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EFFECT OF PEG AND MANNITOL INDUCED WATER STRESS ON REGENERATION IN WHEAT (*Triticum aestivum* L.)

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Responses of the wheat commercial cultivar 'Faisalabad-2008' to various concentrations of 2,4-D and the effect of water stress on callus induction and regeneration efficiency were evaluated. For the assessment of responses to water stress, growing morphogenic calli of 7, 14 and 21 days after culture were exposed to different concentrations of water stress. For induction of stress wheat calli were exposed to different concentrations of PEG 6000 (0, 4, 8, 12 and 16% (w/v) and mannitol (0, 0.5, 1, 1.5 and 2% (w/v). The effect of these stresses on shoot and root length, relative water content and electrolyte leakage were also determined. The results indicated that maximum regeneration was observed at 5mg/L of 2,4-D, increase or decrease in 2,4-D concentration resulted a decrease in regeneration efficiency. Maximum regeneration was observed from 21 days old calli. Significant decreases in the number of shoots regenerated per calli; shoot and root length and relative water contents occurred under both stresses with the highest reduction under mannitol-induced osmotic stress. Electrolyte leakage increased at higher PEG and mannitol concentrations. Furthermore, both PEG and mannitol induced drought stress efficiently but mannitol was more severe. The results here will be helpful for improvement of the wheat crop tolerance against water stress.

Keywords: Wheat, embryos, callus, regeneration, optimization, drought stress

INTRODUCTION

Wheat (*Triticum aestivum* L.) is the leading cereal crop and covers more of the earth than any other crop. It also contributes to vegetable protein and source of calories for more than 2.5 billion people of the developing world (FAO-2012). To feed the 9.1 billion populations projected for the world in 2050 agricultural production needs to be doubled (Tillmen *et al*., 2011; FAO, 2012). Wheat like other cereals is unable to meet the goal as 24-39% of the cropland areas reported no yield improvement (Ray *et al*., 2012, 2013; Wang *et al*., 2013). The yield ceilings are mostly attributed to environmental stresses, which cause adverse physiological changes in plants (Shao *et al*., 2008). Among a number of abiotic factors drought is the most common environmental constraint that prevent crops attaining their maximum yield potentials (Edward and Wright, 2008; Johari *et al.,* 2011). This stress has threatened the world food supply (Nevo and Chen, 2010; Ahmad *et al*., 2014).

In crop plants water stress is induced either due to its excess or shortage (Harb *et al*., 2010). The most common water stress encountered by plants is drought. Drought imparts a negative effect on plants growth and development. These effects become chronic due to unpredictable weather conditions in the regions characterized by low annual rainfall. Drought has remained a challenging issue for plant scientists and breeders. It is expected that about 65% of the world population will face water shortage problems in the year 2025. Thus the situation demands for better understanding of plant mechanisms to thrive with these scarcer water resources (Rosegrant and Cline, 2003).

Drought induced osmotic stress, triggers a wide range of perturbations ranging from growth and development disruption to the modification of ion transport and uptake systems (Karimi *et al.,* 2011). *In vitro* culture technique serves as a useful tool to study the biochemical and physiological response of undifferentiated callus to drought stress at the cellular level (Ghasempour *et al.,* 2007). Moreover, the distinction between different solutes to induce stress in culture media, and the relationship between surviving abilities of cultured cell lines and their growth properties can be served by *in vitro* culture techniques. Undifferentiated cells and callus cultures eliminate complications associated with genetic and morphological variability inherent to different tissues in whole plants (Parida and Das, 2005; Shao *et al.,* 2007; Ghasempour *et al.,* 2007). Understanding of plants ability to tolerate stresses opens a way for crops manipulations to improve tolerance, adaptation or resistance to stresses (Lutts *et al.,* 2004; Parida and Das, 2005; Movahhedy-Dehnavy *et al.,* 2009).

Previous studies on wheat tissue culture have shown that callus induction frequency and regeneration of *in vitro* plants is immensely influenced by the genotype (Filippov *et al*., 2006), type of explants used for regeneration (Patel *et al*.,

2004; Tamas *et al*., 2004; Shariatpanahi *et al*., 2006; Liu *et al.,* 2008), composition of media (Tamas *et al*., 2004) and hormones (Friml, 2003). So, needed are highly efficient and reproducible regeneration protocols for commercial varieties having essential characters (Kumlehn and Hensel, 2009).

Auxin may act as both morphogen and hormone (Friml, 2003). Auxin signals are transduced resulting into a variety of responses including changes in growth directions and differentiation of roots and shoots (Leyser, 2001). Petrasek *et al*. (2002) explained that external source of auxin is required for *in vitro* culture of most of the cell types and internal to external auxin ratio is also vital for regulation of growth cycle. Type and concentration of auxin is crucial (Mendoza and Kaeppler, 2003) and greatly influences the morphology of the culture.

In cereals synthetic auxin 2,4-D is a kind of essential component of culture media used widely for induction and maintenance of callus (Prado *et al*., 2000) or somatic embryogenesis if provided in large concentration. Sosa *et al.* (2005) called 2,4-D the best hormone used for plant growth.

Polyethylene glycol and mannitol have been used to stimulate osmotic stress and these neutral polymers are being widely used to impose water stress in plants (Zgallai *et al.,* 2005). The non-toxic PEG solution is used because of high molecular weight, which cannot enter into cell through plant cell wall (Kaydan *et al*., 2008) as compared to mannitol. Mannitol has a low molecular weight sufficient to enter into cells and cause toxicity. The objective of the present study was to determine the optimum concentration of 2,4-D for friable callus induction and to compare the effect of PEG and Mannitol on regeneration.

MATERIALS AND METHODS

***Explant preparation and callus induction*:** Mature basic seeds of wheat variety 'Faisalabad-2008' were collected from Ayub Agricultural Research Institute, Faisalabad. Matured seeds were surface sterilized with 70% (v/v) ethanol for 2 min, followed by 5% (v/v) sodium hypochlorite for 10 min and thoroughly rinsed for five to six times with sterile distilled water. Disinfected seeds were imbibed in autoclaved water for 24 h. Mature embryos were excised from the imbibed seeds and placed on MS (Murashige and Skoog, 1962) basal medium plates (pH 5.7) supplemented with 30 g/L sucrose, 8 g/L agar and variable concentration of 2,4-D(1, 2, 3, 4, 5 and 6mg/L). Variable concentrations of 2,4-D were used in order to optimize the most suitable concentration for callogenesis and regeneration. Plates were incubated in the dark under controlled temperature 25±2°C. Plates were inspected daily for any contaminations. Calli were transferred to fresh media plates after every two weeks interval.

Callus induction frequency, proliferation efficiency and embryogenic efficiency were estimated using following equations.

Callus induction frequency (%) = (No. of embryos produced calli) / (No. of embryos cultured) x 100

Proliferation efficiency (%) = (No. of proliferating calli) / (No. of incubated embryos) x 100

Embryogenesis efficiency (%) = (No. of calli forming shoots) / (Total number of calli) x 100

***In vitro drought stress*:** Healthy, friable and embryogenic calli of 7, 14 and 21 days were transferred to regeneration media containing MS basal media supplemented with kinetin (1.5mg/L) and incubated at 16/8 h day and night length, respectively. One week after incubation on regeneration media calli were divided into two groups as follows.

Group I= Calli subjected to drought stress using PEG 6000 (0, 4, 8, 12 and 16%).

Group II= Calli subjected to drought stress using mannitol (0, 0.5, 1, 1.5 and 2%).

These levels were evaluated in different treatment combinations (Table 1). Six weeks after drought stress, regenerated plantlets were placed on MS medium supplemented with 1.5 mg/L IAA (Indole Acetic Acid) for root regeneration. Data were recorded for regeneration efficiency.

Regeneration efficiency (%) = (No. of plantlets) / (Total number of calli) x 100

***Shoot and root length (cm)*:** Shoot length was measured with the help of meter stick from the point where root begins to the upper tips of the leaves. Root length was measured from the point where shoot begins to the total length of root and data was averaged.

***Electrolyte leakage*:** This technique is based on the increase of cellular membrane permeability and concomitantly greater electrolyte diffusion out of cells when leaf tissue is injured by a stress situation. After harvest, the uppermost fully expanded leaves of 10 plants per treatment were immediately cut into discs of 0.8 cm diameter. The discs were washed briefly three times in deionized water to remove solutes released during cutting of the discs. Five discs of each leaf were then placed in a vial filled with 10 ml de-ionized water and maintained at 20°C for 4h. Electrolyte leakage was determined by measuring the electrical conductivity of the vial solution, using a conductivity meter (High performance laboratory multi-meter CP-500L, BMS, Medifield, USA) and data were expressed as $\mu S\ cm^{-1}$.

***Leaf relative water content (RWC)*:** RWC were measured for five discs (0.8 cm diameter) of each leaf. The leaf discs were obtained similarly as used for electrolyte leakage. Relative water content (RWC) was estimated using the formula of Schonfeld *et al.* (1988).

RWC= (FW-DW)/ (TW-DW) ×100

Where FW, TW and DW represents weight of freshly collected material, weight after rehydration for 20-24 h at

4°C in the dark and weight after drying at 80°C for 48h, respectively.

Table 1. Different treatment combinations for *in vitro* drought stress.

Treat.	Treatment combinations
T1	Shoot regeneration medium (MS1)+ 0%PEG and 0.0% mannitol
T2	Shoot regeneration medium (MS1)+ 0%PEG and 0.5% mannitol
T3	Shoot regeneration medium (MS1)+ 0%PEG and 1.0% mannitol
T4	Shoot regeneration medium (MS1)+ 0%PEG and 1.5% mannitol
T5	Shoot regeneration medium (MS1)+ 0%PEG and 2.0% mannitol
T6	Shoot regeneration medium (MS1)+ 4%PEG and 0.0% mannitol
T7	Shoot regeneration medium (MS1)+ 4%PEG and 0.5% mannitol
T8	Shoot regeneration medium (MS1)+ 4%PEG and 1.0% mannitol
T9	Shoot regeneration medium (MS1)+ 4%PEG and 1.5% mannitol
T10	Shoot regeneration medium (MS1)+ 4%PEG and 2.0% mannitol
T11	Shoot regeneration medium (MS1)+ 8%PEG and 0.0% mannitol
T12	Shoot regeneration medium (MS1)+ 8%PEG and 0.5% mannitol
T13	Shoot regeneration medium (MS1)+ 8%PEG and 1.0% mannitol
T14	Shoot regeneration medium (MS1)+ 8%PEG and 1.5% mannitol
T15	Shoot regeneration medium (MS1)+ 8%PEG and 2.0% mannitol
T16	Shoot regeneration medium (MS1)+ 12%PEG and 0.0% mannitol
T17	Shoot regeneration medium (MS1)+ 12%PEG and 0.5% mannitol
T18	Shoot regeneration medium (MS1)+ 12%PEG and 1.0% mannitol
T19	Shoot regeneration medium (MS1)+ 12%PEG and 1.5% mannitol
T20	Shoot regeneration medium (MS1)+ 12%PEG and 2.0% mannitol
T21	Shoot regeneration medium (MS1)+ 16%PEG and 0.0% mannitol
T22	Shoot regeneration medium (MS1)+ 16%PEG and 0.5% mannitol
T23	Shoot regeneration medium (MS1)+ 16%PEG and 1.0% mannitol
T24	Shoot regeneration medium (MS1)+ 16%PEG and 1.5% mannitol
T25	Shoot regeneration medium (MS1)+ 16%PEG and 2.0% mannitol

***Experimental design and statistical analysis*:** Drought experiments were arranged in a completely randomized design with factorial arrangements, 10 replicates for each treatment. Data were statistically analyzed using ANOVA using software Statistix 8.1. Experiments were performed in triplicate.

RESULTS

***Optimization of protocol for regeneration*:** During allogenesis calli induced at different concentrations of 2,4-D were subjected to regeneration (Table 2). Regeneration efficiency (no. of shoots/ calli) was observed in calli induced at lower concentration 1mg/L (1.5 shoots per explant) of 2,4-D and gradually increased at higher concentration 5mg/L(6 shoots per explant). A decrease in regeneration efficiency was observed for calli induced at 6mg/L of 2,4-D (Fig. 1).

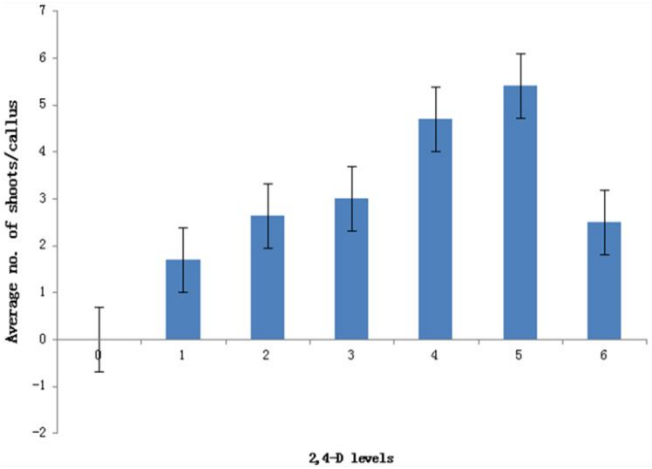

Figure 1. Effect of 2,4-D on regeneration response (average no of shoots/callus). Maximum number of shoots were produced from calli produced on callus induction media containing 5mg/L 2,4-D. T bars show the standard error of the means.

***Age of calli for successful regeneration*:** Reliable plant regeneration from sufficient number of calli enhances the efficiency and use of tissue culture techniques in plant molecular biology. The age of callus is a significant factor that affects differentiation of cells during regeneration. During the present study calli of 7, 14 and 21 days were used and varying levels of regeneration was observed. Seven days old calli showed maximum regeneration. Calli older than 14 days became non-embryogenic (yellowish and wet in appearance (Fig. 2) and kept on proliferating without any regeneration when cultured on regeneration medium. Maximum callus weight (1.46±0.03) was observed in three weeks old calli at 5mg/L of 2,4-D (Table 2). Statistical analysis revealed a significant difference in regeneration for calli of different ages. The maximum regeneration (8 shoots/callus) was observed from 7 weeks old calli initially incubated on medium containing 5mg/L 2,4-D whereas

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

2,4-D concentration (mg/L)	Average fresh weight of calli (g)			Callus induction frequency
	7 days	14 days	21 days	
0	Control	Control	Control	0%
1	0.15±0.02	0.33±0.02	0.30±0.03	71%
2	0.41+0.03	0.69+0.03	0.79+0.03	83%
3	0.94+0.02	1.04+0.05	1.24+0.03	85%
4	1.02±0.03	1.19±0.02	1.36±0.04	89%
5	1.15±0.03	1.38±0.03	1.46±0.03	96%
6	0.52±0.05	0.64±0.03	0.71±0.05	76%

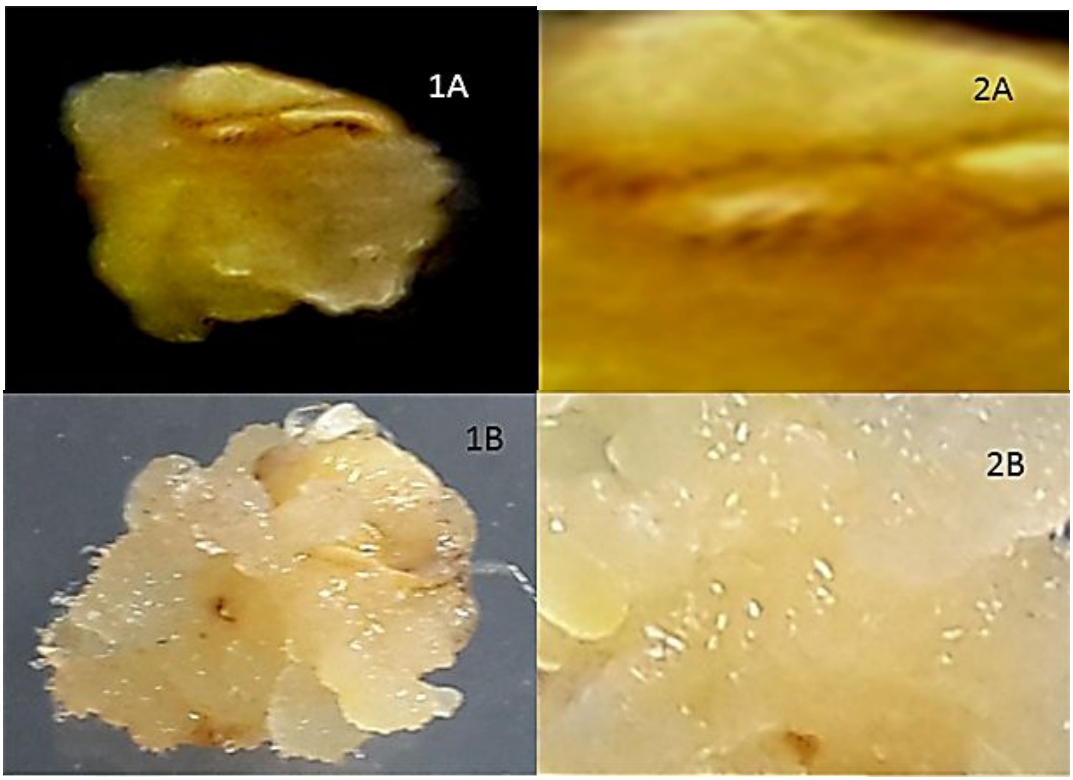

Figure 2. Non-embryogenic and embryogenic callus.
1A: Non-embryogenic callus (at normal resolution) induced from immature embryos, 1B: magnified image, 2A: Embryogenic callus of wheat variety Faisalabad-2008, 2B: magnified image.

minimum (3 shoots/callus) was obtained from 21 days old calli (Fig. 3).

Seven days old calli were highly embryogenic in nature and produced maximum shoots per calli. With increase in calli age number of shoots decreased and minimum number of shoots regenerated from 21 days old calli. T bars show the standard errors.

***Effect of drought on regeneration*:** Use of polyethylene glycol and mannitol in the media significantly decreased regeneration. No regeneration was observed at the highest concentration of polyethylene glycol or mannitol. On the basis of average regeneration response mannitol highly reduced the regeneration rate compared to the PEG6000 (Table 3).

***Shoot and root length*:** Maximum shoot length was recorded in control treatment T1 (8cm) as compared to other treatments while minimum shoot length (2.5cm) was observed at T14 treatment combination and no shoot regeneration was evident at highest level of mannitol (2%) and PEG (16%).

Similarly, root regeneration was also affected by drought stress. Highest root length was observed in T1 treatment (10cm) while minimum at T12 (3cm). At higher treatment levels (T14 to onward) no root regeneration was observed. Both mannitol and PEG induced stress decreased shoot and

root length. Mannitol induced osmotic stress seemed to be more harmful as compared to PEG.

Table 3. Effect of drought stress on root/shoot length, electrolyte leakage and leaf relative water contents.

Treat.	Shoot length (cm)	Root length (cm)	Electrolyte leakage (µS cm^{-1})	Leaf relative water contents (%)
T1	8.0	10	30	95
T2	7.0	8	75	94
T3	6.8	6.5	150	90
T4	6.5	5.5	200	88.5
T5	0	0	0	0
T6	7.5	8	40	93
T7	6.8	6.5	100	90
T8	6.2	4	180	88
T9	5.8	3.5	205	86
T10	0	0	0	0
T11	6.2	6	70	93
T12	5.0	3	135	88
T13	4.0	0	190	85
T14	2. 5	0	220	81
T15	0	0	0	0
T16	6.0	0	80	90
T17	4.8	0	150	86
T18	3.0	0	215	83
T19	0	0	0	0
T20	0	0	0	0
T21	3.5	0	85	82
T22	0	0	0	0
T23	0	0	0	0
T24	0	0	0	0
T25	0	0	0	0

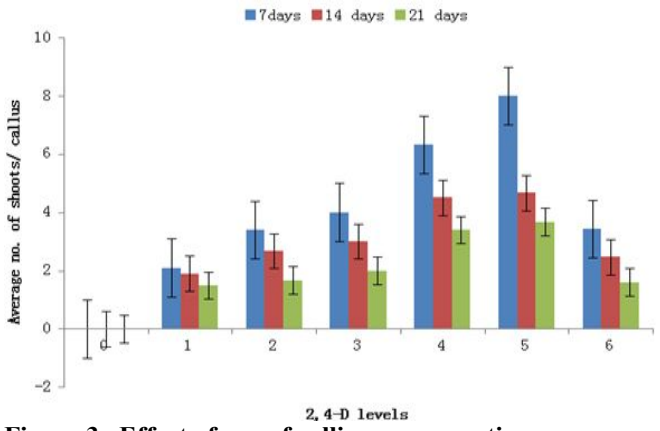

Figure 3. Effect of age of calli on regeneration response.

***Electrolyte leakage*:** The electrolyte leakage was determined from only those treatments where stress allowed shoot regeneration (T1 to T18). No shoot regeneration was evident at T5, T10, T15, T19, T20, T22, T23, T24 and T25 treatment combinations. Solute leakage of leaves under different stress treatment levels differed between regenerated plants ranging from 30 µS cm^{-1} in control plants to 220 µS cm^{-1} at T14 treatment level due to combined effect of PEG and mannitol. The highest level of PEG (T21) proved to be less drastic (85 µS cm^{-1}) as compared to mannitol (T5 , no regeneration was observed).

***Leaf relative water content (RWC)*:** Decrease in leaf relative water contents was observed under both mannitol and PEG induced stress. The highest reduction was noticed under T14 treatment combination. Leaf relative water contents were evaluated in plants with shoot regeneration. Maximum RWC (95%) were measured in leaves obtained from control plants and minimum value (81%) was recorded in T14 treatment combination. At highest level of PEG (16%) RWC were reported to be 82% as compared to 81% at 1.5% of mannitol concentration.

DISCUSSION

***Optimization of regeneration protocol for regeneration*:** Wheat transformation is still a bottleneck in the application of genetic manipulation to this important crop and improvement of efficiency is a priority. Poor tissue culture performance due to wheat recalcitrant behavior *in vitro* is one of the focal reason limiting transformations. As shown by Kumlehn *et al.* (2009) *in vitro* wheat performance is attributed to its genetics. In view of genotypic dependent variation to regeneration in wheat, development of proficient and highly repeatable plant regeneration methodology for each genotype is a pre-requisite (Neelakandan and Wang, 2012) for the successful application of available genetic transformation methods. Therefore, a regeneration protocol was independently optimized for commercial wheat variety Faisalabad-2008 because this variety is among the best as compared to other cultivars on the basis of yield related parameters (Anwar *et al*., 2005; Sultana *et al*., 2013) and commercially grown on large areas of the Punjab Province.

As genotypic effects are unavoidable (Al-Khayri *et al.,* 1996), any strategy to improve plant regeneration must involve the use of apposite explants for initiation of callus cultures and management of culture conditions. In monocots, especially wheat, selection of source material is the major obstacle for *Agrobacterium*-mediated transformation. Regeneration is possible only by using limited number of regenerable and actively dividing cells. In the totipotent cells/ tissues, induction of somatic embryogenesis and regeneration leads to development of transgenic plants.

In wheat, mature embryos derive somatic embryos were amenable to regeneration due to their efficient response to *in vitro* induced stimuli (Chen *et al.,* 2003; Hu *et al.,* 2003; Shewry and Jones, 2005; Jones *et al.,* 2007; Xia *et al.,* 2012). Although other explants have been used for the same

purpose such as shoot tips (Sharma *et al.,* 2005), inflorescences (Ozias-Akins *et al.,* 1982; Caswell *et al.,* 2000; He and Lazzeri, 2001), anthers (Ou *et al.,* 1973), microspores (Liu *et al.,* 2002), immature and mature embryos (Ozias-Akins *et al.,* 1982; Yin *et al.,* 2011), from which some transgenic wheat plants have been generated successfully (Cheng *et al.,* 1997; Wang and Altman, 2009; Li *et al.,* 2012). These tissues varied in regeneration ability *in vitro*; among them the mature embryos were proved to be the most responsive tissues and their availability throughout the year made them more reliable source for *in vitro* manipulation. Elhiti and Stasolla (2011) and Tahir *et al.* (2011) reported that in recent years, most preferred choice of explant for angiosperm and conifer have been mature embryos.

In this study, we illustrated the establishment of regenerable cell cultures in the commercially valuable wheat crop, mature embryos were subjected to exogenously applied auxin 2,4-D, to initiate and maintain undifferentiated growth in plant cell cultures and similar findings have already been reported by Feher (2006). Somatic embryos were formed on nutrient medium with different concentrations of 2,4-D (Delporte *et al.,* 2001). Previous findings have reported that 2,4-D induce the development of embryonic cells by hyper methylation of nuclear DNA (Xiao *et al*., 2006; Legrand *et al*., 2007).

In the present study callus mass proliferated with increase in concentration of 2,4-D up to 5 mg/L but with further increase in its concentration negative growth trend was observed. Our findings are in line with Afzal *et al.* (2010) they reported 76.04% callus induction frequency at 5mg/L of 2, 4-D. Further increase or decrease in its concentration resulted in lower callus induction frequency. Rashid *et al.* (2009) reported that an increase in 2,4-D concentration from 4mg/L to 5mg/L increased calli mass of wheat varieties Inqlab-91, Chakwal-50 and Manthar. At higher concentration of 2,4-D calli mass increased but ratio of non-embryogenic calli was high compared to embryogenic calli.

Munazir *et al.* (2010) reported maximum callus proliferation in Sehar and GA-02 at 2 and 4mg/L 2,4-D using mature seeds as explants. Haliloglu (2006) reported that MS media supplemented with 2mg/L 2,4-D produced 96% embryogenic calli in Bobwhite. These results do not match with our findings probably due to difference in genotypes and suggest that genotypic factor operate in response to particular concentrations of growth regulators.

As far as effect of 2,4-D initially used for callus induction is concerned, it has a significant effect on regeneration efficiency. Mendoza and Kaeppler (2003) reported that concentration and type of auxin used for callus induction also effects regeneration. These results are in agreement with our findings that increase in 2,4-D concentration during callogenesis decreased regeneration efficiency. Nasircilar *et al*. (2006) reported that *T. aestivum* cultivar, Yakar and *T. durum,* Kiziltan gave the highest regeneration response on MS media containing 2mg/L 2,4-D using mature embryos as explant.

Age of calli also depends on the amount of 2,4-D (used for callus induction) for regeneration. Many studies have demonstrated that the use of 2,4-D is critical for induction of somatic embryogenesis (Choi *et al.,*2000; Wang *et al*,, 2004). However, in some species and genotypes, continuous long-term auxin treatment can inhibit somatic embryogenesis and organogenesis completely. The response of explants cultured on media containing 2,4-D depends not only on the concentration of the phytohormone but also, and to a great extent, on the duration of exposure to growth regulators. The meristematic sites probably could develop in roots or shoots only when transferred to regeneration medium. Continuous long term auxin treatment inhibited the morphogenic response of explants, a phenomenon which should be considered when experiments are planned.

The present study was also conducted to investigate the effect of drought on regeneration of plantlets. Drought or water deficit impose many agronomic and physiological effects on plants. This abiotic factor severely limits plant growth and development. Wheat yield is severely limited by drought (Blanco *et al*., 2004) resulting a decrease in shoot and root length (Wardlaw and Willenbrink, 2000). Results of direct exposure to PEG and mannitol revealed that increasing water stress caused a sharp reduction in shoot and root length and they proved to be a good indicator of plant growth.

There are a number of physiological indices for drought tolerance in wheat. Leaf relative water contents (RWC) have been used by many researchers (Chandrasekar *et al.,* 2000; Rampino *et al.,* 2006) as an efficient parameter for screening drought tolerant wheat genotypes. Control treatment showed highest RWC as compared to other treatment combinations. RWC reflects plant metabolic activity and water status as reported by Nayyar and Gupta, (2006). Siddique *et al.* (2000) reported that water deficit conditions can be tolerated by wheat genotypes with higher RWC. Thompson, (1988) reported that drought also effect cell membrane integrity in the subject plants and estimated through electrolyte leakage from membrane. Membrane damage decrease RWC and this might accelerate senescence. Hadi *et al.* (2004) reported that high water content was noted in progressive mild stress than severe stress indicating that plants have the ability to sustain their water content under mild stress, whereas this ability is lost under severe stress.

***Conclusion*:** In the present study effect of concentration of 2,4-D and water stress on callus induction and regeneration efficiency was investigated for wheat variety Faisalabad-2008. Our results indicate that 5mg/L of 2,4-D and 21 days old calli is the best combination for the maximum regeneration. Mannitol and PEG were used to impose

drought stress. PEG and mannitol induced drought stress efficiently but mannitol were more severe. These findings will be helpful to improve tolerance of wheat against water stress.

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