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PRIMARY CALLUS INDUCTION, SOMATIC EMBRYOGENESIS AND REGENERATION STUDIES IN SELECTED ELITE WHEAT VARIETIES FROM PAKISTAN

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Abstract

In the present study, effects of varying concentrations of growth regulators including 2, 4-D (2,4-Dichlorophenoxyacetic acid), BAP (6-benzylaminopurine), IAA (indole-3-acetic acid) and Kin (kinetin) was investigated for primary callus induction, embryogenic callus formation and regeneration of two elite wheat varieties viz., Sahar and GA-02. Mature seeds were used as explants for primary callus induction. Different concentrations of 2, 4-D were investigated to study its effect on callus induction and callus growth frequency (CGF). Compact, whitish, healthy and fluffy calli were induced in Sahar and GA-02 in MS medium supplemented with 5 mg/l and 4 mg/l 2, 4-D respectively. The calli produced were subjected to somatic embryogenesis and regeneration studies. For this purpose, MS Medium was supplemented with different concentrations and combinations of plant growth regulators like IAA and BAP. Maximum embryogenic callus formation was observed in MS medium supplemented with 0.1 mg/l IAA in combination with 1.5 mg/l BAP and it resulted in 73.51% and 62.33% embryogenic callus formation in Sahar and GA-02 respectively. These optimum concentrations of IAA and BAP were further experimented with different concentrations of Kin for efficient regeneration and it was observed that 1 mg/l Kin was optimum for this purpose. Such studies help in understanding the response of wheat to tissue culture conditions and ultimately promise in improving yield by employing various biotechnological techniques.

Introduction

Bread wheat (*Triticum aestivum* L.) is one of the major staple food crops grown worldwide (Zhou *et al*., 2003; Bhalla *et al*., 2006) that covers almost 17% of total area of world under cultivation. It provides basic needs of energy, proteins, vitamins (e.g. Vitamins B and E) and minerals (e.g. magnesium and phosphorous) (Jones, 2005). Recently, there is progressive increase in wheat yield; however, there exists a gap between wheat yield and demand throughout the world for burgeoning human population (Bhalla, 2006). It has been estimated that a significant increase of more than 40% in wheat yield is required by 2020 to meet growing demand of human population (Janakiraman *et al*., 2002).

Conventional plant breeding methods although have been practiced successfully since 1960s for the production of improved wheat varieties but have limited potential to meet such a great challenge due to availability of limited gene pool (Janakiraman *et al*., 2002). Genetic engineering techniques are gaining popularity and any desired gene isolated from any genetic background can be introduced in wheat genome thus imparting in improved characters (Malik *et al*., 2003). Transformation of various cereal crops

including wheat to tackle with various biotic and abiotic stresses that cause reduction in their yield is now in practice in many countries of the world and is contributing in the varietal improvement (Jones *et al*., 2005). However, these methods are entirely dependent upon successful, reliable and reproducible regeneration of transformed explants through tissue culture (Yu *et al*., 2008). So, establishment of reliable tissue culture protocols for callus induction, somatic embryogenesis and regeneration is desired in order to improve wheat yield (Noor *et al*., 2009).

Studies on wheat tissue culture revealed that regeneration can be brought about either by somatic embryogenesis or adventitious bud and shoot development with subsequent rooting (Bhaskaran & Smith, 1990). Direct organogenesis has also been studied in wheat (Li *et al*., 1992). Somatic embryogenesis for regeneration of wheat plants can be brought about in tissue culture through a callus phase initiation from a zygotic embryo. It was observed by various researchers that callus formation was successful in wheat (Shimada *et al*., 1969). Recently various explants of wheat have been reported to be successful in callus induction that include young spikes, stem sections and nodes of wheat (Lu *et al*., 1988); shoot tips (Viertel & Hess, 1996); glumella and lemma (Lu, 1992); mature and immature wheat embryos (Ozgen *et al*., 1996; Sarker *et al*., 2007). Immature embryos have been reported by various researchers to be the most responsive to tissue culture conditions and showed relatively high rate of callus induction as compared with mature embryos (Shah *et al*., 2003). However, mature embryos showed better regeneration response than immature embryos (Ozgen *et al*., 1996). Moreover, mature embryos are readily available throughout the year and being used for transformation studies in the recent years (Patnaik *et al*., 2006), thus necessitating the need for optimization of tissue culture protocols. Growth and morphogenesis is remarkably affected by supplementing tissue culture medium with plant growth regulators (PGR) including auxins and cytokinins. Various studies investigated factors which affect the plant regeneration in tissue culture including composition of culture medium, genotype and environmental factors (Mathias & Simpson, 1986; Fennel *et al*., 1996; Uppal *et al*., 1996; Wang & Wei 2004). The objective of the present study was to optimize a tissue culture protocol for callus induction and regeneration in two elite wheat cultivars viz., Sahar and GA-02.

Materials and Methods

Efficient regeneration system *via* somatic embryogenesis was developed for two local wheat varieties e.g., Sahar and GA-02. Wheat program of National Agricultural Research Center Islamabad provided seeds of these varieties. Mature and healthy seeds were first surface sterilized by washing with a detergent (Tween-20) for 2-3 minutes, rinsed properly with autoclaved distilled water and then with 50% Clorox (sodium hypochlorite) for 20 minutes for surface sterilization of explants. These were then thoroughly washed with autoclaved distilled water four to five times for complete removal of Clorox. Surface sterilized seeds were then inoculated on MS (Murashige & Skoog, 1962) medium which was supplemented with 3% (w/v) sucrose as a source of carbon and subsequently solidified by adding 0.6% (w/v) agar. The pH of this medium was adjusted at 5.75 and then autoclaved at 121°C at a pressure of 105 kPa for 20 min. The seeds were inoculated on MS medium which was supplemented with different concentrations of 2,4-D corresponding to a total of 8 treatments i.e., 1mg/l, 2mg/l, 3mg/l, 4mg/l, 5mg/l, 6mg/l, 7mg/l and 8mg/l each replicated 4 times for callus induction. After inoculation, cultures were incubated in dark for 4-5 days at 25±1°C. Contaminated explants were removed from cultures. For callus proliferation, cultures were shifted to

growth chamber and kept for 16 hours in white fluorescent light with eight hours dark interval in every 24 hours for two weeks. The best concentration of 2,4-D that resulted in optimum callus induction was recorded. Healthy, friable and compact calli initiated from zygotic explants (seeds) were carefully separated from other tissues with a sharp sterilized blade under aseptic conditions, cut into small manageable sizes (3-5 pieces of each callus) and sub-cultured onto fresh MS medium containing already optimized concentration of 2,4-D for a continued supply of healthy calli for further studies. The healthy calli were aseptically transferred to fresh MS medium containing different concentrations and combinations of IAA and BAP (Table 1), to find out the most appropriate treatment for somatic embryogenesis. The cultures were maintained in controlled environment of growth chamber.

For plant regeneration, somatic embryos were aseptically cut from embryogenic calli and cultured in MS medium containing optimized concentrations of IAA and BAP (i.e. 0.1 mg/l IAA and 1.5 mg/l BAP) along with different concentrations of Kin (i.e.0mg/l, 0.1mg/l, 0.5mg/l and 1mg/l.) Cultures were maintained in the same controlled conditions of light and temperature as embryogenic calli were kept.

Results and Discussion

Callus induction: In the present study, different concentrations of 2, 4-D (i.e., from 1mg/l. to 8 mg/l.) were experimented to find out the best concentration for successful callus induction. It was found that a concentration of 5mg/l and 4 mg/l 2,4-D was optimum for callus induction in Sahar and GA-02 respectively i.e., 71% in Sahar and 82.60% in GA-02. The results revealed that at very high and very low concentrations of 2, 4-D, there was a reduction in callus induction frequency and subsequent growth of calli. This indicates that a suitable concentration of 2, 4-D is necessary for callus induction and subsequent proliferation in tissue culture. It has already been documented that no callus formation takes place in mature wheat embryos at lower concentrations of 2,4-D (Yasmin *et al*., 2001). Callus induction media have been optimized by various researchers at varying concentrations of 2,4-D like 2mg/l (Farooq *et al*., 2004; Rashid *et al*., 2009), 3mg/l (Noor *et al*., 2009, Rashid *et al*., 2009), 3.5 mg/l (Shah *et al*., 2003), 5.5 mg/l (Sarkar & Biswas, 2002) and 6 mg/l (Rahman *et al*., 2008). This difference in results in all these cases may rightly be regarded to difference in varieties, sources of explants and tissue culture conditions.

It was observed that at different concentrations of 2,4-D, tendency of direct shoot regeneration and callus induction have an inverse relationship i.e., with a gradual increase in concentration of 2,4-D, frequency of direct shoot regeneration decreased and the tendency for callus induction gradually increased (Alizadeh *et al*., 2004). By using optimum concentration of 2,4-D, maximum callus induction was observed and no shoots were developed in any explant. It is suggested that supplementation of growth medium with different concentrations of growth regulators affects endogenous concentration and control of biochemical aspects of plant growth regulators.

Comparison of Callus Induction Frequency (CIF) and Callus Growth Frequency (CGF): Proper callus induction and its rapid proliferation to form a healthy callus is vital to tissue culture. If calli are induced but no proliferation takes place then such calli are of no use and are discarded. In the present study, it was observed that at different concentrations of 2,4-D, callus induction and callus growth frequencies were different in both the varieties under investigation. The results revealed that at very high and very low

concentrations of 2,4-D (i.e., at 2 mg/l. and 8 mg/l.), callus induction and growth frequencies were low (Table 2). At these concentrations, an initial swelling was observed and smaller, un-healthy, brownish and watery calli were produced which did not proliferate further. Such calli were of no use and discarded. On the other hand, at a concentration of 4 mg/l of 2,4-D, callus induction and callus growth frequencies were higher. The calli produced at 4 mg/l were whitish, compact, healthy and fluffy (Fig. 2*A*, 2B, 2C and 2D). These results show that an optimum concentration of 2,4-D is required for proper callus induction and subsequent growth.

Table. 1. Different concentrations and combinations of IAA and BAP used for somatic embryogenesis in wheat varieties viz., Sahar and GA-02.

Treatments	IAA	BAP	Treatments	IAA	BAP
T1	0	0	T7	0.1	1.5
T2	0	0.5	T8	0.1	2.5
T3	0	1.5	T9	0.2	0
T4	0	2.5	T10	0.2	0.5
T5	0.1	0	T11	0.2	1.5
T6	0.1	0.5	T12	0.2	2.5

Table 2. Comparison of callus induction and callus growth frequencies in Sahar and GA 02 at different concentrations of 2,4-D.

Concentration of 2,4-D (mg/liter)	Callus induction frequency (CIF)		Callus growth frequency (CGF)	
	Sahar	GA-2002	Sahar	GA-2002
8	17	15	0	0
7	20	14	0	0
6	22	20	2	5
5	51	49	10	3
4	55	60.56	40	53.52
3	51	48	30	23
2	7	4	0	0

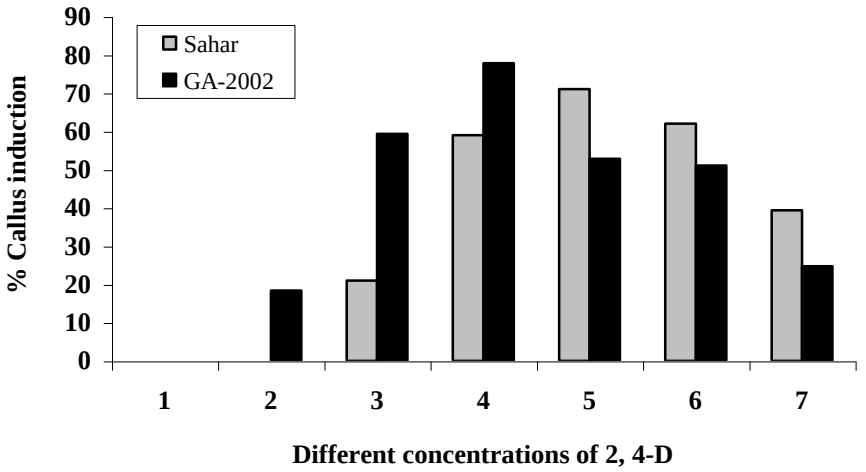

Fig. 1. Comparison of callus induction frequency in Sahar and GA-02 at different concentration of 2,4-D ranging from 1-8mg/l corresponding to seven treatments.

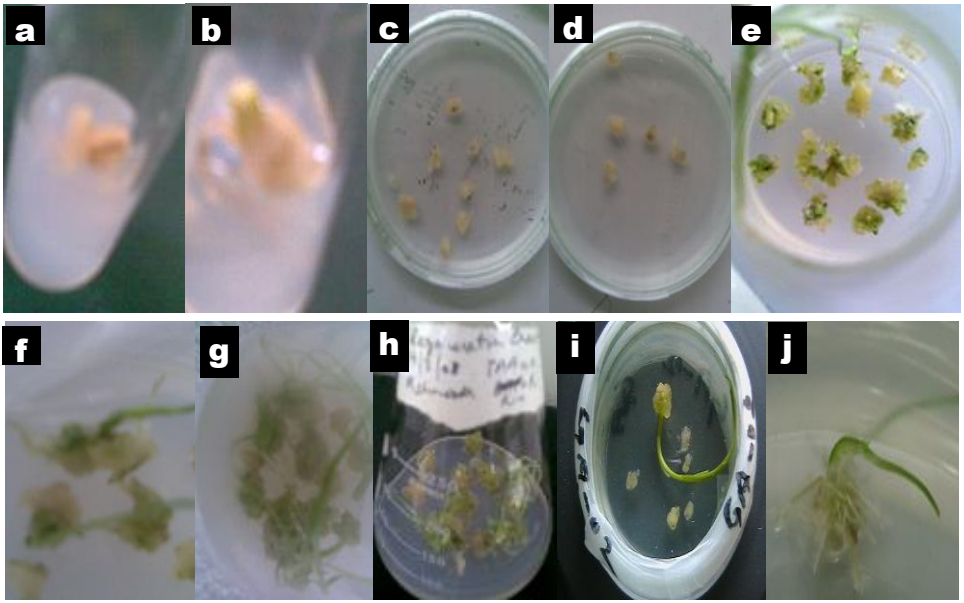

Fig. 2. Effect of different concentrations of growth regulators on callus induction, somatic embryogenesis and regeneration in two elite wheat varieties, GA-02 and Sahar; *A* callus formation in Sahar; *B* callus formation in GA-02; *C* maintained calli of Sahar; *D* maintained calli of GA-02; e embryogenic calli of Sahar showing green spots; f embryogenic calli of GA-02; *G* and *H* calli of Sahar showing regeneration; *I* and *J* calli of GA-02 showing regeneration

Somatic Embryogenesis and Regeneration: Different concentrations of IAA and BAP were experimented to optimize the best concentrations of these hormones for the formation of embryogenic callus in both the varieties under study. It was observed that somatic embryo formation occurred in primary calli within 2 weeks thus confirming the role of these plant growth regulators in embryogenesis. A combination of IAA and BAP (0.1 mg/l IAA + 1.5 mg/l BAP) resulted in highest frequency of embryogenic callus formation (Fig. 3). The calli thus produced were healthy and were capable of undergoing regeneration (Fig. 2*E* and 2*F*).

Regeneration is a crucial step in a successful transformation system because transformed healthy explants should produce a fertile transgenic plant (Jones, 2005). Various different explants can be used for *In vitro* regeneration in wheat including immature embryos, mature embryos, seeds, endosperm, root tips, leaves and shoot bases (Sarker & Biswas, 2002). Among all these explants, immature embryos have been reported to be the most efficient in callus induction and shoot regeneration (Sarker & Biswas, 2002). But immature embryos can be available in wheat growing season only. To ensure their continued availability throughout the year, expensive and sophisticated growth chambers are required which is a serious disadvantage associated with their use as explants. In contrast, mature embryos are readily available throughout the year and hence they are a suitable alternative as explants for tissue culture studies (Rahman *et al*., 2008).

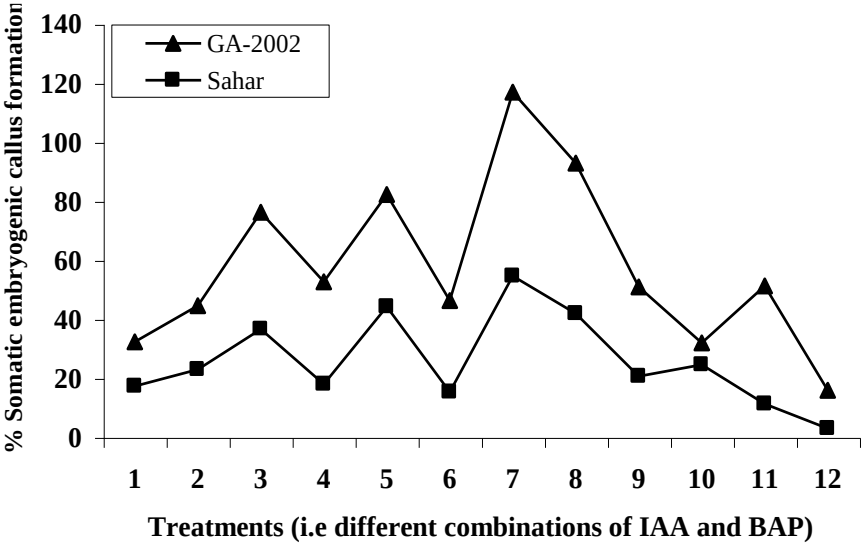

Fig. 3. Comparison of somatic embryogenesis in wheat varieties at different concentrations and combinations of growth regulators.

Auxins and cytokinins are the major growth regulators that affect various aspects of plant cell division, differenciation and organogenesis (Tang *et al*., 2000; Fehe'r *et al*., 2003; Nikolic *et al*., 2006). In present study, effects of varying concentrations of a cytokinin i.e., Kinetin (Kin) on regeneration was studied. For this purpose, 3-weeks old healthy embryogenic calli initiated from seeds of both varieties were transferred to MS medium (containing already optimized concentrations of IAA and BAP) that was supplemented with different concentrations of Kin and maintained in the growth chamber. It was observed that Kin at a concentration of 1 mg/l resulted in maximum regeneration in embryogenic calli (Fig. 2*G, H, I* and *J*). Effect of Kin has been investigated in wheat regeneration but different results obtained regarding optimization of regeneration medium. On one hand, its effectiveness in wheat regeneration has been appreciated and it resulted in maximum regeneration at a concentration of 1 mg/l (Rahman *et al*., 2008), 3 mg/l (Sikandar *et al*., 2007) and 0.5 mg/l (Shah *et al*., 2003) and on the other hand, its role in wheat regeneration has been negated as it had no effect on regeneration when different concentrations of it i.e. 0 to 5 mg/l were employed (Anju *et al*., 2003). The variation in results may be due to difference in explant sources.

In the present study, it has also been observed that GA-02 was more responsive to regeneration as compared to Sahar, which clearly shows that there exists variation among different varieties in their tissue culture response (Fig. 4). Effect of genotype in regeneration has been intensively studied and appreciated not only in wheat but other cereal crops as well (Sears & Deckard, 1982; Maddock *et al*., 1983; Ma *et al*., 1987; Chowdhury *et al*., 1991; Fennel *et al*., 1996). It is believed that various genes regulate regeneration in all plant species but studies are lacking on this aspect and need to be conducted in the future.

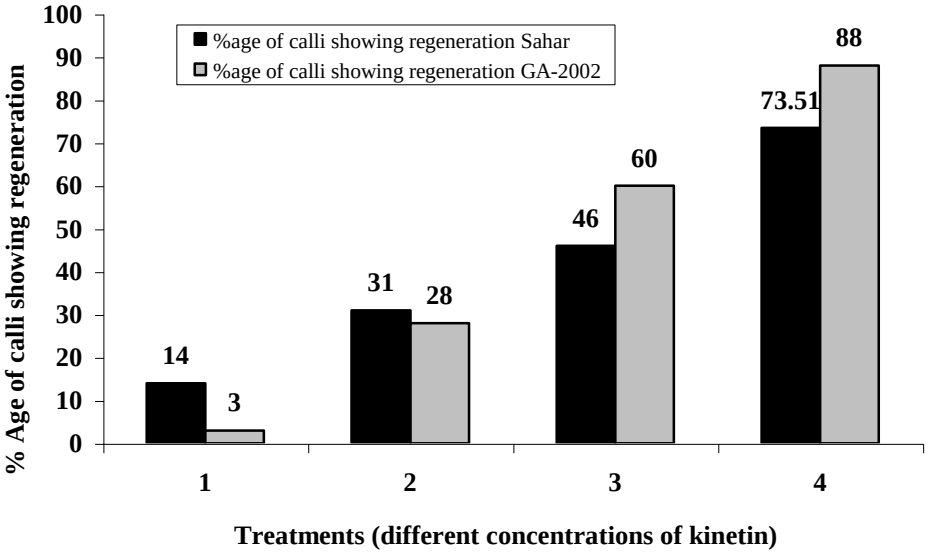

Fig. 4. Comparison of regeneration in wheat varieties, Sahar and GA-02 at different concentrations of kinetin used in combination with optimized concentrations of IAA and BAP.

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