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Optimization of *in vitro* Conditions for Callus Induction, Proliferation and Regeneration in Wheat (*Triticum aestivum* L.) Cultivars

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Abstract: Tissue culture conditions for two varieties of wheat (*Triticum aestivum* L.), Fakhr-e-Sarhad and Gandam-2002 were optimized using mature seeds as source of explant. Both of these varieties were tested for their callus production, callus proliferation and regeneration abilities. Different concentrations of 2,4-D (2,4-Dichlorophenoxy acetic acid) were tested for callus induction. Both of these varieties showed maximum callus induction (46.87% for Fakhr-e-Sarhad and 62.5% for gandam-2002) on MS medium supplemented with 3.5 mg L^{-1} 2,4-D. Excellent callus proliferation (87.5% for Fakhr-e-Sarhad and 95.31% for Gandam-2002) was obtained on MS medium containing 4 mg L^{-1} 2,4-D, also callus proliferation (81.25% for Fakhr-e-Sarhad and 93.75% for Gandam-2002) was found on 3 mg L^{-1} BAP (Benzyl aminopurine). Both varieties were regenerated into whole plant on medium containing 1 mg L^{-1} IAA (Indol, 3-acetic acid) in combination with 2 mg L^{-1} BAP after a culture period of 3 weeks. The regeneration was 50 and 60% for Fakhr-e-Sarhad and Gandam-2002, respectively on medium containing 3 mg L^{-1} Kinetin. The Gandam-2002 produced an average of 4 plantlets per callus, while the Fakhr-e-Sarhad produced an average of 2 plantlets per callus on medium fortified with 1 mg L^{-1} IAA along with 2 mg L^{-1} BAP.

Key words: Tissue culture, *in vitro*, wheat, callus induction, proliferation, regeneration

INTRODUCTION

Wheat (*Triticum aestivum* L.) is a grass that is cultivated worldwide (Belderok and Donner, 2000). Wheat is a member of the family Poaceae, tribe Hordeae and placed in the genus *Triticu*m. It is an annual, long day and self-pollinated plant. Wheat occupies 70% of Rabi (winter season) and 37% of total cropped area in Pakistan. In NWFP, wheat is grown on 40% of total cropped area. Average yield of 2375 kg ha^{-1} in our country (Ministry of Food, Agriculture and Livestock (MinFAL), Annual Report, 2003-2004), however, is very low compared to world production of 2793 kg ha^{-1} in 1992 (Vasil, 1998). There are several species of *Triticum*. These species fall into three distinct groups, such as Diploids, Tetraploids and Hexaploids with 14, 28 and 42 chromosomes, respectively (Martin and Leonard, 1963). Globally, it is the most important human food grain and ranks second in total production as a cereal crop behind maize; the third being rice (Food and Agriculture Organization of the State (FAOSTAT) database of World Agriculture, 2006).

One of the important steps in application of biotechnology for crop improvement is the successful plant regeneration from cells, organs or tissues. Many investigations on tissue culture and plant regeneration in a variety of plants including wheat have been well documented. Plant regeneration from wheat tissue culture has mainly been obtained from callus derived from immature embryos. Callus induction from immature wheat embryos are commonly used for genetic transformation or tissue culture. Thus, first of all, for biotechnological research on wheat requires reliable callus induction and then plant regeneration. Especially, for an efficient genetic transformation, establishment of a successful wheat tissue culture system using immature or mature embryos is essential (Turhan and Baser, 2004). Tissue culture of dicots is simple as compared to monocots (Power and Cocking, 1976). The regeneration of whole plant is possible today from cereal species; such as maize (Duncan *et al*., 1985), rice (Yamada *et al*., 1986), barley (Luhrs and Lorz, 1987) and bread wheat (Redway *et al*., 1990). The first successful tissue culture in cereal crops was raised by La Rue (1949) from endosperm. Suspension cultures of wheat can be achieved by using a defined medium consisting of mineral salts containing sucrose, vit B and 2,4-D (Gamborg and Eleveigh, 1968). Callus formation and single cell cultures in wheat were developed by Shimada *et al*. (1969).

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Immature embryos in wheat have been reported as a good source of callus induction (Redway *et al*., 1990). However, immature embryo production is time-dependent procedure and difficult to obtain all seasons of the year. Mature embryos are thus an alternative explant for production of calli which can be used as target tissue for genetic transformation studies, mature embryo-derived calli also show a regeneration response comparable to that achieved from the calli initiated from immature embryos (Khurana *et al*., 2002). The maturity stage of explants is also important for embryogenic callus formation (Varshney *et al*., 1996). Therefore, this problem led the researchers to develop mature embryo derived callus. Although mature embryos from dried seeds are available at all times throughout the year, but the problem is low callus induction frequency. In order to overcome this problem, Ozgen *et al*. (1998) successfully used endosperm supported callus induction method from mature embryo cultures. He found that mature embryos had low frequency of callus formation but a high regeneration capacity as compared to immature embryos.

A less genotype dependent *in vitro* regeneration system capable of producing multiple shoot clumps and whole plants in four different genotypes of wheat were reported. All four genotypes responded positively to shoot multiplication depending upon media composition (Ahmad *et al*., 2002). The present study was designed to focus on the tissue culture conditions i.e., to look for the genotype, which is most responsive to callus induction and regeneration media. One of the objectives was to develop a reproducible regeneration system, which is essential for establishing *Agrobacterium* mediated transformation in wheat.

MATERIALS AND METHODS

Varieties of wheat (*Triticum aestivum* L.): Two varieties of wheat (*Triticum aestivum* L.), Fakhr-e-Sarhad and Gandam-2002 obtained from CCRI (Cereal Crop Research Institute, Pirsabak, Nowshera) were used in the present study for callus induction and callus proliferation. After successful callus induction and callus proliferation, these were then advanced to regeneration stage. All the experiments were conducted at the laboratory of Biotechnology University of Malakand from 1st September 2006 to 15th January 2007.

Preparation of tissue culture media: MS (Murashige and Skoog, 1962) media was used for callus induction, callus proliferation and regeneration in the present study. For callus induction, 2,4-D (2,4-Dichlorophenoxy acetic acid) a plant growth regulator was used alone in different concentrations. Where as for callus proliferation purpose the 2,4-D (2,4-Dichlorophenoxy acetic acid) and BAP (Benzyl aminopurine) were used alone in different concentrations, as well as a combination of 2,4-D and BAP were also used for the same purpose. For regeneration, varying ratios of IAA (Indol, 3-acetic acid) and BAP were used in combination, while Kn (Kinetin) was also used alone in different concentrations. This medium was modified with 3% sucrose (30 g L^{-1}) as an organic carbon source, required concentration of growth regulators and 1% (10 g L^{-1}) Agar, the solidifying agent. The pH of medium was adjusted as 5.7 and autoclaved on 121°C for 20 min at 15 PSI.

Explant preparation and sterilization: Mature seeds of the before mentioned wheat varieties were used as source of explant. For surface sterilization of plant material i.e., seed explants were soaked in 1% (w/v) mercuric chloride ($HgCl_2$) solution for 2-3 min. Before culturing, the seeds were washed 3-4 times with sterilized distilled water. All the operations of inoculation were carried out under strict aseptic conditions in a laminar airflow cabinet (Shah *et al*., 2003). After inoculation the flasks were transferred to growth room and were kept under 16 h light and 8 h dark photoperiod at 25±1°C.

Data analysis: The response of two wheat varieties to callus induction media was compared in terms of their callus induction frequencies. Response of both the varieties to callus proliferation media and regeneration media were also compared in the same way as callus induction. The frequencies of the callus induction, callus proliferation and regeneration were determined as the percentage of explants producing callus, percentage of calli proliferated and the percentage of calli producing fully regenerated plants, respectively.

RESULTS

Callus induction: Callus was induced invariably from mature seed explants and was visible with in one week (6-8 days). Five concentrations of the hormone 2,4-D were used to induce callus in the mature seeds: 2.5, 3, 3.5, 4 and 4.5 mg L^{-1}. After 21 days of inoculation the callus induction frequency were calculated as a percentage of explants producing calli for both of the varieties (Table 1). Calli of Fakhr-e-Sarhad and Gandam-2002 induced at 4 mg L^{-1} of 2,4-D and 3.5 mg L^{-1} of 2,4-D, respectively are shown in Fig. 1.

Callus proliferation: Callus was proliferated on all the three combinations of growth regulators used, which

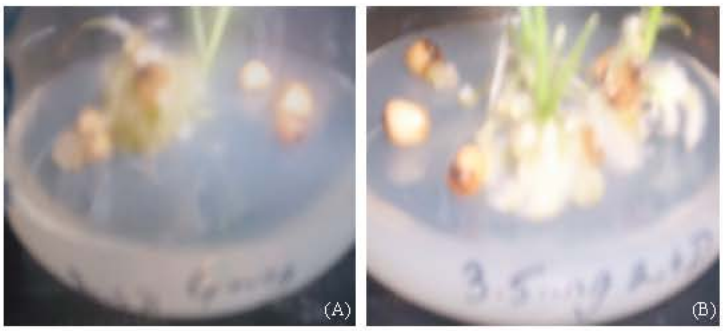

Fig. 1: The calli with axillary shoots induced from mature seed explants in both varieties of wheat (*Triticum aestivum* L.), (A) 21 days old callus of Fakhr-e-Sarhad induced at 4 mg L^{-1} of 2,4-D. (B) 21 days old callus of Gandam-2002 induced at 3.5 mg L^{-1} of 2,4-D

Fig. 2: A) Fully regenerated shoots with well developed roots of Fakhr-e-Sarhad after 22 days on 1:2 mg L^{-1} (IAA:BAP). B) Complete regenerated plantlets of Fakhr-e-Sarhad after 22 days on 3 mg L^{-1} (Kinetin). C) Complete regenerated plantlets of Gandam-2002 after 22 days on 1:2 mg L^{-1} (IAA:BAP) and D) Complete regenerated shoots with well developed roots of Gandam-2002 after 22 days on 3 mg L^{-1} (Kinetin)

were 2,4-D and BAP singly as well as both in combination (2,4-D:BAP). Both of the varieties gave different response to each combination of growth regulators used. Best callus proliferation was obtained on medium containing 4 mg L^{-1} 2,4-D (Table 2).

Regeneration: For regeneration of both varieties, IAA: BAP were used in combinations at a ratio 1:0.5, 1:1, 1:2, 2:0.5, 2:1 and 2:2 mg L^{-1}. Another growth regulator used for regeneration was Kinetin. The response of Gandam-2002 was higher as compared to Fakhr-e-Sarhad. Complete regeneration of shoots with well developed roots was observed after 3 weeks (22 days). Regeneration frequencies were calculated as a percentage of calli producing complete plantlets for both varieties (Table 3).

Gandam-2002 produced an average of 4 plantlets per callus, while Fakhr-e-Sarhad produced an average of 2 plantlets per callus on medium fortified with

Table 1: Comparison of callus induction frequencies of two varieties of wheat (*Triticum aestivum* L.) on MS medium containing different concentrations of 2,4-D

Conc. of 2,4-D* (mg L^{-1})	Frequency of callus induction (%)	
	Fakhr-e-Sarhad	Gandam-2002
2.5	-	7.5
3	31.25	45.0
3.5	46.87	62.5
4	29.68	50.0
4.5	-	12.5

*2,4-D (2,4-Dichlorophenoxy acetic acid), -(No callus induction)

Table 2: Comparison of callus proliferation frequencies of two varieties of wheat (*Triticum aestivum* L.) on MS medium containing different hormonal concentrations

Hormone	Hormonal concentrations: Present (mg L^{-1})	Hormonal concentrations: Previous (2,4-D) mg L^{-1}	Frequency of callus proliferation (%): Fakhr-e-Sarhad	Frequency of callus proliferation (%): Gandam-2002
2,4-D: BAP	1.5 : 0.5	4.0	62.50	50.00
	2 : 0.5	4.0	56.25	75.00
	2.5 : 0.5	3.0	12.50	31.25
BAP $^{\alpha}$	2	3.5	-	18.75
	3	3.5	81.25	93.75
	4	4.0	12.50	6.25
2,4-D*	3	3.5	37.50	43.75
	3.5	3.0	50.00	56.25
	4	3.5	87.50	95.31

*2,4-D (2,4-Dichlorophenoxy acetic acid), $^{\alpha}$BAP (Benzyl aminopurine), - (No proliferation), previous (concentrations of 2,4-D on which callus was induced), present (hormonal concentrations on which calli were placed for proliferation)

Table 3: Regeneration frequencies of two varieties of wheat (*Triticum aestivum* L.) on MS medium containing different hormonal concentrations

Hormone	Hormonal Conc. (mg L^{-1})	Regeneration frequencies (%): Fakhr-e-Sarhad	Regeneration frequencies (%): Gandam-2002
IAA : BAP*	1 : 0.5	-	10
	1: 1	-	20
	1 : 2	60	70
	2 : 0.5	20	-
	2 : 1	-	10
	2 : 2	-	20
Kinetin	2	-	-
	3	50	60
	4	10	-

* IAA:BAP (Indol, 3-acetic acid : Benzyl aminopurine), - (No regeneration)

1 mg L^{-1} IAA along with 2 mg L^{-1} BAP. On the other hand both varieties produced an average of 6 plantlets per callus on medium containing 3 mg L^{-1} Kinetin. The fully regenerated plantlets of both varieties are shown in Fig. 2.

DISCUSSION

Callus induction: Two varieties of wheat (*Triticum aestivum* L.), Fakhr-e-Sarhad and Gandam-2002 were tested for their callus production ability. In embryogenic wheat callus formation, 2,4-D seemed an effective growth regulator (Turhan and Baser, 2004). Therefore different concentrations of 2,4-D were used in the present study, to evaluate the callogenic response in seed explants of wheat (Table 1). The results obtained show that callus induction frequency was high at 3.5 mg L^{-1} 2,4-D, in both of the wheat varieties, i.e., Fakhr-e-Sarhad (46.87%) and Gandam-2002 (62.5%), after a culture period of 3 weeks. These results are supported by those of Shah *et al.* (2003) who obtained excellent callus induction at the same concentration of 2,4-D, using mature seed explants of wheat variety Lu-26S. On the other hand these results are contradictory to the findings of Satyavathi *et al.* (2004) who found 64.1% callus induction on MS media containing 2.5 mg L^{-1} 2,4-D in durum wheat (*Triticum turgidum* L.), however, they modified the MS media by adding an additional component i.e., Casein hydrolysate at the rate of 100 mg L^{-1}. The difference in the composition of culture medium as well as genotypic variation can lead to varying frequencies of callus induction (Torbet *et al.*, 1998).

Callus proliferation: The highest callus proliferation frequencies for both the varieties (95.31% for Gandam-2002 and 87.5% for Fakhr-e-Sarhad) was obtained on MS media containing 4 mg L^{-1} 2,4-D (Table 2). Present results are contradictory to those of Ogura and Shimada, (1978) who reported the calli maintenance of *Triticum aestivum* cultivar Chinese spring on 3 mg L^{-1} 2,4-D, under continuous fluorescent illumination. The slight difference in both results is possibly due to change in culture conditions provided and due to genotypic variations. In contrast to present findings Dornelles *et al.* (1997) proliferated calli of three wheat genotypes on MS medium containing 0.5 mg L^{-1} 2,4-D under complete darkness.

Excellent callus proliferation was observed in both varieties (93.75% for Gandam-2002 and 81.25% for Fakhr-e-Sarhad) on media containing 3 mg L^{-1} BAP (Table 2). In contrast Shah *et al.* (2003) and Tanzarella and Greco (1985) obtained best results on 4 and 5 mg L^{-1} BAP, respectively. The contradiction in results is due to varying genotypes as well as source of explants, as Shah *et al.* (2003) used *Triticum aestivum* var. Lu-26S while Tanzarella and Greco (1985) used immature embryos and shoot bases of *Triticum durum* as sources of explants.

The combined effect of Auxin (2,4-D) and cytokinin (BAP) was also observed. Callus proliferation was found 75 and 56.25% for Gandam-2002 and Fakhr-e-Sarhad, respectively on medium containing 2 mg L^{-1} 2,4-D and 0.5 mg L^{-1} BAP (Table 2). Present results are in agreement with those of Shah *et al.* (2003) who obtained good callus proliferation on medium containing 2 mg L^{-1} 2,4-D and 0.5 mg L^{-1} BAP. Vasil (1987) also reported similar results in cereals.

Regeneration: The highest regeneration frequency for both of the varieties (70% for Gandam-2002 and 60% for Fakhr-e-Sarhad) was obtained on MS medium containing 1 mg L^{-1} IAA in combination with 2 mg L^{-1} BAP (Table 3). These results are supported by those of Shah *et al.* (2003) who obtained plantlet regeneration in wheat calli on 1 mg L^{-1} IAA in combination with 2 mg L^{-1} BAP. Similar results were also reported by Varshney *et al.* (1996), who worked on immature embryos of *Triticum aestivum* and *Triticum durum*.

Using different concentrations of Kinetin, it was found that 3 mg L^{-1} Kinetin was effective for regeneration in wheat, as regeneration frequencies for both of the varieties obtained were 60% for Gandam-2002 and 50% for Fakhr-e-Sarhad (Table 3). These results are in agreement with those of Shah *et al.* (2003). In contrast Anju *et al.* (2003) failed to induce regeneration in wheat using Kinetin at all levels (0-5 mg L^{-1}). This contradiction in results is possibly due to alteration in the source of explant as they used shoot tips of wheat as source of explant instead of mature seeds.

From the results of this study it is concluded that Gandam-2002 was found to be better than Fakhr-e-Sarhad, but as a whole both of the varieties gave excellent response. The results of the present study are encouraging and show that there is a tremendous potential for transformation studies in both of the varieties.

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