) was observed in Chakwal-97 with 3.0mg/l of BAP while least regeneration was observed in Khyber (17.33%) at 4.0mg/l among all cultivars. It was also observed that all cultivars showed shoot as well as root development with 3 and 5mg/l of BAP. Using 8mg/l agar rather than 6 and 10 mg/l significantly enhanced regeneration ability of cultivars. The results of present findings will be helpful for selecting the most tissue culture responsive cultivars for genetic transformation against different biotic and abiotic stresses as well as for improvement of important agronomic traits of wheat crop (Fig. 2).

Introduction

Wheat (*Triticum aestivum* L.) belonging to family Poaceae is growing in the most parts of the world. In Pakistan it is the most important cereal crop and major staple food grown all over the country. Its average yield is very low and uncertain due to biotic and abiotic stresses (Rashid *et al.,* 2012). The population growth rate increasing day by day so there exists a gap between wheat yield and its demand throughout the world (Bhalla, 2006).

Conventional breeding methods in Pakistan are being used for enhancing the production and quality improvement of wheat crop. However, limited gene pool availability and long duration of these methods are the major limitations for improvement of the crop through conventional methods. Genetic engineering techniques are gaining popularity because the desired gene can be introduced from any source without species barrier in wheat genome in short time to improve its characters (Malik *et al.,* 2003). Transformation of wheat crop entirely depends upon regeneration of transformed explants through tissue culture (Yu *et al.,* 2008). Consequently, establishment of reliable tissue culture protocols for callus induction and regeneration is desired in order to improve wheat yield (Noor *et al.,* 2009) through genetic transformation.

Tissue culture of wheat depends upon genotype of wheat (Mahmood *et a.,* l 2012; Sears & Deckard, 1982), culture medium (Mahmood *et al.,* 2012; Mathias & Simpson, 1986) and growth regulators. (Saad *et al.,* 2004). Many explants such as immature and mature embryos, shoot bases, leaves, whole seeds and roots tips (Sarkar & Biswas, 2002) have been used for this purpose. Among these explants immature embryos are nominated the best for callus as well as regeneration (Sarkar & Biswas, 2002; Arzani & Mirodjagh, 1999) but availability of immature embryos as compared to mature embryos throughout the year is restricted. Therefore, present research was conducted to determine the tissue culture response of all six wheat cultivars for callus induction and regeneration by using mature embryos as explants source. Objective of this study was to determine suitable protocol and the most tissue culture responsive wheat cultivars.

Materials and Methods

Experimental sites and source of explants: This study was carried out in Plant Transformation Lab of National Institute for Genetics and Advanced Biotechnology, NARC Islamabad, Pakistan. Seeds of six wheat cultivars namely Lasani-08, Inqalab 91, Tatara, Chakwal 97, GA-02 and Khyber were collected from Crop Science Institute NARC Islamabad.

Seeds sterilization: Mature seeds of selected wheat cultivars were selected as an explant and they were washed under running tap water with detergent. Then they were disinfected with 70% ethanol for 30 seconds followed by 60% clorox for 15 minutes with continuous shaking under laminar air flow hood. Seeds were washed six times with autoclaved distilled water to remove clorox and then transferred on autoclaved filter papers in sterilized petri plates for drying.

Callus induction and regeneration: Seeds were inoculated in test tubes having callus induction media (CIM) i.e., MS or N6 containing different concentrations of 2, 4 D (Table 1). The pH of media was adjusted to 5.75-5.8 before autoclaving. Cultures were kept in growth room at temperature of 25±1°C. After 2 weeks, callus induction data was recorded and callus were separated from seeds and then transferred to fresh maintenance medium (half of optimized 2,4 D for callus induction media of respective cultivars) for further proliferation and growth up to one

week. After proliferation calli were transferred to regeneration media (RM) i.e., MS or N6 supplemented with different levels of BAP (Table 1) to get maximum regeneration. Each medium was supplemented with 3% sucrose, 1mg/L myo-inositol, vitamins and growth regulators. Media were solidified with 6 to 10g/l agar. Regenerated plants were shifted in hydroponic for further root elongation and healthy root development. When roots were developed then they were shifted in soil filled pots (Figs. 3 & 4).

Table 1. Media used for callus induction and regeneration of wheat cultivars.

Callus induction Treatments (2,4 D)	Regeneration Treatments (BAP)
T1=1.5 mg/L with MS or N6 media	T1=1 mg/L BAP in MS
T2=2.0 mg/ with MS or N6 media	T2=2.0 mg/ BAP in MS
T3=2.5 mg/L with MS or N6 media	T3=3 mg/L BAP in MS
T4=3.0 mg/L with MS or N6 media	T4=4 mg/L BAP in MS
T5=3.5 mg/L with MS or N6 media	T5=5 mg/L BAP in MS
T6=4.0 mg/L with MS or N6 media	

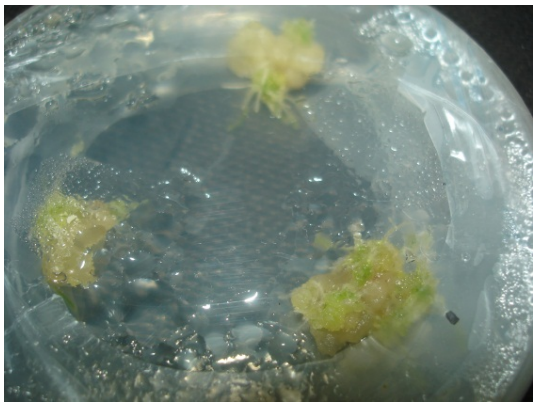

Fig. 1. Embryogenic callus.

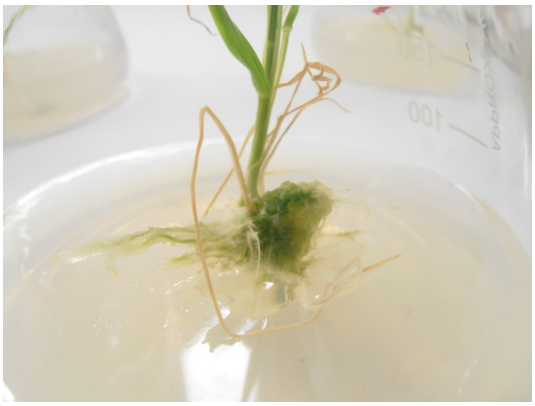

Fig. 2. Shoots and roots development.

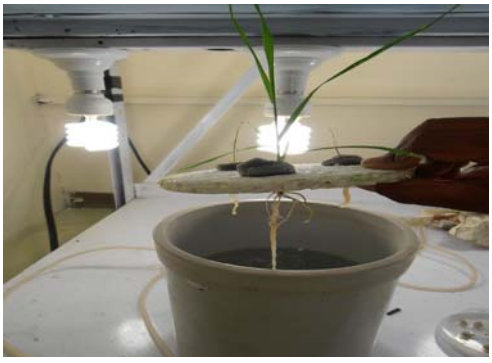

Fig. 3. Roots elongation in hydroponic.

Fig. 4. Plant shifted in soil.

Results and Discussion

Callus induction

Effects of 2, 4-D on callus induction: Callus induction is the fundamental step of tissue culture. Callus formation and regeneration response of six wheat cultivars were evaluated. Different levels of 2, 4-D was used to obtained its optimum level for maximum callus induction frequency of each cultivar under study. Callus initiated after 3 to 7 days of culturing under light having temperature ±25^{o}C. Similar observations were reported by (Satyavathi *et al.,* 2004) compact and nodular structures were observed on the surface of callus which is the

characteristic of embryogenic callus (Fig. 1). Non-embryogenic callus was fleshy and whitish in color along with shoots. These observations were also recorded by (Munazir *et al.,* 2010; Rashid *et al.,* 2009).

After three weeks data were recorded for each cultivar in percentage form which is shown in (Table 2). The data indicates that cultivars differed significantly in their potential to produce callus under same culture conditions and medium. Maximum callus induction frequency (90%) was recorded in Inqalab-91 with 3 mg/l 2, 4–D. However, highest callus induction frequency (84.43%) in Tatara was recorded with 2 mg/l 2, 4–D. Maximum callus induction frequency in lasani-08 (78.78%), Chakwal 97 (77.08%), GA-02 (74.3%) and Khyber (65.97%) was observed with 3.0, 2.5, and 3.5 mg/l 2, 4–D, respectively. Callus induction response of cultivars to 2, 4–D indicates that cultivars respond differently to concentration of 2, 4–D. Cultivars vary in their requirement of 2. 4–D for maximum callus induction. Inqalab-91 and tatara may be considered the most tissue culture responsive cultivars and show maximum callus formation at 3.0 and 2.0 mg/l of 2, 4–D, respectively.

Data recording: The frequency of callus induction and regeneration were recorded according to following formulas:

$$\text{Callus induction frequency (\%)} = \frac{\text{No. of seeds produced calli}}{\text{No. of seeds cultured}} \times 100$$

$$\text{Plant regeneration (\%)} = \frac{\text{No. of calli produced plants}}{\text{No. of plants plated}} \times 100$$

Table 2. Callus induction frequency (%) of wheat cultivars at various levels of 2, 4-D.

	Cultivars					
2, 4-D (mg/l)	**Lasani-08**	**Inqalab-91**	**Tatara**	**Chakwal-97**	**GA-02**	**Khyber**
1.5	6 0.41 ^{m*}	72.22 ^{i}	76.38 ^{efg}	70.13 ^{j}	38.19 ^{q}	27.77 ^{r}
2.0	65.97 ^{l}	77.77 ^{de}	**84.43** ^{b}	75.00 ^{gh}	47.91 ^{o}	38.88 ^{q}
2.5	72.44 ^{i}	83.52 ^{b}	79.86 ^{c}	**77.08** ^{def}	54.86 ^{n}	45.13 ^{p}
3.0	**78.78** ^{cd}	**90.00** ^{a}	75.69 ^{fgh}	74.30 ^{h}	61.80 ^{m}	54.16 ^{n}
3.5	68.75 ^{jk}	84.72 ^{b}	72.22 ^{i}	70.13 ^{j}	**74.30** ^{h}	**65.97** ^{l}
4.0	61.80 ^{m}	76.00 ^{fgh}	68.05 ^{k}	65.97 ^{l}	70.13 ^{j}	54.86n

* Means not sharing a letter in common differ significantly at 5% probability level

Table 3. Callus induction frequency (%) of wheat cultivars with MS and N6 medium.

Media	Lasani-08	Inqalab-91	Tatara	Chakwal-97	Ga-02	Khyber
MS	78.78^{c*}	90.0^{a}	84.43^{b}	77.08^{c}	74.30^{d}	65.97^{f}
N6	63.88^{g}	77.08^{c}	69.44^{e}	61.80^{h}	57.63^{i}	50.0^{j}

*Means not sharing a letter in common differ significantly at 5% probability level

Statistical analysis: Data were analyzed by MSTAT-C statistical software applying ANOVA with two factorial CRD fashion to find out variation response of cultivars and treatments. The significance of treatments means was further analyzed by using LSD test.

The variability in callus induction frequency in response to various levels of 2, 4-D may be due to differences in genes that control callusing. Our results suggested that variable response of cultivars to tissue culture might be due to genotype and media interaction as postulated by (Yasmin *et al.,* 2009). These results are similar with those of Hassan *et al.,* (2009), Shah *et al.,* (2009) and Kilinc (2004) who suggested that callus induction depends upon genotype of wheat. Many investigators have reported different levels of 2,4-D (2.0, 3.0, 3.5. 4.0 and 6 mg/l) for maximum callus induction (Rashid *et al.,* 2009; Farooq *et al.,* 2004; Noor *et al.,* 2009. These differences in results might be due to differences in varieties, types and source of explants, types of media and tissue culture conditions. Our results also indicates that all cultivars showed different callus induction response at different levels of 2, 4-D. It was also observed that with lower level of 2,4 - D, tendency of direct shoot regeneration increased and callus induction decreased while by increasing its level, shoots development decreased and callus induction frequency increased. Similar results were also reported by Alizadeh *et al.,* (2004).

Effect of medium on callus induction: For maximum callus induction media standardization is very crucial. Two types of media i.e. MS and N6 were compared for callus induction for six wheat cultivars. Data presented in Table 3 indicate that MS medium induced significantly higher callus in all cultivars as compared N6 medium. This reflects that MS medium is better than N6 medium

for callus induction in all wheat cultivars. Similar results were also reported by Ozgen *et al.,* (1998), He *et al.,* (1989) and Maddock *et al.,* (1983). These results indicate that tissue culture response of wheat is influenced by types and composition of culture medium.

Regeneration

Effects of BAP and media on regeneration: After one week of maintenance, calli were transferred to regeneration medium i.e. MS supplemented with different levels of BAP and agars. Data given in Table 4 showed that all cultivars exhibited best response for regeneration at MS media rather than N6 medium. The same was reported by Raziuddin *et al.,* (2010). They concluded that some cultivars have best response on MS while others perform better on LS media. It means that every genotype behaves differently at different type of medium.

Different levels of BAP were used for regeneration. It is an important cytokinin used for regeneration of cereals crops (Raja *et al.,* 2009). The results (Table 5) indicated that all cultivars responded differently to different levels of BAP. Differences observed in regeneration were significant among wheat cultivars. Chakwal-97 showed highest regeneration percentage (59.33 %) at 3mg/l of BAP, while least regeneration (17.33%) was observed in Khyber cultivar. Regeneration depends upon genotype of wheat so that each genotype behaved differently at different levels of growth regulators. Tatara and GA-02 had maximum regeneration at 2mg/L of BAP, lasani-08, inqalab-91 and chakwal 97 at 3mg/L of BAP while Khyber at 4mg/L of BAP. Optimized level of growth regulator for plant regeneration of one cultivars fails to develop plants in another cultivar of same species (Bhaskaran & Smith, 1990) because regeneration frequency depends on genotype and it might be controlled by genetic system.

Excellent regeneration was reported by many researchers by using different combination of growth regulators. Maximum regeneration was observed with MS medium supplemented with 0.5mg/l BAP, 25.0mg/l tyrosine and 0.5mg/l Kn (Sarker & Biswas, 2002). Rashid *et al.,* (2009) reported highest regeneration in cultivars chakwal-97, inqalab-91 and manthar on media containing 0.40mg/l Kn, .1 mg/l IAA and 0.50 mg/l of 2 iP.

Effect of agar concentration on shoot regeneration: Agar is mostly used as gelling agent for solidifying culture medium. It is complex polysaccharide which is obtained from algae. In present study comparison was made between three different levels of agar. The calli showed the variable response at different concentration of agar in regeneration medium of all varieties (Table 6). Higher concentration of agar enhances the regeneration ability of all calli. It was observed that with lower concentration of agar, calli take time for regeneration and most calli become fluffy while in higher concentration calli become compact and hard so from green spots, shoots emerged within short time. It was also observed that if agar concentration was increased from 6 g/l to 8 g/l then chances of regeneration increased. It might be that stress enhanced the regeneration ability but high concentration of agar above 8g/l reduced the regeneration ability. These results were in line with those of (Ali *et al.,* 2004; Afrasiab & Jafar 2011). It is thought that agar has agropectin with its sulphate side groups and with some other organic impurities due to which it might have inhibitory effects on callus proliferation and explants growth (Bhojani & Razdan 1996).

Table 4. Effects of MS and N6 media on regeneration.

Media	Lasani-08	Inqalab-91	Tatara	Chakwal-97	Ga02	Khyber
MS	48^{c*}	52^{b}	38^{d}	59.33^{a}	46.66^{c}	17.33^{g}
N6	32^{e}	38^{d}	28^{f}	41.33^{d}	31.33^{ef}	10^{h}

*Means not sharing a letter in common differ significantly at 5% probability level

Table 5. Effects of BAP on regeneration of wheat cultivars.

BAP (mg/l)	Lasani-08	Inqalab-91	Tatara	Chakwal-97	Ga02	Khyber
1	14^{op*}	18^{m}	11.33^{q}	28^{h}	20^{l}	04^{r}
2	24.66^{j}	35.33^{e}	38^{d}	47^{c}	46.66^{c}	10^{q}
3	48^{c}	52^{b}	33^{f}	59.33^{a}	36^{e}	13.33^{p}
4	34.66^{ef}	38.66^{d}	22^{k}	52.66^{b}	26.66^{hi}	17.33^{m}
5	25.33^{ij}	30^{g}	15.33^{no}	38.66^{d}	16.66^{mn}	10.66^{q}

*Means not sharing a letter in common differ significantly at 5% probability level

Table 6. Effects of agar concentration on regeneration.

Agar	Lasani-08	Inqalab-91	Tatara	Chakwal-97	Ga02	Khyber
6	37.33^{e*}	41.33^{d}	27.33^{i}	38^{e}	34^{f}	10^{l}
8	48^{c}	52^{b}	38^{e}	59.33^{a}	46.66^{c}	17.33^{k}
10	31.33^{g}	40^{d}	24^{j}	32^{g}	29.33^{h}	06^{m}

* Means not sharing a letter in common differ significantly at 5% probability level

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(Received for publication 1 September 2012)