

OPTIMIZATION OF REGENERATION PROTOCOLS FOR WHEAT UNDER DROUGHT AND SALT STRESS

Sehar Nawaz¹, Nisar Ahmed^{1,*}, Ahsan Iqbal¹ and Ihsan Khaliq²

¹Centre of Agricultural Biochemistry and Biotechnology (CABB), University of Agriculture, Faisalabad, 38000, Pakistan;

²Plant Breeding and Genetics Department (PBG), University of Agriculture, Faisalabad, 38000, Pakistan

*Corresponding author's e.mail: drmiannisar@yahoo.com

Responses of the wheat genotype 'Faisalabad-2008' to plant regeneration using immature embryos and *in vitro* drought and salt stress tolerance were investigated. For this study immature embryos 15 DAP (Days After Pollination) were used and after 6 weeks healthy calli were transferred to media containing variable concentrations of NaCl (0, 5, 10, and 15g/L) and drought stress intensities using PEG 6000 (0, 5, 10, 15 and 20% (w/v)). The findings suggested that the embryogenic capacity of callus depended on concentrations of exogenous hormones and the genetic makeup of the genotype. Faisalabad-2008 showed good callus induction and ability to proliferate and regenerate seedlings under drought and salt stress conditions. This study will serve as a guide line in future for *in vitro* screening of wheat varieties for their regeneration ability using indirect methods. Further establishment of *in-vitro* screening protocols for abiotic stress tolerance will facilitate screening of genotypes during early developmental stages.

Keywords: Wheat, salt, drought, regeneration

INTRODUCTION

Wheat (*Triticum aestivum* L.), is one of the most important cereal crops though out the world, according to an estimate (FAO, 2010). Wheat food calories consumption is almost 20 percent. In Pakistan this crop is a staple food. In spite of its critical position wheat production is following a negative trend for the last few years. Its production declined from 23,517 thousand tons in 2011-12 from 25,214 thousand tons in 2010-11 showing a decrease of 6.7 percent (Anonymous, 2011-2012). A number of factors are responsible for low yield but the main reasons for the decline were abiotic stresses (Jain *et al.*, 2010; Shamsi *et al.*, 2010; Khavarinejad and Karimov, 2012). Drought and salt stress are the most common environmental limitations that cause significant reduction of growth, development and yield on present cultivated lands. In addition, they are major problems in the cultivation of wheat on arid and semiarid areas (Savin and Nicolas, 1999; Richards *et al.*, 2001; Kimurto *et al.*, 2003; Edward and Wright, 2008; Johari *et al.*, 2011).

Conventional plant breeding techniques were fruitful for a long period of time to improve yield related parameters by increasing the drought and salt tolerance of wheat (Jauhar and Chibbar, 1999; Bruce *et al.*, 2002). The green revolution was characterized by the use of input intensive cropping systems which showed maximum genotypic to environmental interactions. Now for further yield related revolutions new genes stacks are needed related to traits like drought stress. Genetic transformation techniques made it possible to introduce few novel genes into adapted commercial genotypes to produce genetically modified varieties (Jones *et al.*, 2005). These techniques require

establishment of efficient regeneration protocol depending on specific genotypic requirements. Several *in vitro* regeneration and selection protocols have been developed in different plants for drought and salt stress tolerance (Jain, 2010). Cereal species, particularly wheat, has lagged behind dicots in their response to *in vitro* techniques and remain relatively genotype-dependent (Bhalla *et al.*, 2006). A number of explants sources have been used in wheat but mature and immature embryos proved to be most reliable among them (Özgen *et al.*, 1998). When immature embryos were compared to mature embryos, the former were a better choice for regeneration. Immature embryos are highly regenerative as they contain embryogenic totipotent cells (Zale *et al.*, 2004). In contrast, mature embryos, either pretreated before culture (Yu *et al.*, 2008) or used directly (Özgen *et al.*, 1998), show regeneration potentials that were very low (Ozias-Akins and Vasil, 1983).

In addition to plant regeneration tissue culture also provide an opportunity to screen genotypes against drought and salt stress. For the evaluation against stresses a number of tissue culture based techniques have been used (Cano *et al.*, 1998; Gholamin *et al.*, 2010). The salt stress tolerance mechanisms of whole plants may be quite different from those involved at the plant cell level. An ontogenic evolution of salt tolerance was clearly demonstrated (Adam *et al.*, 1992). Application of salt stress during the regeneration process constitutes a convenient way to study the effects of salinity on the morphogenic steps of development. Similarly high molecular weight PEG 6000 (Polyethylene Glycol) treatments have been used by many scientists (Ruf *et al.*, 1967; Kaufmann and Eckard, 1971) to stimulate drought conditions in plants. It is an inert, non- phytotoxic and non-

penetrating osmoticum that decreases the osmotic potential of nutrient solutions (Lawlor, 1970). It creates water stress in the same way both *in vivo* and *in vitro* plants. So, death of callus in medium with PEG 6000 was not caused by its absorption to the cells, but decreasing water potential in the medium. Several studies showed that the selective pressure can be applied during the phase of callus formation and /or regeneration (Flowers, 2004; Saleem *et al.*, 2005).

The present research was, therefore, planned to evaluate the comparative effects of salt and drought stress at the cellular level. Callus proliferation, callus embryogenesis and regeneration were the parameters under consideration to determine calli viability. The main objectives to be achieved in present research project were to (i) evaluate the ability of genotype to induce callus by immature embryo culture; and (ii) *in vitro* screening of bread wheat genotype for drought and salt tolerance.

MATERIALS AND METHODS

Plant materials and growth condition: Seeds of wheat variety Faisalabad-2008 (*Triticum aestivum* L.) were collected from Ayub Agricultural Research Institute, Faisalabad. This variety has been approved for general cultivation in Pakistan due to its high yield, performance and adaptability. Seed were sterilized with 70% (v/v) ethanol for 1 min, then with 3% (v/v) sodium hypochlorite (NaOCl) solution for 20 min, and finally washed five times with sterile deionized double distilled water.

Seeds (20 seeds /dish) were incubated on two layers of filter paper (Whatman No.10) moistened with 6ml of sterile water in a Petri dish at 4°C in dark to synchronize germination. These seeds were transferred to growth chamber for germination at 23°C with 16/8 hr day and night (4000 lux) for seven days. Germination began within 24h after incubation; seven days after germinated seedlings were transferred to pots under controlled conditions of light (4000 lux) and temperature (23±2°C). At pollination spikes were tagged and covered with craft paper bags. Spikes were harvested and seeds were collected at 15 DAP (Days After Pollination).

Embryo excision and callus induction: Seeds were threshed from spikes and surface-sterilization using 70% (v/v) ethanol for 50 second with continuous stirring increased the efficiency of sterilization, then 3% (v/v) sodium hypochlorite solution was used to disinfect for 20 min, and finally washed five to six times with autoclaved deionized double distilled water. Immature embryos were excised from seeds, in a laminar flow hood, using a sterilized metallic scalpel. They were placed in the Petri dishes scutellum side up after damaging the embryonic axis. Petri dishes containing Murashige and Skoog (1962) culture medium supplemented with 30 g/L sucrose, 8 g/L agar, and different concentrations (0, 1, 2, 3, 4, 5, 6 mg/L) of 2,4-D for callus

induction were used. Petri dishes were sealed using Parafilm™ and placed in growth chamber in darkness. The temperature was maintained at 23±2°C. Plates were changed after 15 days to refresh the media and regularly checked for contamination. For the determination of callus growth rate, 6 week old calli were transferred to a sterile Petri dish and their weight was measured in aseptic conditions. Afterwards, callus pieces were re-transferred to callus culture medium. Data were also recorded related to callus induction frequency according to the formula.

Callus induction frequency (%) =

$$\frac{\text{No. of embryos produced calli}}{\text{No. of embryos cultured}} \times 100$$

No. of embryos cultured

***In vitro* salt and drought stress:** Six weeks after the incubation of calli in dark, the healthy calli were selected and sub-cultured on MS₂ medium supplemented with variable amount of NaCl (0, 5, 10, and 15 g/L), and under different drought stress intensities (0, 5, 10, 15 and 20% (w/v)) using PEG 6000 (Merck, Germany). Four weeks after salt and drought stress, calli were transferred to magenta box on MS media supplemented with 30 g/L of sucrose, 8 g/L of agar, 1 mg/L IAA (Indole Acetic Acid) for shoot regeneration.

When sufficient shoots were developed, regenerated calli were placed on MS medium supplemented with 30 g/L of sucrose, 8 g/L of agar, 1 mg/L kinetin for root regeneration. Data were recorded related to callus proliferation efficiency, embryogenic efficiency, regeneration efficiency, Callus fresh weight, callus relative growth, callus water content and callus necrosis were recorded. After the development of roots the plantlets were transferred to pots under optimum conditions of light and temperature and data were recorded.

RESULTS

Callus induction: Immature embryos cultured on MS medium supplemented with different concentration of 2,4-D were incubated in a controlled environment. Irrespective of different concentrations of 2,4-D callus formation was initiated on the first day of incubation. Callus was allowed to proliferate for six weeks to gain sufficient masses, with regular checks for contamination. Sprouting of shoots was not observed due to the damaged embryonic axes. After six week of embryo culture callus frequency and size were calculated.

Callus production frequency was more than 95% except in the control medium (without hormone) and weight varied in line with the concentration of 2,4-D (Table 1).

In the control media (media without 2,4-D) no callus formation was observed. At low concentration of 2,4-D (1mg/L) callus formation was negligible, except that the embryos swelled and became enlarged in the first few days after incubation. Callus initiation was evident at a minimum concentration of 2mg/L (0.289±0.079) and positive growth

trend was observed with further increase in 2,4-D concentrations. Maximum callus production was observed at 4mg/L 2,4- D (1.158 ± 0.315) but with further increases in concentration (5mg/L to 6mg/L) decrease in growth (1.078 ± 0.293 - 0.604 ± 0.164) was observed. Morphology of generated calli was also different at various concentrations of 2, 4- D (Fig. 1). At the lower concentration, 3mg/L, friable, nodular, embryogenic, creamy callus was produced (Type I). Callus obtained at highest concentration was yellowish with watery appearance and more compact (Type II). Embryogenic type I calli produced at 2, 3, 4, 5 and 6 mg/L (2,4-D) were 50, 60, 58, 50 and 20 percent of all calli, respectively (Fig. 2A and B).

Table 1. Effect of different concentrations of 2,4-D on callus induction

2, 4-D concentration (mg/l)	Average fresh weight (g)
0	Control
1	Only embryos swelled
2	0.289 ± 0.079
3	1.078 ± 0.293
4	1.158 ± 0.315
5	1.078 ± 0.293
6	0.604 ± 0.164

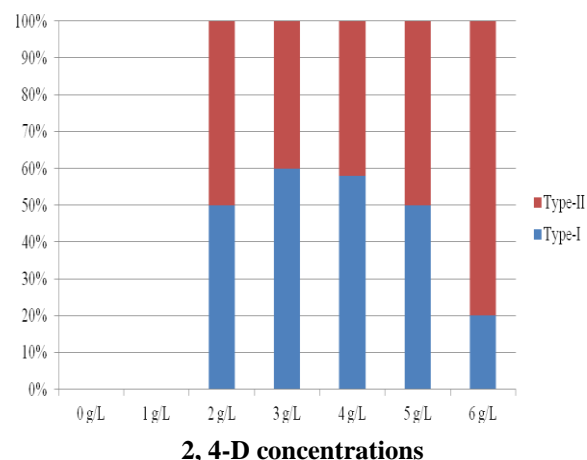


Figure 1. Developmental potential of Type I and Type II nature of calli as a function of 2,4-D

Effect of salt stress: After four weeks when sufficient callus mass was formed it was placed on MS2 media supplemented with varying concentrations of NaCl (0, 5, 10, and 15 g/L). The presence of NaCl, in the media resulted in necrosis of cells and its intensity increased with increase in salt concentration. At lower concentrations, 5g/L, few calli were affected and some showed sectional/partial necrosis. At higher concentrations (10g/L) the number of necrotic calli increased and reached maximum at 15g/L. A decrease of

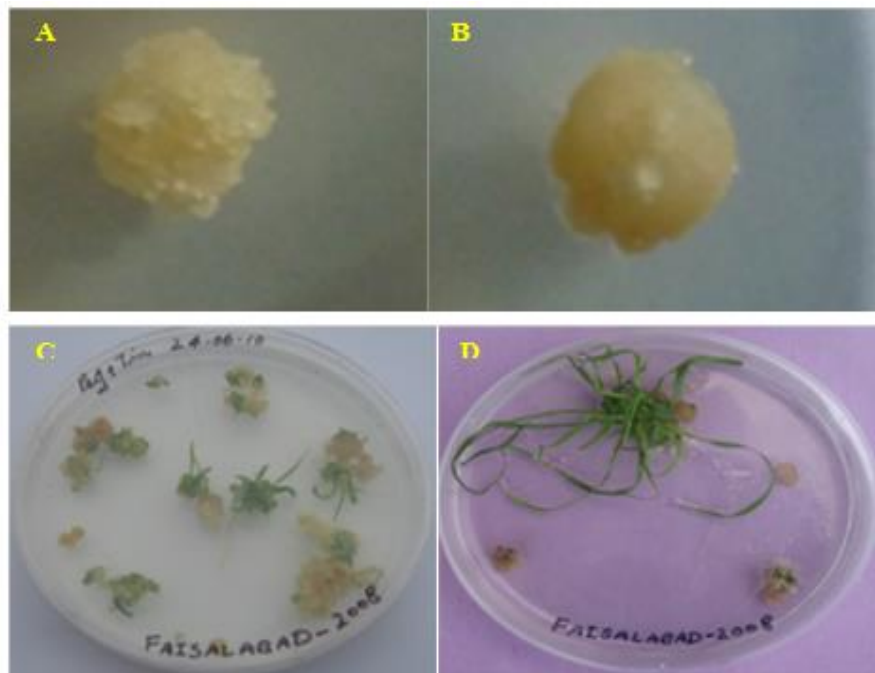


Figure 2. (A) Type-I embryogenic callus with nodule formation, (B) Type-II non-embryogenic compact callus, (C) Plant regeneration after 15 weeks treated with 5g/L NaCl, (D) Plant regeneration after 15 weeks treated with 5% PEG.

72% in calli proliferation rate was observed with 15g/L of NaCl in the medium.

Six weeks after induction of salt stress calli mass was measured and transferred to regeneration media. Embryogenic and regenerative efficiency from callus were also influenced by the NaCl. Thus, the embryogenic capacity of callus, evaluated by the number of shoots regenerating from calli varied with respect to salt concentrations. The number of shoot producing calli decreased from 22 to 10 at 5g/L NaCl concentration. This number was further reduced with increasing concentrations of NaCl. At 10g/L this number was 2 and further decreased to 1 at 15 g/L.

Results of this study showed that 5 g/L of NaCl does not affect the callus proliferation rate (Fig. 2C) but decreased the number of shoots (Table 2). Higher concentrations of NaCl drastically decreased callus regeneration from 82% to 13%.

Effect of drought stress: After four week of incubation on callus multiplication media, calli were separately transferred to MS₂ media supplemented with different concentrations of PEG 6000 (0, 5,10,15 and 20% (w/v)). The callus relative growth (CRG) in stress and unstressed medium was measured in terms of percent increase in fresh weight (Table 3).

CRG = (final fresh weight-initial fresh weight)/ initial fresh weight.

Callus water content (CWC %) = (callus fresh weight (CFW) - callus dry weight (CDW))/CFW.CDW)

Callus dry weight was determined after 48 h of incubation in the oven at 80°C and callus fresh weight was taken just before drying.

Although it was difficult to quantify the level of callus

necrosis, the following 5 categories based on visual observation were used during the present study: A: 81-100%; B: 61-80%; C: 41-60%; D: 21- 40%; E: 0-20% (Table 3).

Callus fresh weight was significantly affected by different concentrations of PEG 6000 in media. At lower concentration i.e. 5% there was no considerable decrease in callus mass (4.00g as compared to 5.42g on control media) but at higher concentrations the decrease was drastic (10, 15 and 20% PEG 6000 resulted in 1.98g, 1.52g and 1.24g of callus fresh weight respectively). The callus relative growth percentage decreased from 3.68 to 0.07 (3.68, 2.45, 0.52, 0.31 and 0.07 from 0 to 20% PEG 6000 concentration) and CWC from 83.58 to 60.70 (83.58 at control, 80.12 at 5%, 67.54 at 10%, 63.42 at 15% and 60.70 at 20% PEG 6000 concentration) when higher concentrations of PEG 6000 were used. Callus treated with 5% PEG show regeneration (Fig. 2D) but at higher concentration it was not observed.

DISCUSSION

In cereal tissue culture, according to available reports, low concentration of 2,4-D failed to initiate any type of calli, when immature embryos were the source of explants (Padmaja *et al.*, 1992; Abdrabou and Moustafa, 1993; Akashi *et al.*, 1993; Mohammad, 1993; Pius *et al.*, 1994; Kosulina, 1995). In view of these reports, for callus initiation, the requirement was at least 2.0 mg/L 2,4-D, the only exogenously added hormone. In wheat accessions, the immature embryos also failed to initiate any type of calli at low concentration of 2,4-D. At these concentrations, only

Table 2. Effect of different salt treatment on callus induction and regeneration

Parameters	NaCl (g/L)			
	0	5	10	15
No. of incubated embryos	32	32	32	32
No. Proliferating calli	32	30	14	9
Proliferation efficiency (%)	100	93	43	28
No. of shoot producing calli	22	10	2	1
Embryogenic efficiency (%)	68	31	6	3
No. of root giving calli with shoots	18	07	0	0
Regeneration efficiency (%)	56	21	0	0
No. of shoots/ plantlet	8	4	0	0
No. of roots/ plantlet	2	2	0	0

Table 3. Effect of PEG 6000 concentrations on callus development

PEG 6000 concentration (%)	Callus fresh weight (g)	Callus relative growth	Callus water contents (%)	Callus Necrosis
0	5.42	3.68	83.58	E
5	4.00	2.45	80.12	D
10	1.98	0.52	67.54	D
15	1.52	0.31	63.42	A
20	1.24	0.07	60.70	A

initial swelling was observed. Higher concentration of 2,4-D was found responsible for non-embryogenic calli in cereal tissue cultures (Kim *et al.*, 1992). The data here are inconsistent with the previous findings.

Ozias-Atkins and Vasil (1982) observed that formation of discreet nodules prior to germination of the somatic embryos in immature embryo derived calli. Later on, trichomes formed on the smooth nodular areas. They differentiated leafy structures by the formation of a notch on the surface of a nodule. Finally the embryo-like structures were observed. In Faisalabad-2008 callus, at the end of 6 weeks period the similar globular structures were observed. These structures can be interpreted as those “notches” as previously described can be considered as globular stage which is the first sequential stage of embryo formation in the somatic embryogenesis process. From these results it can be concluded that proliferation of embryogenic callus is genotype, hormonal concentration and time dependent.

In vitro culture has also been widely adopted as the most adequate technique in cereals for the selection of salt tolerant genotypes (Oudija and Ismaili, 2002). This technique allows obtaining plantlets by direct regeneration (Oudija and Ismaili, 2002; Saleem *et al.*, 2005) or by indirect regeneration *via* callus induction (Hassanein, 2004; Messai *et al.*, 2004; Satyavathi *et al.*, 2004; Raveendar *et al.*, 2008; Feng-Ling *et al.*, 2011). In case of direct regeneration, the stressing agent is embedded in culture medium while in the indirect regeneration, the application of the stressing agent is done during the callus initiation (Khaleda *et al.*, 2007), or during the different stages of callus growth, after their initiation on a medium without salt (Saleem *et al.*, 2005; Nwe *et al.*, 2011), or in the regeneration medium (El-Sayed *et al.*, 2004). The present study was conducted to investigate indirect regeneration of plantlets to salinity *via* callus. Salt stress was applied during callus induction phase and the results showed that salinity causes a significant reduction in callus regenerative capacity (56 to 0%). The regeneration rate was reduced severely at higher concentrations of NaCl. Similar findings have been reported in wheat (Alvarez *et al.*, 2003; Oudija and Ismaili, 2002), tomato (Saleem *et al.*, 2005) and rice (Khaleda *et al.*, 2007; Nwe *et al.*, 2011). Among genotypes of rice, Nwe *et al.* (2011) observed that NaCl concentrations of 8.8, 11.7 and 14.6 g/L result more than 70% decrease in regeneration rate. They concluded that tissue culture derived from these concentrations can be used to conduct studies on the physiological mechanisms associated with *in vitro* tolerance to salinity. Similarly, Raveendar *et al.* (2008) showed that the addition of 50, 75 and 100‰ of sea water in the medium culture inhibits the *in vitro* regeneration of rice callus, whereas 10 and 25‰ of sea water did not affect the regeneration rate. He also found that depressive effect accentuated by high concentration of salt, led to destruction of regeneration capacity of calli. In contrast, Oudija and Ismaili (2002) obtained albino plantlets

on media containing 15 and 20 g/L NaCl from callus initiated in the presence of NaCl. They also showed that at high salt concentrations, some callus produced roots only. On the other hand, Messai *et al.* (2004) showed that 6 g/L NaCl was toxic to regenerating tomato callus.

Calli cultured on stress free medium (without PEG 6000) were found to be healthier with unrestricted growth (0-20% necrosis) than those on the stressed media (80-100% necrosis). Calli subjected to drought stress (-1.2 MPa) for four weeks exhibited very poor health. Callus culture for two weeks on media containing 5% PEG 6000 did not affect callus health and it looked light yellow to whitish in color with no browning. Whole callus tissues were turned brown when calli were cultured on the same media with 20% PEG 6000 for two, three or four weeks.

Callus browning rate was found to be good indicator of callus sensitivity to PEG 6000 induced osmotic stress. Necrosis was more on the surface of the callus facing medium. These observations are in line with those of Hassan *et al.* (2004). They reported that *in vitro* osmotic stress of -1.0 MPa for eight months of culture onto PEG 6000 containing media is lethal for sunflower calli with evident necrotic tissues on callus surface.

The major effect of PEG 6000 stress on callus growth was mainly observed in the form of decreasing callus fresh weight which is a typical response in callus tissue of many crop plants (Lutts *et al.*, 1996; Khalequzzaman *et al.*, 2005; Sakthivelu *et al.*, 2008; Wani *et al.*, 2010). Such a decrease in callus fresh weight in response to PEG 6000 stress might be due to water shortage which affects development and growth of cells as increase in PEG 6000 concentration reduced callus water contents. Addition of PEG 6000 in solid media lowers water potential of the medium that adversely affect cell division leading to reduced callus growth (Ehsanpour and Razavizadeh, 2005; Sakthivelu *et al.*, 2008). Cell division and cell growth are the two primary processes involved in increase of fresh weight. In general, cell division is considered to be less sensitive to drought when compared with cell enlargement or growth (Sakthivelu *et al.*, 2008). However, both cell expansion and cell division can be influenced by relatively mild osmotic stresses. This is evident from decline in CRG % in media containing higher PEG 6000 (-1.2 MPa) induced osmotic stress might be due to reduced cell division, shrinking cytoplasmic volumes and loss of cell turgor, nutritional imbalances due to reduced uptake of water, an increase in electrolyte leakage and decrease in cell water contents with increasing stress (Lokhande *et al.*, 2010). Generally, the results of this experiment showed that the callus proliferation rates are drastically affected by induced drought stress and this mechanism is genetically determined in wheat like other cereals.

Conclusion: *In vitro* tissue culture is an important means of improving crop tolerance and yield through genetic transformation. Therefore, it is important to devise an

efficient protocol of callus proliferation to start *in vitro* selection for salt and drought stress tolerance, and to broaden opportunities for genetic manipulation of wheat through tissue culture, using explants and hormonal concentration. The results of this study indicated that Faisalabad-2008 showed a good callus induction when immature embryos were used as an explants and maximum callus induction was observed when 4mg/L 2,4-D was used. Differential responses were also noted in callus ability to proliferate and regenerate seedling under drought and salt stress conditions. Callus mass, shoot and root regeneration was considerably affected by higher salt and drought concentrations in media. Callus showed visible sign of necrosis and reduced regeneration when exposed to these stresses. Therefore, to establish an efficient and cost effective regeneration protocol of wheat for its genetic manipulation, it is necessary to screen cultivar in question for its ability of callus induction and plant regeneration. Screening genotypes for a biotic stresses at the cell level will also facilitate early and efficient plant selection for these traits.

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