INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
16–0748/2017/19–5–969–975
DOI: 10.17957/IJAB/15.0324
http://www.fspublishers.org

Full Length Article

Improving Regeneration from Mature Embryo Derived Callus in Wheat (*Triticum aestivum*) by Pre- and Post-callus Induction Treatments

Usman Saeed[1], Iqrar Ahmad Rana[1*], Siddra Ijaz[1] and Zulfiqar Ali[2]
[1]*Centre of Agricultural Biochemistry and Biotechnology (CABB), University of Agriculture, Faisalabad-38040, Pakistan*
[2]*Department of Plant Breeding and Genetics, University of Agriculture, Faisalabad-38040, Pakistan*
[*]For Corresponding: Iqrar_rana@uaf.edu.pk

Abstract

Immature embryos are preferred explant source to get regeneration frequency compatible for genetic transformation in wheat. Under natural conditions, immature embryos can be obtained only once a year. Either controlled conditions to grow wheat in off season should be developed or alternative explant should be optimized. To develop mature embryos as explant, two commercial wheat varieties "AARI-11" and "Galaxy-13" were used for the development of regeneration system. Seeds were imbibed with five different concentrations of 2,4-D for 24 h. Embryos were isolated from imbibed seeds, subjected to seven callus induction media (CIM) containing different commercial auxins, and regeneration was achieved on two regeneration media (RM). Effects of genotypes, 2,4-D in imbibition solutions, CIM, and their interactions were studied. Imbibition produced significant effects on callus induction and embryogenesis. Imbibition solution with 8 mg/L 2, 4-D and CIM4 (2,4-D+Dicamba as growth regulator) was found to be most favorable combination for callus induction, and embryogenesis. Both regeneration media responded excellent for regeneration with 81.10% and 80.62% regeneration frequency. Our results also showed that this is not only the regeneration media but also the genotype, CIM and imbibition solutions that played their role for embryogenic callus induction and regeneration. To further improve regeneration the effects of starvation stress, and extended time at CIM were analyzed for embryogenesis induction. The best embryogenesis was observed after 3 weeks of stress and 9 weeks old calli maintained on CIM through sub-culturing.

Keywords: Wheat; Mature embryo; 2,4-D; Callus induction; Stress; Regeneration

Introduction

Being recalcitrant for *in vitro* regeneration, wheat has been a challenging cereal crop for plant biotechnologists (Bhalla, 2006; Ganeshan *et al.*, 2006; Vasil, 2007). Immature embryos and immature inflorescence are the most preferably used explants for regeneration as well as genetic transformation studies (Jones, 2005; Chauhan *et al.*, 2007). However, immature tissues need more labor and expenses in maintaining the plant source. Moreover, the most suitable time for culturing immature tissues also limits their use (Repellin *et al.*, 2001). In contrast, the use of mature embryos has been quite easy, compared to immature embryos because of their ease in isolation and their availability round the year (Chugh and Khurana, 2003; Patnaik *et al.*, 2006; Ding *et al.*, 2009). Additionally, the physiological state of mature embryos shows minimum variation (Yu *et al.*, 2008). Many scientists have used mature embryos in multiple ways, like; endosperm-supported embryo (Ozgen *et al.*, 1998; Filippov *et al.*, 2006), thin mature embryo fragments (Delporte *et al.*, 2001; Mendoza and Kaeppler, 2002), longitudinally bisected mature embryos (Yu *et al.*, 2008) and pretreatment of embryos with moderate to high levels of 2, 4-D before culture or during culture for callus induction and plantlet regeneration. Since, it has been proven that high levels of 2, 4-D cause somaclonal variation, so the protocols with low levels of auxins have been recommended (Mendoza and Kaeppler, 2002). Moreover, the effects of various factors, such as genotype (Ozgen *et al.*, 1996, 1998; Zale *et al.*, 2004), type and concentration of different auxins (Mendoza and Kaeppler, 2002; Filippov *et al.*, 2006) and components of media (Redway *et al.*, 1990; Mendoza and Kaeppler, 2002; Greer *et al.*, 2009) either solely or in combinations has been assessed for callus induction and plant regeneration. Despite these attempts, low frequency of regeneration has been found (Ozgen *et al.*, 1998; Delporte *et al.*, 2001; Mendoza and Kaeppler, 2002), which is again genotype dependent (Zale *et al.*, 2004; Filippov *et al.*, 2006; Yu *et al.*, 2008). Among the important factors, which has not been well under consideration is the appearance of non-embryogenic callus along with embryogenic callus in regeneration from mature embryos (Redway *et al.*, 1990; Delporte *et al.*, 2001; Mendoza and Kaeppler, 2002). Mac

To cite this paper: Saeed, U., I.A. Rana, S. Ijaz and Z. Ali, 2017. Improving regeneration from mature embryo derived callus in wheat (*Triticum aestivum*) by pre- and post-callus induction treatments. *Int. J. Agric. Biol.*, 19: 969–975

Kinnon *et al.* (1987) showed that different parts of mature embryo vary in their capability to generate embryogenic calli. Therefore, it is inevitable to clearly identify embryogenic callus from non-embryogenic callus to obtain maximum and efficient plantlet regeneration. Redway *et al.* (1990) demonstrated that non-embryogenic calli can be distinguished from embryogenic calli based on morphology and colour and are normally removed during subculture. However, this distinction based on morphology and colour is not always clear when callus become aged. Furthermore, colour of callus is also dependent on genotype as well as type of auxins used for callus induction. In this study, we present a novel, highly reproducible, efficient and cost effective approach for best callus induction and regeneration from mature embryo, for two commercial wheat varieties viz AARI-11 and Galaxy-13, and how to minimize the chances of non-embryogenic callus during culturing and sub culturing. In this study, the effects of different auxins; 2, 4-D, Dicamba and Picloram alone and in combinations have been analyzed for embryogenic callus induction and plant regeneration. Further we also used extended period for improving callus quantity and provided starvation stress to improve morphogenesis. Once the regeneration is optimized, agrobacterium transformation can be employed on induced calli.

Materials and Methods

Imbibition of Explant with 2, 4-D Solutions

Seeds of commercial wheat cultivars "AARI-11" and "Galaxy-13" were obtained from Ayub Agricultural Research Institute (AARI), Faisalabad, Pakistan. Mature seeds were surface sterilized with 0.1% (w/v) aqueous Mercuric Chloride by adding 2–3 drops of Tween-20, followed by 20 minutes of incubation in a thermal shaker at room temperature. Then seeds were washed with autoclaved distilled water 4–5 times, and soaked in aqueous solutions of 2, 4-D at 5 different levels (2–10 mg/L) along with control which was autoclaved distilled water. Seeds were incubated at 30°C in thermal shaker overnight for proper imbibition. Mature embryos were excised with a pair of forceps and fine scalpel under aseptic conditions in laminar airflow chamber. The excised embryos were cut horizontally into two halves with coleoptile completely removed (Fig. 1), to minimize the chance of shoot formation during callus induction and proliferation (Modified from Delporte *et al.*, 2001). The dissected embryos were placed on callus induction media in 9 mm petri plates.

Callus Induction Media

A simple MS (Murashige and Skoog, 1962) medium without vitamins containing micro and macro salts (ready to use), sucrose (30 g/L), combinations of three auxins; 2, 4-D, Dicamba (3,6 dichloro-o-anisic acid), and Picloram (4-amino-3,5,6-trichloropicolinic acid), in 0.4% gellan gum were used for callus induction (Table 1). pH 5.7–5.8 for all the media combinations was adjusted with the help of KOH and HCl. All the media were sterilized by autoclaving at 121°C for 21 min and 15 psi. Mature embryos were excised aseptically and shifted to callus induction media which later shifted to dark at 25±2°C for 2 weeks. The cultured embryos were checked for pseudo callus and off shoots regularly. A few shoots and pseudo calli (Fig. 2) were observed and immediately removed with forceps and scalpel under aseptic conditions during sub culturing.

Plantlet Regeneration

For regeneration, 8–10 weeks old highly embryogenic calli were transferred to 2 regeneration media containing MS basal salts, sucrose and 0.1 mg/L 2, 4-D termed RM1 (Rana *et al.*, 2012) and MS basal salts, sucrose, 0.1 mg/L 2, 4-D and 5 mg/L Zeatin, termed as RM2, (Ding *et al.*, 2009) solidified with 0.4% gellan gum under 16/8 h light/dark period. Calli were sub cultured every 15 days and regenerated shoots were transferred to ½ MS with no growth regulators for root development. Further, best stage of callus age for regeneration was determined statistically in which calli of different ages, such as (4–10 weeks old), were used for obtaining best plantlet regeneration. Calli were also subjected to starvation stress by stopping subculture to fresh medium after 15 days and exposed in the same medium for at least 3–4 weeks and the effects were evaluated statistically.

Hardening

Efficiently developed plants were taken out of the jars and Magenta boxes carefully, washed with distilled water and immediately shifted to small pots containing compost. The pots were carefully covered with clear polythene bags for proper humidity. The pots were kept in light at 25–28°C under growth room conditions. Then acclimatized plants were transferred to normal greenhouse conditions (Rana *et al.*, 2012).

Statistical Analysis

All the experiments were repeated thrice until stated otherwise. Mature seeds were soaked in different levels of 2, 4-D overnight. Embryos were isolated and cultured on each CIM with 40 explants in each Petri plate. Callus mass was recorded for each replication for 8–10 weeks and their mean was taken. The best CIM was chosen on the basis of individual callus mass data and greater number of embryogenic calli. Afterwards, calli from each CIM were shifted to RM1 and RM2 and regeneration efficiency was calculated (number of calli showing regeneration/total number of calli x 100).

Fig. 1: Best stage for embryo excision. (1a), excised embryo (1b), embryo with coleoptile removed (1c), embryo cut horizontally into two halves (1d)

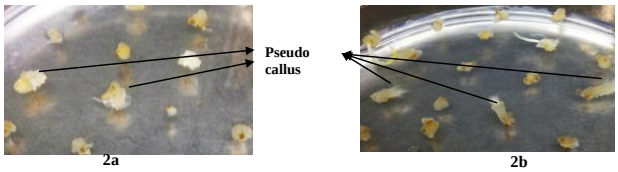

Fig. 2: True callus along with pseudo callus

Factorial Analysis using ANOVA and LSD test at 5% significance level was carried out to determine the sole effect of each treatment and their interactions, both on callus induction and regeneration.

Results

Callus Induction (Mass Calculated in Terms of Grams)

For callus induction, seeds were washed and imbibed overnight at 5 different levels of 2, 4-D solutions prior to embryo excision. The embryos were isolated from imbibed seeds and cultured on seven CIM. The data were taken on callus mass induced per explant cultured. The analysis of variance showed highly significant effects of imbibition treatment, and interaction of imbibition with genotypes, on callus induction. The impact of genotypes, CIM, and interaction of CIM into genotypes and the interaction of three factors was non-significant.

Effects of Imbibition Solutions on Callus Induction

As compared to control (imbibition with H_2O), the use of different levels of 2, 4-D for imbibition showed significant effects. The embryos isolated from seeds imbibed with distilled water induced at 0.099 g per explant cultured which was statistically same from 2, 4-D treatments of 2, and 4 mg/L. The callus induction increased slightly at 2 mg/L and 4 mg/L but the increase was statistically same to control. At 6 mg/L and 8 mg/L, the response was statistically different and it resulted in increased callus mass. The trend of increased callus mass shown by both the genotypes and the highest response was seen at 8 mg/L and 10 mg/L with an average of 0.133 g per explant and was statistically similar to the response at 6 mg/L (0.124 g). The mean value of callus induction for Galaxy-13 was the higher at 10 mg/L (0.137 g), while for AARI-11 it was at 8 mg/L (0.129 g) (Table 3).

Table 1: Composition of callus induction media

	CIM1	CIM2	CIM3	CIM4	CIM5	CIM6	CIM7
MS salt (g/L)	4.33	4.33	4.33	4.33	4.33	4.33	4.33
Sucrose (g/L)	30	30	30	30	30	30	30
2,4-D (mg/L)	2	_	_	1	1	_	1
Dicamba (mg/L)	_	2	_	1	_	1	1
Picloram (mg/L)	_	_	2	_	1	1	1
Gellan Gum (%)	0.4	0.4	0.4	0.4	0.4	0.4	0.4

Table 2: Media composition for plantlet regeneration

	RM1	RM2
MS salt (g/L)	4.33	4.33
Sucrose (g/L)	30	30
2,4-D (mg/L)	0.1	0.1
Zeatin (mg/L)	_	5
Gellan gum (%)	0.4	0.4

Table 3: Effects of imbibition on callus induction calculated in terms of grams

Treatment	Genotype/Variety		Mean
	Galaxy-13	AARI-11	Callus mass
Control	0.092±0.004c	0.105±0.001bc	0.099±0.002B
2 mg/L 2,4-D	0.100±0.003c	0.103±0.002bc	0.102±0.002B
4 mg/L 2,4-D	0.105±0.002bc	0.107±0.002bc	0.106±0.001B
6 mg/L 2,4-D	0.128±0.005a	0.119±0.004ab	0.124±0.003A
8 mg/L 2,4-D	0.136±0.005a	0.129±0.004a	0.133±0.003A
10 mg/L 2,4-D	0.137±0.005a	0.128±0.003a	0.133±0.003A
Mean for callus mass	0.116±0.002A	0.115±0.001A	

Means sharing similar letter in a row or in a column are statistically non-significant (P>0.05). Small letters represent comparison among interaction means and capital letters are used for overall mean. Capital letters show effects individual treatments while small letters show impact of interactions

Table 4: Effects of genotypes and imbibition on embryogenesis

Treatment	Genotype/variety		Mean
	Galaxy-13	AARI-11	
Control	62.96±1.05g	59.81±1.14g	61.38±0.81E
2 mg/L 2,4-D	69.77±1.09f	79.32±0.95d	74.54±1.03D
4 mg/L 2,4-D	74.01±0.62e	85.10±0.95bc	79.56±1.03C
6 mg/L 2,4-D	81.25±1.21cd	81.61±0.98cd	81.43±0.77C
8 mg/L 2,4-D	93.39±0.64a	88.35±0.88b	90.87±0.66A
10 mg/L 2,4-D	87.68±0.83b	86.02±1.10b	86.85±0.69B
Mean	78.18±1.00B	80.03±0.94A	

Means sharing similar letter in a row or in a column are statistically non-significant (P>0.05)

Embryogenic Callus Induction (Embryogenesis Calculated in Terms of Percentage)

The callus induction studies showed that imbibition with 2, 4-D increases callus mass. When it was tried to see the embryogenesis in the induced calli and to differentiate callus clumps with embryoids, more pronounced impact of the imbibitions was observed. The calli induced from embryos without 2, 4-D imbibition showed the least number of embryoids with average percentage of 61.38%. For embryogenic efficiency

genotype, imbibition, CIM and genotype x imbibition interaction had highly significant effects. Galaxy-13 showed a highest mean value of 93.39% and AARI-11 of 88.3% at imbibition level of 8 mg/L of 2, 4-D (Table 4). As far as CIM are concerned, CIM4 showed the highest overall mean value of 83.04% (Table 5) for both the genotypes, although, the genotype x CIM interaction for the embryogenic efficiency was non-significant. On each imbibition level of 2, 4-D, CIM4 produced best embryogenesis for both varieties and on each CIM, imbibition level of 8 mg/L 2, 4-D produced best embryogenesis (Tables 4 and 5). Thus, for further studies imbibition level of 8 mg/L 2, 4-D and CIM4 were used.

Regeneration Studies

Calli from each CIM were shifted to RM1 and RM2. Analysis of variance showed significant effects of genotype and highly significant effects of CIM on regeneration, while RM had no significant effects which clearly depicted that regeneration was dependent on genotype and quality of callus. As callus from each CIM was shifted to each RM therefore, genotype × CIM with highest mean regeneration efficiency of 85.50% for both genotypes and RM × CIM interactions were also significant (data not shown). These results are summarized in Tables 6 and 7.

Effect of Imbibition and Callus Age on Embryogenesis and Regeneration

To determine the best suitable age of callus for regeneration, calli of different ages, from 4 to 10 weeks old, were cultured on two regeneration media (Table 2). Calli from each imbibition level and CIM were shifted to RM1 and RM2 media. The analysis of variance showed that genotype, callus age and imbibition had highly significant effects on plantlet regeneration. Furthermore, genotype × imbibition with highest mean efficiency value of 72.67% at 8 mg/L for both genotypes and callus age × imbibition interactions (data not shown) had also significant effects on regeneration. Both genotypes showed significant increase in regeneration with highest individual and overall mean value at 8 mg/L imbibition level (Table 8). Galaxy-13 however was more responsive (77.24%) than AARI-11 (68.10%) (Table 8). Similarly, callus age had significant effects on the regeneration of both genotypes. Although both genotypes showed maximum regeneration when callus was 9 weeks old (67.39%) but mean values of 8 and 9 weeks old callus showed no significant effects and as the callus age prolonged to 10 weeks the effects started to decline (Table 9). Both genotypes were significantly different and Galaxy-13 was more responsive than AARI-11 towards regeneration in terms of callus age with highest mean regeneration efficiency of 70.56% and 64.22% respectively when calli were 9 weeks old (Table 9, Fig. 3).

Table 5: Effects of genotypes and CIM on embryogenesis

CIM	Genotype/Variety		Mean
	Galaxy-13	AARI-11	
CIM1	77.22±2.86	78.40±2.66	77.81±1.93CD
CIM2	76.30±2.89	76.22±2.31	76.26±1.82D
CIM3	75.40±2.54	77.31±2.64	76.36±1.82D
CIM4	82.37±2.65	83.72±2.05	83.04±1.65A
CIM5	78.95±2.17	82.93±2.44	80.94±1.64AB
CIM6	77.87±2.70	80.21±2.43	79.04±1.80BCD
CIM7	79.11±2.83	81.46±2.69	80.28±1.93ABC

Means sharing similar letter in a row or in a column are statistically non-significant (P>0.05)

Table 6: Effects of genotypes X regeneration media on regeneration

RM	Variety		Mean
	Galaxy-13	AARI-11	
RM1	80.38±0.51	81.81±0.69	81.10±0.44A
RM2	80.10±0.94	81.14±0.88	80.62±0.64A
Mean	80.24±0.53B	81.48±0.55A	

Means sharing similar letter in a row or in a column are statistically non-significant (P>0.05)

Table 7: Effects of genotypes and CIM on regeneration

Treatment	Variety		Mean
	Galaxy-13	AARI-11	
CIM1	80.33±0.80bc	84.33±1.41ab	82.33±0.98B
CIM2	78.33±1.20c	81.67±1.20abc	80.00±0.95BC
CIM3	77.00±1.98c	78.67±0.84c	77.83±1.06C
CIM4	84.67±0.67ab	86.33±1.09a	85.50±0.66A
CIM5	79.00±0.68c	80.67±0.84bc	79.83±0.58BC
CIM6	81.67±0.95abc	79.33±1.23c	80.50±0.82BC
CIM7	80.67±0.84bc	79.33±0.42c	80.00±0.49BC

Means sharing similar letter in a row or in a column are statistically non-significant (P>0.05)

Table 8: Effects of genotypes and imbibition on regeneration mean±SE

Treatment	Variety		Mean
	Galaxy-13	AARI-11	
Control	44.95±2.84e	38.67±2.27f	41.81±1.86E
2 mg/L	48.19±2.80e	44.57±2.54e	46.38±1.89D
4 mg/L	52.86±2.99d	52.95±3.17d	52.90±2.15C
6 mg/L	69.05±4.36b	60.95±3.87c	65.00±2.95B
8 mg/L	77.24±4.32a	68.10±4.19b	72.67±3.06A
10 mg/L	71.52±4.01b	62.76±4.00c	67.14±2.88B
Mean	60.63±1.83A	54.67±1.65B	

Means sharing similar letter in a row or in a column are statistically non-significant (P>0.05)

Effect of Starvation Stress on Embryogenesis and Regeneration

Calli were kept under starvation stress and its effects on embryogenesis and regeneration were statistically analyzed. Analysis of variance showed that stress period had highly significant effects while genotype and genotype × stress period interaction were non-significant. Means of control and one week stress period were statistically similar i.e., 80.71% and 84.76%, respectively.

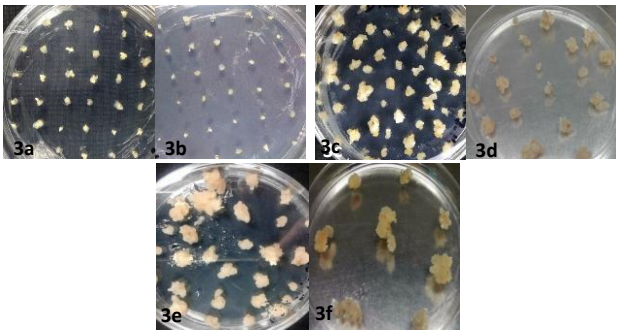

Fig. 3: Comparison between calli of Galaxy-13 and AARI-11 at different stages. (3a) Galaxy-13, one week old (3b) AARI-11, one week old (3c) Galaxy-13, 5weeks old (3d) AARI-11, 5 weeks old (3e) Galaxy-13, 10 weeks old (3f) AARI-11, 10 weeks old

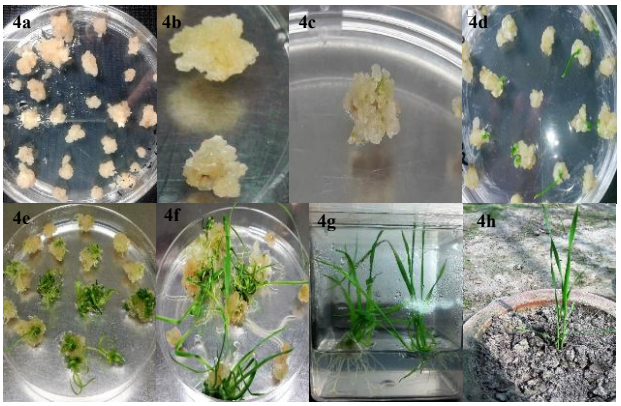

Fig. 4: Stages of plant development from callus to complete plant. (4a & 4b), callus induction, (4c) callus embryogenesis, (4d-4f) regeneration, (4g) rooting, (4h) shifting to soil

The effects were positive when stress period extended to 2 to 3 weeks, although statistically similar. However, higher effects were achieved for both genotypes at stress period of 3 weeks (93.10%). Galaxy-13 showed the highest mean value of 92.38% while AARI-11 of 92.38% when starvation period was 3 weeks (Table 10). Calli of both genotypes showed higher rate of embryogenesis and regeneration as the stress period prolonged with highest rate and efficiency of embryogenesis and regeneration achieved when the stress period reached 3 weeks. Calli under the stress period of 4 weeks showed significant decrease in the rate of embryogenesis and thus regeneration (Table 10).

Discussion

Efficient regeneration system is a key to successful transformation of plants including wheat. In order to get transgenics through tissue culture in wheat, one needs to grow wheat throughout the year to have immature embryos available at any time. Immature embryos are established as

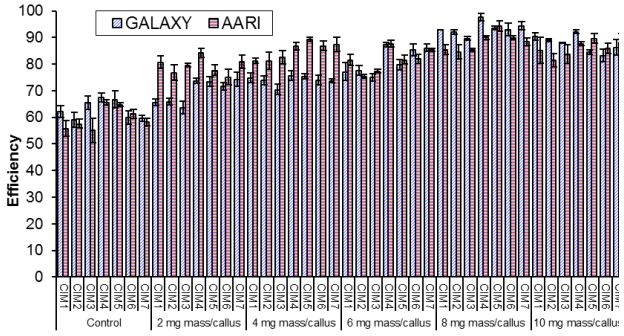

Fig. 5: Effect of genotypes, soaking and callus induction media on embryogenic efficiency

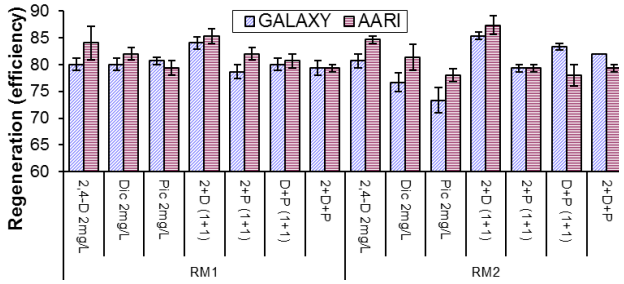

Fig. 6: Effect of genotypes, CIM and RM on regeneration efficiency

the most prolific starting material to get excellent regeneration needed for recovering transgenic plants. This is extremely difficult to get immature embryos throughout the year, if not impossible, in a country like Pakistan where summer temperature touches 50ºC. The other possibility is to identify a tissue which does not need to grow wheat throughout the year. This study was conducted to optimize an improved regeneration system using mature embryos (we do not need to grow wheat round the year to get mature embryos), which can be successfully deployed for wheat transformation programs.

Achieving the proper stage of mature embryo (Fig. 1a) is an important land mark in callus induction, proliferation, embryogenesis and regeneration. This proper stage was achieved by imbibing seeds in 2, 4-D solution overnight at 30ºC. The seeds not showing swelling after overnight incubation were not taken further for callus induction. This in turn enhanced callus mass (induction and proliferation) as compared to control with no 2, 4-D. A significant effect of imbibition of seeds with 2, 4-D solutions indicated that callus induction, proliferation and embryogenesis are enhanced compared to untreated seeds. Though, callus induction was improved with increasing 2, 4-D concentration in the imbibition media but statistically the impact of 2 mg/L, and 4 mg/L was same, and similarly 6, 8 and 10 mg/L (Table 3) with highest on 8 mg/L 2, 4-D for Galaxy-13 and 10 mg/L for AARI-11. The concept of imbibing the seeds with 2, 4-D before culturing on CIM was

taken from Razzaq *et al.* (2011) who used different levels of 2, 4-D for *in planta* transformation of wheat. The concept behind this activity was to trigger cell division and synthesis of fresh cells resembling cells in immature embryos. The callus induction was less at lower 2, 4-D, which may be due to less access of growth regulator to target tissues, at the same time low level of auxins is demonstrated to be effective in obtaining good quality callus (Jones, 2005; Rana *et al.,* 2012). For embryogenesis in the obtained calli, we found that genotype, imbibition levels and CIM were highly significant which means that embryogenesis depends on above factors. The effects of seven different media on callus induction, (CIM-1 to CIM-7) were statistically non-significant, CIM4 produced better callus with improved embryogenesis (Table 5) and the results are in accordance with Chauhan *et al.* (2007) and Rana *et al.* (2012) in terms of quality of callus. It was found that imbibition level of 8 mg/L 2, 4-D produced callus of best quality in terms of embryogenesis (Fig. 5). The imbibition at 8 mg/L 2, 4-D and CIM4 were found as the most suitable combination for callus induction, and embryogenesis (Table 4 and 5, Fig. 5). It was also found that embryogenesis was achieved 45–50 days after explant excision. During callus induction, two different kinds of calli, embryogenic and non-embryogenic were found (Parmar *et al.,* 2012) Non-embryogenic calli were characterized as creamy, membranous and hairy which we termed it as pseudo callus (NRS; non-regenerated structures described by Parmar *et al.,* 2012). This pseudo callus was removed during culturing and subculturing. Moreover, the effect of culturing embryos by removing coleoptile segment and then cutting the remaining horizontally into two halves and without removing and cutting in order to determine the callus proliferation, mass and embryogenesis was studied (Fig. 1a–d). It was found that the former showed more callus proliferation, and embryogenesis and very less shoot development as compared to latter. This method is modification of Delporte *et al.* (2001) in which the mature embryos were ground to pieces through a sterile nylon mesh. Plantlet regeneration was found to be highly dependent on genotype and embryogenesis while RM1 and RM2 showed non-significant variation. There was a significant effect of imbibition and CIM on regeneration. The calli generated on CIM4 showed highest rate of plantlet regeneration on each RM (Fig. 6) because calli on CIM4 were highly embryogenic. Additionally, the effect of callus age on regeneration efficiency was determined. Callus from each CIM was analyzed on each RM in triplicated manner. Seven age levels of callus (4–10 weeks old) were studied and it was found that 8 and 9 weeks old calli were best for regeneration (85–90%) as shown in (Fig. 6), as 8–9 weeks old callus was highly embryogenic (Fig. 4a). Another milestone we achieved was the starvation stress on callus. Normally calli are shifted to fresh media after two weeks, as nutrients level in media plates deplete gradually. If media is not changed, calli come under starvation stress. One month old calli were used for this experiment in triplicated manner. Four stress levels (1 week, 2 week, 3 week and 4 week along with control) were studied and found that calli under stress for 3 weeks showed highest embryogenesis and thus regeneration (Fig. 4b–f) and efficiency of ~90% (Fig. 6). The calli with controlled conditions also showed greater embryogenesis and more than 80% regeneration. Thus, starvation stress may be one of the major factors contributing to an efficient regeneration system from mature embryo. Furthermore, in this whole study apart from three auxins and Zeatin, there was no use of special or additional growth regulators and vitamins which make this protocol quite cost effective.

Conclusion

Two major milestones were achieved in this study. First, imbibition of seeds in different 2, 4-D solutions prior embryo isolation results in production of embryogenic callus. Second, the stress enhanced the rate of embryogenesis. This optimized protocol is cost effective and more efficient than previously published protocols till now and can be used in any wheat regeneration and transformation program.

Acknowledgements

The corresponding author, who is also Principle Investigator of this research, acknowledges the financial support for this research and fellowship to the first author for his PhD by Higher Education Commission of Pakistan.

References

Bhalla, P.L., 2006. Genetic engineering of wheat-current challenges and opportunities. *Trends Biotechnol*., 24: 305–311

Chauhan, H., S.A. Desai and P. Khurana, 2007. Comparative analysis of the differential regeneration response of various genotypes of *Triticum aestivum, Triticum durum* and *Triticum dicoccum. Plant Cell Tiss. Org. Cult.*, 91: 191–199

Chugh, A. and P. Khurana, 2003. Regeneration via somatic embryogenesis from leaf basal segments and genetic transformation of bread and emmer wheat by particle bombardment. *Plant Cell Tiss. Org. Cult.,* 74: 151–161

Delporte, F., O. Mostade and J.M. Jacquemin, 2001. Plant regeneration through callus initiation from thin mature embryo fragments of wheat. *Plant Cell Tiss. Org. Cult.*, 67: 73–80

Ding, L., S. Li, J. Gao, Y. Wang, G. Yang and G. He, 2009. Optimization of *Agrobacterium*-mediated transformation conditions in mature embryos of elite wheat. *Mol. Biol. Rep.*, 36: 29–36

Filippov, M., D. Miroshnichenko, D. Vernikovskaya and S. Dolgov, 2006. The effect of auxins, time exposure to auxin and genotype on somatic embryogenesis from mature embryo of wheat. *Plant Cell Tiss. Org. Cult.*, 84: 63–73

Ganeshan, S., S.V. Chodaparambil, M. Baga, D.B. Fowler, P. Huel, B. Rossnagel and R.N. Chibber, 2006. *In vitro* regeneration of cereals based on multiple shoot induction from mature embryos in response to thidiazuron. *Plant Cell Tiss. Org. Cult.*, 86: 63–73

Greer, M.S., I. Kovalchuk and F. Eudes, 2009. Ammonium nitrate improves direct somatic embryogenesis and biolistic transformation of *Triticum aestivum*. *New Biotechnol.,* 26: 1–2

Jones, H.D., 2005. Wheat transformation: current technology and applications to grain development and composition. *J. Cereal Sci.*, 41: 137–147

Mac Kinnon, C., G. Gunderson and M.W. Nabors, 1987. High efficiency plant regeneration by somatic embryogenesis from callus of mature embryo explants of bread wheat (*Triticum aestivum L.*) and grain sorgum (*Sorghum bicolor*). *In Vitro Cell. Dev. Biol. Plant.*, 23: 443–447

Mendoza, M.G. and H.F. Kaeppler, 2002. Auxin and sugar effects on callus induction and plant regeneration frequencies from mature embryo of wheat (*Triticum aestivum* L.). *In Vitro Cell Dev. Biol. Plants*, 38: 39–45

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

Ozgen, M., M. Turet, S. Ozcan and C. Sancak, 1996. Callus induction and plant regeneration from immature and mature embryos of winter durum wheat genotypes. *Plant Breed.*, 115: 455–458

Ozgen, M., M. Turet, S. Altmok and C. Sancak, 1998. Efficient callus induction and plant regeneration from mature embryo culture of winter wheat (*Triticum aestivum L.*) genotypes. *Plant Cell Rep.*, 18: 331–335

Parmar, S.S., S. Manish, C. Darshna and K.J. Pawan, 2012. Plant regeneration from mature embryo of commercial Indian bread wheat (*Triticum aestivum L.*) cultivars. *Physiol. Mol. Biol. Plants*, 18: 177–183

Patnaik, D., D. Vishnudasan and P. Khurana, 2006. *Agrobacterium*-mediated transformation of mature embryos of *Triticum aestivum* and *Triticum durum. Curr. Sci.*, 91: 307–317

Rana, I.A., H. Loerz, W. Schaefer and D. Becker, 2012. Over expression of chitinase and chitosanase genes from Trichoderma harzianum under constitutive promoters in order to increase disease resistance in wheat (*Triticum aestivum L*). *Mol. Plant Breed.*, 3: 37–49

Redway, F.A., V. Vasil, D. Lu and I.K. Vasil, 1990. Identification of callus types for long-term maintenance and regeneration from commercial cultivars of wheat (*Triticum aestivum* L.). *Theor. Appl. Genet.*, 79: 609–617

Repellin, A., M. Baga, P.P. Jauhar and R.N. Chibbar, 2001. Genetic enrichment of cereal crops via alien gene transfer: new challenges. inorganic nutrient manipulation in the induction of embryogenic callus from immature embryos of wheat. *Plant Cell Tiss. Org. Cult.*, 64: 159–183

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.*, 127: 249–255

Vasil, I.K., 2007. Molecular genetic improvement of cereals: transgenic wheat (*Triticum aestivum L.*). *Plant Cell Rep*., 26: 1133–1154

Zale, J.M., H. Borchardt-Wier, K.K. Kidwell and C.M. Steber, 2004. Callus induction and plant regeneration from mature embryos of a diverse set of wheat genotypes. *Plant Cell Tiss. Organ Cult.*, 76: 277–281

(Received 23 November 2016; Accepted 13 March 2017)