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Optimization of Somatic Embryogenesis and *Agrobacterium*-mediated Transformation of Elite Wheat (*Triticum aestivum*) Cultivars of Pakistan

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Abstract

Immature embryos of twenty six wheat genotypes were regenerated on Murashige and Skoog (MS) medium supplemented with different levels of plant growth regulator (PGRs). 2, 4-D at 2 mg L^{-1} level was found to be the most favorable callus inducer. Bobwhite exhibited maximum embryogenic callus production followed by 'Ufaq-2000', 'Punjab-2011' and 'Sehar-2006'. Regeneration potential of selected wheat genotypes was evaluated on two PGRs indole-3-acetic acid (IAA) and kinetin (N6-furfuryladenine) in 4 different combinations. The auxin-cytokinin combination in IK4 (1 mg L^{-1} IAA and 1 mg L^{-1} Kinetin) media was the most promising for regeneration as variety Bobwhite presented the highest plantlet frequency. Immature embryos of cultivar 'Sehar-2006' were utilized for *Agrobacterium* mediated transformation and GUS activity was observed in callus to determine the expression pattern. Transgenic plants of 'Sehar-2006' were confirmed by PCR and Southern blotting. Transformation efficiency of 2.45 % was achieved which reinstated the viability of optimized regeneration system and this particular wheat genotype for utilization in crop improvement program. © 2014 Friends Science Publishers

Keywords: *Agrobacterium*; Callus; IAA; Kinetin; Regeneration, Transformation, Wheat

Introduction

Employing state of the art crop improvement techniques like biotechnology and genetic engineering is promising. Emphasis has been shifted for deployment of genetic modification systems in modern day crops for rapid and sustainable crop improvement. Plant tissue culture is the foremost tool in any plant transformation system (Debnath *et al*., 2006; Noor *et al*., 2009). Despite all the efforts, most cereal crop in particular, wheat has proven to be very tedious for regeneration of embryogenic callus (Fazali-nasab *et al*., 2012; Bouiamrine *et al.,* 2012).

The choice of regenerable and highly transformation efficient genetic material is very limited and confined to only a few specialized varieties like bobwhite. One way to overcome these bottle necks is to screen and utilize our indigenously adaptive and high yielding wheat cultivars for the purpose of exploring their regeneration potential and transformation efficiency. This will reduce the development period of a desirable transgenic material. Moreover, the exact concentration and combination of these auxins and cytokinins in the nutrient media are critical and play a decisive role in regeneration initiation particularly in crops like wheat (Saad *et al*., 2004; Zarif *et al.,* 2013).

This study describes a highly efficient callus induction, embryogenic callus production and plant regeneration system for local elite wheat cultivars. Immature embryos were utilized as explant and cultured on different levels and combinations of phytohormons to optimize the PGR concentrations and to establish best responsive wheat genotypes for *Agrobacterium* mediated transformation. This optimized high frequency callus induction and regeneration system will therefore provide ample embryogenic material for plant transformation and higher rates of plantlet regeneration for better transformation efficiency of local and exotic wheat genotypes.

Materials and Methods

Plant Material and Explant Preparation

Twenty six wheat genotypes including twelve commercial cultivars ('TD-1', 'Uqab-2000', 'SH-2002', 'AS-2002', 'GA-2002', 'GD-2002', 'Bhakkar-2002', 'Sehar-2006', 'Shafaq-2006', 'Inquilab-91', 'Lasani-2008', 'Punjab-2011'), seven old varieties ('Punjab-76', 'Chakwal-86', 'Pak-81', 'Watan', 'Blue Silver', 'TW-471' and 'Ufaq-2000'), six advanced breeding lines ('V-03079', 'V-04188', 'Millat-2011', 'V-07076', 'Galaxy-2013' and 'G-98-4') and one exotic highly regenerable check 'Bobwhite' were evaluated for regeneration potential. After 14-16 days post anthesis freshly removed seeds were surface sterilized with 70% ethanol for one min. After rinsing with sterilized distilled water, these seeds were washed with 20% Clorox bleach (5.25% Sodium hypochlorite) and 0.1% Tween 20 for 10 min. Seeds were thoroughly rinsed three times with sterile distilled water and excess water was removed by placing the seeds on sterile filter paper under aseptic conditions.

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Optimization of Callus Induction Media

For initiation of callus from immature embryos, MS basal medium (Murashige and Skoog, 1962) was supplemented with 40 g L^{-1} Maltose, 10 g L^{-1} glucose, 100 mg L^{-1} myo-inositol, 0.5 g L^{-1} glutamine, 100 mg L^{-1} casein hydrolysate and 2 g L^{-1} MES. For callus induction, three concentrations of 2, 4-D (2 mg L^{-1}, 3 mg L^{-1} and 4 mg L^{-1}) were added to above nutrient media and pH was adjusted at 5.8. To solidify the media, 5 g L^{-1} gellan gum powder (Phytotechnology, USA, Cat # G434) was added and autoclaved at 121°C for 20 min. Under aseptic conditions immature embryos (size 1-1.5 mm) were excised and placed on nutrient media scutellum side up whereas axis sides were kept towards the media surface. Hundred embryos per genotype in three replicates were cultured and placed at a controlled temperature of 25±2ºC in the dark.

Optimization of Regeneration Media

Ten best embryogenic callus producing wheat genotypes were taken for examining the regeneration potential and optimizing the auxin: cytokinin combination. For this purpose MS media was supplemented with 20 mg L^{-1} myo-inositol, 30 g L^{-1} maltose, 0.25 mg L^{-1} 2,4-D and four combinations of IAA and kinetin. Embryogenic calli of 6-7 mm size were divided into three equal pieces and plated in three replicates having 15 calli $plate^{-1}$ (45 calli $genotype^{-1}$) on four PGR combinations i.e., IK1 (0.5 mg L^{-1} IAA and 0.5 mg L^{-1} kinetin), IK2 (1 mg L^{-1} IAA and 0.5 mg L^{-1} kinetin), IK3 (0.5 mg L^{-1} IAA and 1 mg L^{-1} kinetin) and IK4 (1 mg L^{-1} IAA and 1 mg L^{-1} kinetin). The cultured plates were kept at 23 ± 2°C in diffused light under 14 h day and 10 h dark regime. After 4-6 weeks of sub-culturing, when globular structures and green spots started to appear on the callus, these plates were shifted to normal florescent light with 16 h light and 8 h dark photo period at 25-27°C.

***Agrobacterium* Strain, Plant Expression Vector and Explant Culturing**

A hyper virulent *Agrobacterium* strain AGL-1 harboring the plant expression vector *pCAMBIA2301* having *npt*II and *gusA* genes was utilized for the optimization of *Agrobacterium*-mediated transformation of 'Sehar-2006' wheat cultivar through co-cultivation of 14 days post anthesis immature embryos. These immature embryos were aseptically isolated and cultured on callus induction media for one week prior to transformation.

Inoculation, co-cultivation, Selection and Regeneration of Putative Transformants

Immature embryos plated on callus induction media were immersed in 15 mL *Agrobacterium* suspension media (OD_{600nm} ~ 0.5). After 45 min of co-cultivation with *Agrobacterium*, embryos were placed on sterile blotting paper to remove excess bacteria and incubated for 48 h in the dark on callus induction media supplemented with 400 µ*M* (w/v) acetosyringone (Phytotechnology Labs cat # A104) and semi-solidified with 2.5 g L^{-1} gellan gum powder (Phytotechnology Labs cat # G434) at a constant temperature of 25°C. After co-cultivation the inoculated embryos were subjected to two sub culturing cycles of 15 days each on callus induction media supplemented with 160 mg L^{-1} Timentin (15:1 Ticarcillin disodium to Potassium Clavulanate, cat # 42010012-4, bioplus, USA) for the suppression of *Agrobacterium*. Tissues indicating embryogenesis were shifted to IK4 regeneration media having 50 mg L^{-1} geneticin (plant selection) and 160 mg L^{-1} Timentin antibiotics. Regenerated plantlets surviving the selection medium were shifted to half strength MS media whereas, plantlets with 1-2 inch long roots were further transferred to small plastic pots containing Coco peat and compost in 3:1 ratio.

Histochemical Assay for *β*-glucuronidase (GUS) Activity

Transient histochemical GUS assay was performed to check the *gusA* gene expression in transformed wheat calli and plantlets in accordance with the method described by Jefferson *et al*. (1987). Prior to use the GUS solution was filter sterilized and stored in a tinted bottle at -20°C. Sixty randomly selected calli were placed in 10 mL X-Gluc (5-bromo-4-chlloro-3-indoyl-*β*-D-glucuronide) solution and incubated at 37°C overnight. Non transformed calli were kept as check to monitor false positive expression. GUS staining solution was discarded and replaced with 80% (v/v) ethanol to stop the reaction. The GUS activity in the form of blue spots was observed under stereo microscope.

Polymerase Chain Reaction and Southern Blot Analysis

Wheat genomic DNA from putative transgenic plants was isolated by a high throughput DNA extraction protocol described by Xin and Chen (2012). PCR amplification of 750 bp fragment of *npt*II gene was used for the screening of putative plantlets. The sequences of *npt*II specific primers were: npt-F: 5´-TCGGCTATGACTGGGCACAACAGA-3´ and npt-R: 5´-AAGAAGGCGATAGAAGGCGATGGG-3´. The following PCR profile was used; initial DNA denaturing by running 1 cycle at 94°C for 5 min followed by 35 cycles of denaturing at 94°C for 1 min, primer annealing at 58°C for 1 min and 1 min at 72°C for primer extension. These steps were followed by a final extension at 72°C for 10 min. Southern blot hybridization was performed for checking the gene integration and copy number analysis by following the protocol described by Sambrook and Russell (2001). For this purpose 30 µg of restricted genomic DNA was used while blot was hybridized with 450 bp long *npt*II specific

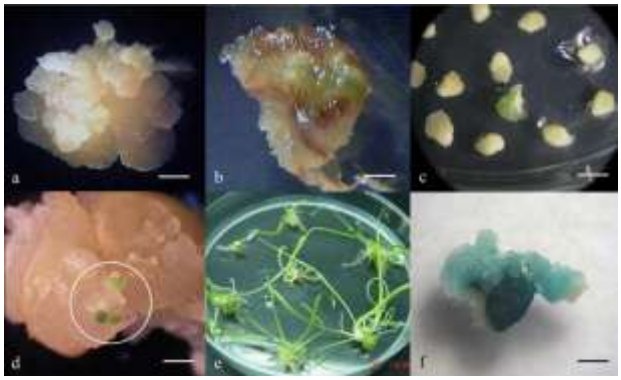

Fig. 1: Various stages during wheat tissue culturing and transformation: (a) Embryogenic callus (b) Non-embryogenic callus (c) Callus induction from immature embryos at MS media supplemented with 2 mgL^{-1} 2,4-D (d) green spot initiation from globular structures on embryogenic callus (e) profuse regeneration from embryogenic callus at IK4 regeneration media (f) GUS stained explant transformed with *pCAMBIA2301* vector. *Scale bars*: **a** = 0.5 mm; **b** = 0.5 mm; **c** = 1 mm; **d** = 2 mm; **f** = 1 mm

Table 1: Mean square values of two way ANOVA for callus induction and regeneration potential of various wheat genotypes at relevant optimized nutrient media

Source of variation	DF	Callus induction	DF	Regeneration potential
Genotype	25	1767.6**	9	28.8370**
Treatment	2	31384.1**	3	70.4444**
Genotype × Treatment	50	130.4**	27	1.1975**
Error	156	15.0	80	2.6833
Total	233		119	

**=Significant at P≤0.05 probability

probe generated with the help of DIG Probe Synthesis Kit (Roche Applied Science, Germany, cat # 11636090910) and chemiluminescent detection through DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Science, Germany, cat # 11585614910).

Statistical Analysis

This experiment was designed in complete randomize design (CRD). Callus induction experiment was performed in three replicates with 100 embryos $genotype^{-1}$. Similarly regeneration potential experiment was conducted in three replications and each replicate contained 15 calli pieces per genotype. Statistical analysis were performed through analysis of variance (ANOVA) using the Statistix software v8.1. Mean and standard deviations were descriptive measures of quantitative data using the analysis of variance for independent samples. P-values of P≤0.05 were considered as significant.

Results

Response of Wheat Genotypes to Callus Induction Media

Callus of 'Punjab-76', 'Watan', 'GA-2002', 'GD-2002', 'V-3079', 'V-04188', 'Uqab-2000', 'Sehar-2006', 'Punjab-2011' and 'Bobwhite' was more crystalline, compact and embryogenic in nature (Fig. 1a) whereas it was mostly brownish, loose, semi-translucent and non-embryogenic in 'TD-1', 'SH-2002', 'AS-2002', 'Bhakkar-2002', 'Shafaq-2006', 'Inquilab-91', 'Lasani-2008', 'Chakwal-86', 'Pak-81', 'Blue Silver', 'TW-471', 'Millat-2011', 'V-07076', 'Galaxy-2013' and 'G-98-4' (Fig. 1b). Analysis of variance of mean callus induction of all wheat genotypes (Table 1) showed highly significant differences among the tested genotypes and given treatments (P≤0.05). Overall highest callus induction was observed on MS media supplemented with 2 mg L^{-1} 2,4-D (Fig. 1c). Bobwhite showed maximum callus induction rate of 92% followed by 'Ufaq-2000' and 'Sehar-2006' with 88 and 85 % respectively (Fig. 2a). On the other hand, MS medium supplemented with 3 and 4 mg L^{-1} 2,4-D gave comparatively lower callus induction rates for all the genotypes (Fig. 2b, 2c).

Embryogenic Callus Production

On the basis of best callus inducing and proliferating media, calli plated on MS media supplemented with 2 mg L^{-1} 2,4-D were chosen for further experimentation and data analysis for embryogenic callus production. Callus production ranged from 15-74% while maximum embryogenic callus frequency of 74% was observed in Bobwhite followed by 71% in Ufaq-2000, 69.7% in 'Punjab-2011' and 67.7% in 'Sehar-2006' (Fig. 2d).

Plant Regeneration

Ten best embryogenic callus producing wheat genotypes (Fig. 2d) were further investigated for superior regeneration ability. Analysis of variance of mean regeneration rate of all ten wheat genotypes showed significant differences (P≤0.05) among the tested genotypes and given treatments (Table 1). On IK4 media, significant gains in regeneration frequency were observed for all the cultured calli (Fig. 1e). At this auxin-cytokinin combination, wheat variety Bobwhite showed maximum regeneration potential of 88.9% while cultivars 'Ufaq-2000' and 'Sehar-2006' showed 80% regeneration capacity (Fig. 3d). Whereas, on 'IK1', 'IK2' and 'IK3' media, the phytohormone combinations were unable to significantly increase in the frequency of green spot appearance on cultured calli (Fig. 3a, 3b, 3c).

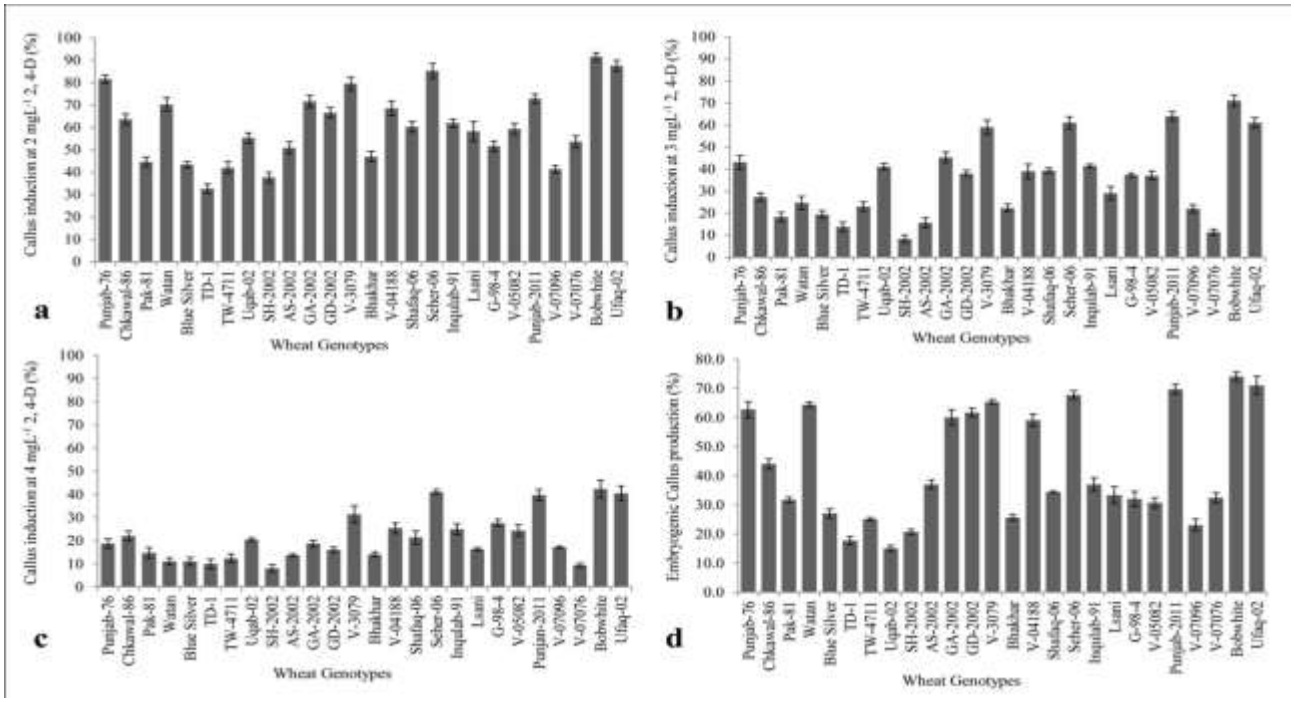

Fig. 2: Callus induction response of 26 wheat genotypes at MS media supplemented with (a) 2 mgL^{-1} 2, 4-D (b) 3 mgL^{-1} 2, 4-D (c) 4 mgL^{-1} 2, 4-D (d) Embryogenic callus production frequency of 26 wheat genotypes on 2 mgL^{-1} 2, 4-D auxin

Table 2: Transformation efficiency of wheat cv. Seher-2006 transformed by *Agrobacterium* mediated transformation having *pCAMBIA2301* plasmid.

Batch	Total explants cultured	Explants checked for GUS expression	GUS positive explants	Total regenerated plantlets	Plantlets survived on selection medium	PCR Amplification of selection survived plantlets	Southern Blot analysis of PCR +ve plantlets	Transformation efficiency (% age)
						nptII +ve	*nptII* +ve	
1	100	20	15	68	5	1	1	1.47
2	100	20	12	61	4	2	2	3.28
3	100	20	11	77	7	2	2	2.60
Total	300	60	38	186	16	5	5	2.45

Agrobacterium Mediated Transformation of Wheat Explants

Fourteen days after anthesis immature embryos of wheat cv. Seher-2006 were inoculated and co-cultivated with AGL-1 strain of *Agrobacterium tumefaciens* harboring *pCAMBIA2301* vector for expression of GUS gene. Wheat cv. 'Sehar-2006' responded favorably in terms of number of immature embryos showing GUS activity in the form of blue colored GUS foci (Table 2). GUS gene expression was visible as blue colored patches on embryogenic calli (Fig. 1f). Out of 60 calli tested for GUS activity, 38 showed varying degrees of expression in the transformed explants (Table 2).

After GUS expression analysis of 60 selected explants, the remaining 240 immature embryos were able to produce 108 plantlets on IK4 regeneration media. After 3 selection cycles on Geneticin, 16 plantlets were able to survive and produced roots in cultured plates and jars as indicated in Table 2.

Molecular Analysis for Transgenic Plants

Sixteen putative transgenic plants having normal phenotypes were further tested for gene integration and copy number through PCR and Southern blot hybridization. An amplification of 750 bp DNA fragment representing *npt*II gene was observed in 5 transgenic plants by employing *npt*II gene specific primers while non-inoculated wild type plants showed no amplification (Fig. 4a). Out of 16 putative transgenes, 5 were found positive for *npt*II gene and mean transformation efficiency of 2.45% was observed (Table 2). Southern blot hybridization of these five PCR positive T_0 transgenes with *npt*II gene specific DIG labeled probe indicated the presence of single insert in transgenes P1, P2, P4 and P5 while in case of P3, two copies of *npt*II

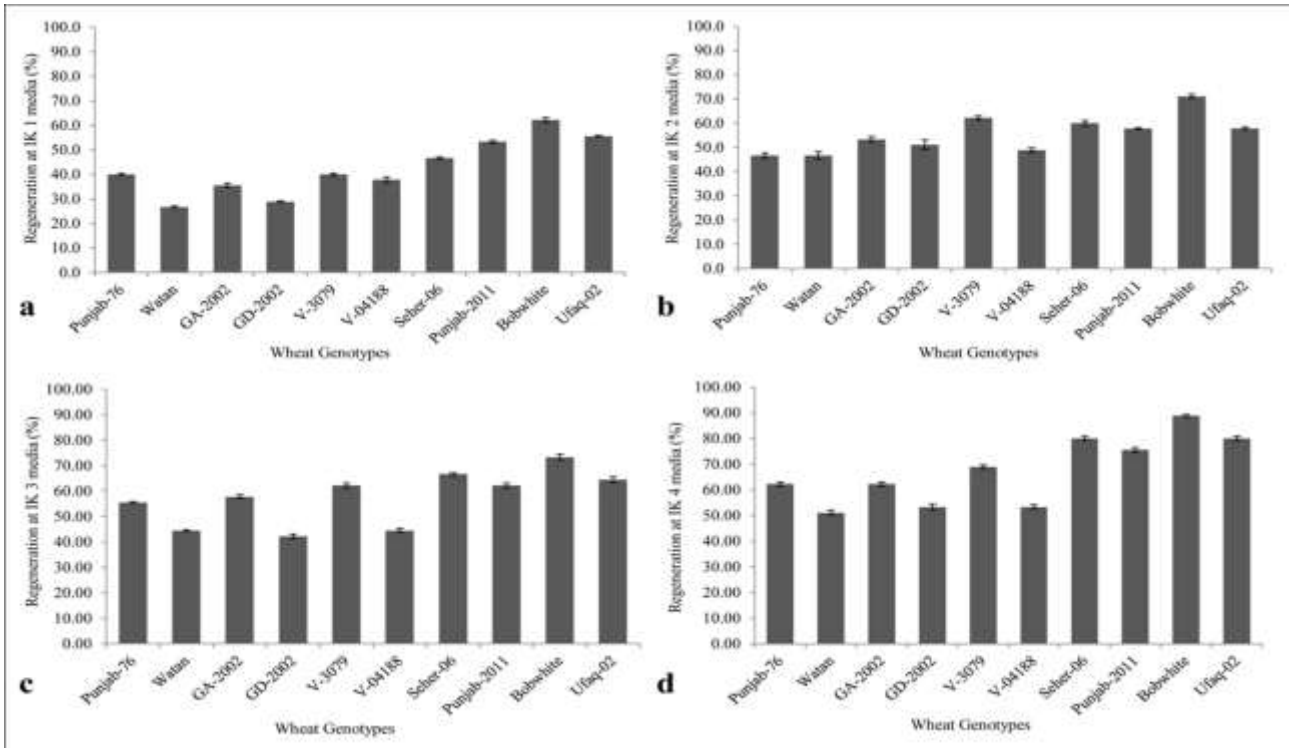

Fig. 3: Regeneration potential of wheat genotypes on MS media supplemented with 4 auxin-cytokinin combinations: (a) Regeneration at IK1 (0.5 mg L^{-1} IAA and 0.5 mg L^{-1} kinetin) (b) IK2 (1 mg L^{-1} IAA and 0.5 mg L^{-1} kinetin) (c) IK3 (0.5 mg L^{-1} IAA and 1 mg L^{-1} kinetin) (d) IK4 (1 mg L^{-1} IAA and 1 mg L^{-1} kinetin)

gene were evident from hybridized blot as illustrated in Fig. 4b.

From these results, it was concluded that MS medium supplemented with 2 mg L^{-1} 2,4-D was the best medium for callus induction of wheat varieties while MS medium supplemented with 1 mg L^{-1} IAA and 1 mg L^{-1} kinetin was the best medium for the promotion of regeneration. *Agrobacterium* mediated transformation proved useful for the integration of low gene copy number into high value commercial wheat cultivars.

Discussion

Tissue culturing media plays a vital role for callus formation and proliferation especially in cereals (Wang and Wei, 2004) whereas, 2,4-D is known to be a vital phytohormone for *in vitro* cell proliferation as it has been proved that nutrient media devoid of 2,4-D can result in false plantlet formation from cultured embryos without callus initiation (Raja *et al*., 2008). In this study, multiple doses of 2,4-D were employed to find out the best callus inducing concentration and the highest callus producing wheat genotype. According to the results, 2 mg L^{-1} 2,4-D proved to be the optimum level for achieving the callus induction for most of the cultured wheat genotypes. Increase of 2,4-D concentration showed a sharp decline in callus proliferation. It is worth mentioning that in case of mature wheat seed, much higher 2,4-D levels are required for callus induction from mature embryos (Raja *et al*., 2008). In contrast, Haliloglu (2002) observed that in immature embryos, at lower 2,4-D concentration of 1 mg L^{-1}, premature germination and root development can occur, which is also highly detrimental for embryogenesis.

As far as callus quality was concerned, all ten selected wheat genotypes showed preference for MS media supplemented with 2 mg L^{-1} 2,4-D. Higher the auxin level, lower the quality and quantity of embryogenic callus. There was a correlation between higher callus induction and higher somatic embryogenesis as genotypes with maximum callus production frequency ('Bobwhite', 'Seher-2006' and 'Ufaq-2000') were also better embryogenic callus producers (Fig. 2a).

In a plant transformation system, the worth of any genotype is judged by its ability to regenerate into viable plantlets (Jones, 2005). Regeneration from plant tissues can be achieved in any tissue culture system by an appropriate balance of auxin-cytokinin combination (Raja *et al*., 2008). Therefore four different combinations of auxin (IAA) and cytokinin (kinetin) were formulated for achieving highest regeneration frequency from tested wheat genotypes. The

vitality of these phytohormones has also been reinstated by Yadav *et al*. (2000) who reported maximum of 19 regenerated shoots from a wheat genotype 'UP2338' when plated on MS media supplemented with both these PGRs.

In the current study, a combination of 1 mg L^{-1} IAA and 1 mg L^{-1} kinetin was found paramount for achieving maximum regeneration frequency from tested wheat varieties and lines. Nayal *et al.* (2002) demonstrated plantlet formation in a wheat variety using 0.5 mg L^{-1} kinetin and 1.5 mg L^{-1} IAA which is in contrast to our results. Similarly quite contrasting findings were reported by Noor *et al.* (2009) where they obtained 62.2 to 80.5 % regeneration from wheat on a hormonal combination of 0.1 mg L^{-1} IAA + 0.5 mg L^{-1} kinetin. Another study suggested wheat variety Kohsar-95 to be the best regenerator on IAA-kinetin combination of 1.0 mg L^{-1} IAA + 0.1 mg L^{-1} Kinetin (Raja *et al.*, 2008). Furthermore, Rahman *et al*. (2008) suggested that MS media supplemented with 1 mg L^{-1} kinetin alone was enough to induce maximum regeneration of wheat varieties. The reason for contrasting results may be due to the difference in the callus age, genotypes and dissimilar culturing media and conditions. In addition, regeneration media in this study was continuously supplemented with 0.25 mg L^{-1} 2,4-D which enables the simultaneous production of fresh callus and regeneration and this incessant supply of embryogenic callus may have a profound effect on the final count of regenerated plantlets.

Optimization of callus induction and plant regeneration from immature embryos is highly desirable for *Agrobacterium* mediated transformation of high yielding wheat varieties. Therefore it was vital to check the transformation efficiency of selected wheat cultivar Seher-2006. Hyper-virulent AGL-1 strain of *Agrobacterium* having monocot specificity was especially employed to enhance the infection rate. *pCAMBIA2301* vector harboring *gusA* gene was exploited for evaluating the scope and efficiency of the devised transformation system. T_0 putative transformed plants exhibited varying levels and intensities of *β*-glucuronidase activity. The variation in GUS expression may be the result of arbitrary gene integration and nucleotide rearrangements in the genome of transformed T_0 plants. The *β*-glucuronidase activity, PCR analysis and high frequency of single gene insert in Southern blot analysis indicated stable transformation of *gusA* and *nptII* genes. On the basis of these molecular parameters, impressively higher transformation efficiency of 2.45% was achieved form immature wheat embryos. These results suggested the feasibility of utilization of Seher-2006 variety for high efficiency *Agrobacterium* mediated transformation for routine genetic transformation experiments.

In conclusion, here we reported a unique auxin-cytokinin blend which has not been reported earlier for the improvement of wheat regeneration potential. These results clearly indicated that Bobwhite which is highly renowned for its callusing and regeneration potential, proved to be best performer out of all genotypes tested. But the auxin and auxin-cytokinin combinations and exploitation of the inbuilt genetic potential devised in this study enabled our local high yielding but genetically diverse commercial cultivars like Seher-2006 and Punjab-2011 to perform at their full embryogenic and morphogenic potential. Currently wheat tissue culturing and genetic transformation is confined to a very small number of selective but non-adaptive regenerable genotypes that are not suited to grow in our environment. However, higher somatic embryogenesis and transformation frequency of wheat explants through *Agrobacterium* mediated transformation of cv. Seher-2006 has reiterated the feasibility of utilization of this optimized transformation protocol for more adaptive and agronomically valuable genetic material for wheat crop improvement through genetic engineering.

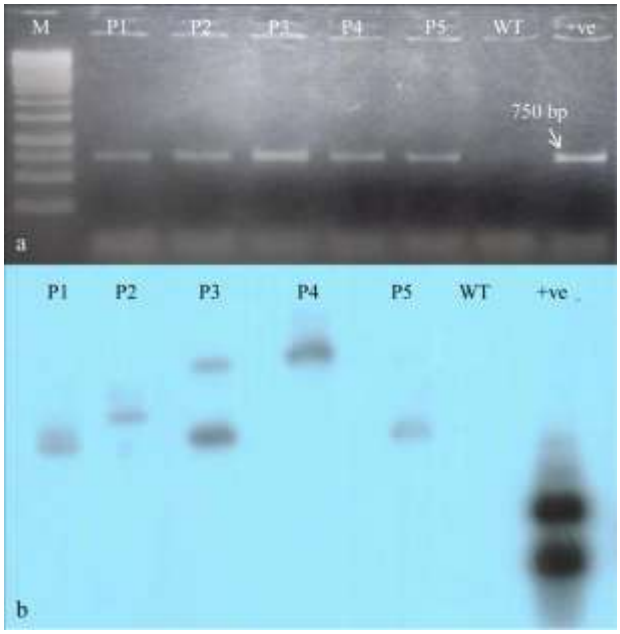

Fig. 4: Molecular confirmation of genetic transformation of cv. Seher-2006: (a) PCR amplification of 750 bp size DNA fragment of representative plants of cv. Seher-2006 with *npt*II gene specific primers (b) Southern blot hybridization of transformed wheat plants

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