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IN VITRO PLANT REGENERATION IN BREAD WHEAT (*TRITICUM AESTIVUM* L.)

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Abstract

Callus was induced on immature embryos of wheat *(Triticum aestivum* L.) cvs. Sarsabz, Khirman and Soghat-90. The application of different phytohormone concentrations yielded differential behaviour of genotypes for callus induction, mainly due to the genotype and media interaction. Variety Khirman produced highest callus on media (M1). Callus proliferation was also influenced by genotype and media interaction. Best callus proliferation was observed in Khirman on media (M4). Data revealed that Soghat-90 showed weakest interaction with present media compositions used in this study. Similarly regeneration potential was also subjective to media and genotype interaction. Data on chlorophyll mutants were also collected to study the frequency of genetic variation produced by each variety due to media effect. Green plantlets were transplanted in the field after weaning for evaluation of genetic variability.

Introduction

Wheat (*Triticum aestivum* L.) is one of the most important staple food crop of the family *Poaceae.* Among the food crops, wheat is a common source of energy and proteins for the world population. A number of environmental factors such as temperature, moisture, soil and light intensity affect the growth and yield of wheat (Keresa *et al.*, 2000). It is characterized by a large genome size (approximately 17000 Mb) thus making the improvement process by any method genetically challenging. However, as is the case for many members of the monocot species, not all wheat species respond to the *In vitro* conditions. Therefore, selection of appropriate genotype for *in vitro* manipulation is the primary task for any *In vitro* study. Callus induction and plant regeneration both are independent phenomenon in wheat (Papenfus & Carman, 1987; Ozgen *et al*., 1998; Benkirane *et al*., 2000). It has been observed that wheat produces two types of calli viz., embryogenic and non-embryogenic (Benkirane *et al*., 2000). Redway *et al*., (1990) established stable cell suspension cultures from two type of callus: one compact, nodular and embryogenic and the other friable and embryogenic, both derived from cultured immature embryos of wheat cultivars. Ozgen *et al.*,(1998) reported that mature and immature embryos of durum wheat when cultured on MS medium supplemented with 2, 4-D, the mature embryos had low frequency of callus formation but a high regeneration capacity as compared to immature embryos. Immature embryos when cultured in liquid MS supplemented with $2mg1^{-1}$ 2,4-D produced more genetic variation as compared to solid media (Ahmed & Sagi, 1993; Zheng & Konzak, 1999; Arun *et al*., 1994). According to Benkirane *et al*., (2000), immature inflorescences gave high frequencies of embryogenic callus when BAP was added in the media along with 2,4-D.

Callus induction and regeneration from immature and mature embryos and immature inflorescence have proved to be genotype –dependent and strongly influenced by the components of the medium used (Carman *et al.,* 1988; Hanzel *et al*., 1985). Organogenesis from callus tissue depends upon the plant species, type of explant from which the callus was derived, age of callus tissue and composition of the nutritional

medium (Elwafa & Ismail, 1999; Alok *et al*., 1999). Plant regeneration is one of the critical steps of plant transformation (Keresa *et al*., 2000). A number of workers have reported the regeneration of wheat plants from callus culture derived from various plant parts but the frequency of green plant regeneration was very low (Yurkova *et al.*, 1981; Ahmad *et al*., 2002; Ayes and Kenanturgut, 2006). Green plantlet production has been improved by cold treatment of the explant (Galiba 1994; Jain, 2001). A number of useful wheat variants has been developed through tissue culture for drought tolerance (Gawande *et. al*., 2005; Bajji *et al.,* 2004), salt tolerance (Zair *et al*., 2003; Yadav *et al*., 2004), disease resistance (Svabova & Lebeda, 2005), herbicide resistance (Saunders *et al*., 1992), resistance against *Helminthosporium sativum* (Chawla & Wenzel, 1987; Yadav *et al*., 2000; Ye *et al.,* 1998; Machii *et al*., 1998; Cai *et al*.,1999); increase in the gliadin and glutenin subunits and protein content in the seed (Hu *et al.,* 1998; Villareal *et al.*1999), developed six new wheat lines for resistance to powdery mildew through *In vitro* culture selection.

The present study was undertaken to develop efficient *In vitro* plant regeneration from immature wheat embryos of three cvs. Khirman, Sarsabz and Soghat-90. All the approaches were focused on the establishment of a regeneration system *via* callus induction which requires a long time interval for the development of whole plant.

Material and Method

Three cvs., Khriman, Sarsabz and Soghat-90 of bread wheat (*Triticum* aestivum L.) were studied for *In vitro* manipulation. The spikes were surface sterilized with 70% alcohol for 5 minutes. The immature embryos were aseptically removed from the imbibed seeds. Ten embryos were cultured per bottle on modified Murashige & Skoog media (Murashige & Skoog, 1962) containing different concentrations of auxins and cytokinin.

Following media were used:

Callus induction

M1 = 2,4-D 1mg/l+Kin 1mg/l+ NAA 2mg/l
M2 = 2,4-D 4mg/l
M3 = 2,4-D 4mg/l+Kin 1mg/l +NAA 2 mg/l

Callus proliferation

M4 = 2,4-D 4mg/l+Kin 1.5mg/l+ NAA 2mg/l
M5 = 2,4-D 1mg/l+Kin 1mg/l +BAP 0.5mg/l
M6 = 2,4-D 1mg/l+IAA 1mg/l +BAP 0.5 mg/l

Plantlet regeneration

M7 = IAA 5.7 mg/l+ Kin 5mg/
M8 = IAA 2mg/l + BAP 2mg/l + IBA 2mg/l
M9 = Kin 1.5mg/l + NAA 2mg/l
M10 = IBA 2mg/l + IAA 2mg/l + Kin 2mg/l
M11 = Kin 2mg/l+ 2ip 5mg/l

The media contain 3% commercial sugar solidified with 3g/liter gelrite. The pH was adjusted to 5.7 before autoclaving at 120^{o}C for 15 minutes. Immature embryos were placed with scutellum in up-ward direction on a solid agar medium. All the cultures were incubated at 25 ± 2^{o}C with 16 hours photoperiod with a photosynthetic photon flux density (PPFD) of 83.6 (E 8m^{-2} s^{-1} provided by white fluorescent tubes. Regenerated plants bearing well developed roots were transferred to jiffy pots containing soil and organic manure (3:1) and kept in a screen house under shade for 15-20 days. In the first week of transfer, the plantlets were covered with polythene covers to maintain humidity. After 15-20 days of hardening, the plantlets were subsequently transferred into the earthen pots. Finally well developed plantlets were transplanted in the field.

Statistical analysis: Each treatment was repeated in triplicate with 50 explants per treatment. Green shoots on callus were counted for calculating the shoot organogenesis. The mean and standard deviations were computed from each treatment. Data were analysed and means were compared during Duncan Multiple range test.

Results and Discussion

Callus induction: The different combinations of phytohormones (2.4-D, NAA, Kin) used for callus induction for three different wheat cvs., revealed that callus induction was dependent on genotype and medium interaction (Table 1). Best callus was observed in Khirman on media containing 1mg/l 2, 4-D + 1mg/lKin+ 2mg/l NAA (Fig. 1). In variety Sarsabz, maximum callus induction was observed on M3 medium containing 2, 4-D 4mg/l+ Kin 1mg/l+ NAA 2mg/l whereas, media M3 did not produce good callus of Soghat-90 and Khirman. Khirman produced maximum callus (1.62g) on M1 media followed by Sarsabz (1.58g) in M3 media. Mohammad (1993), observed good callus from matured seed embryos of spring and winter genotypes of wheat. On contrary, Bartok & Sagi (1990) obtained high amount of callus on low concentration of 2,4-D (2mg/l).

Table 1. Callus induction (g) in *Triticum aestivum* of immature embryos on MS medium supplemented with different concentrations of phytohormones.

Concentrations of auxins	Sarsabz	Soghat-90	Khirman	Mean of callus
2,4-D 1mg/l+ Kin 1mg/l+ NAA 2mg/l (M1)	1.23 bc	1.34 abc	1.62 a	1.39
2,4-D 4mg/l (M2)	1.30 abc	1.40 abc	1.24bc	1.31
2,4-D 4mg/l+Kin 1mg/l +NAA 2 mg/l (M3)	1.58 ab	1.16c	1.17 c	1.30
Mean of varieties	**1.37**	**1.30**	**1.34**	**1.33**

L.S.D =0.05 = 0.39

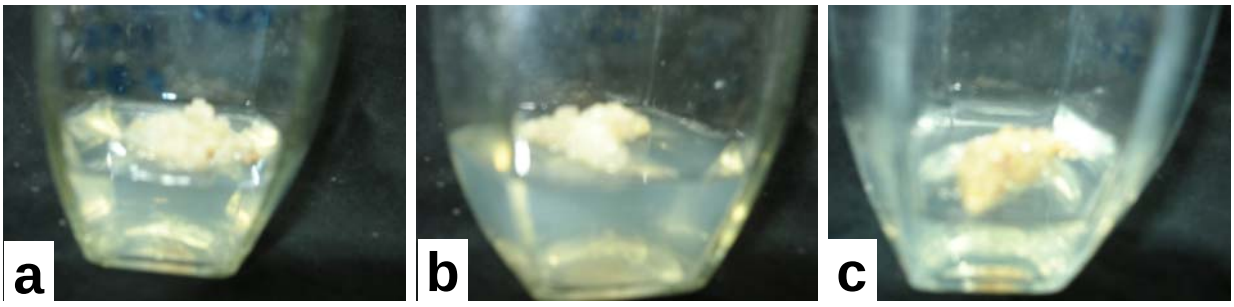

Fig. 1. Callus induction in *Triticum aestivum* of immature embryos on MS supplemented with different concentrations of phytohormones a. 2,4-D 1mg/l +Kin 1mg/l+NAA 2mg/l, b. 2,4-D 4mg/l, c. 2,4-D 4 mg/l +Kin1 mg/l+ NAA 2mg/l.

Callus proliferation: Three different combinations of phytohormones (2.4-D, NAA, Kin and BAP) were used for callus proliferation (Table 2). The data revealed that callus proliferation was affected by phytohormone combination/concentration in wheat genotypes (Fig. 2). The best callus proliferation was observed on medium containing 2,4-D 4mg/l + Kin 1.5mg/l + NAA 2mg/l, followed by media containing 2,4-D 1mg/l + Kin 1mg/l + BAP 0.5mg/l. The low callus proliferation was observed in Soghat-90 and Khirman on media containing 2,4-D 1mg/l + IAA 1mg/l + BAP 0.5mg/l. The maximum callus proliferation was observed in Khirman (1.70g) followed by Sarsabz (1.65g). The analysis of variance for callus proliferation of wheat showed that in varieties Sarsabz and Soghat-90, auxins concentrations and their interactions were non significant. Good callus growth was observed on MS medium containing 2,4-D 4mg/l and similar result was reported by Tanzarella & Greco (1985).

Table 2. Callus proliferation (g) in *Triticum aestivum* of immature embryos on MS medium under different concentration of phytohormones.

Concentrations of auxins	Sarsabz	Soghat-90	Khirman	Mean of callus proliferation
2,4-D 4mg/l+Kin 1.5mg/l+ NAA 2mg/l (M4)	1.30abc	1.42abc	1.70 a	1.47
2,4D1mg/l+Kin1mg/l+BAP0.5mg/l (M5)	1.36 abc	1.49 abc	1.35 bc	1.4
2,4-D 1mg/l+IAA 1mg/l +BAP 0.5 mg/l(M6)	1.65 ab	1.26 bc	1.25 c	1.38
Mean of varieties	**1.43**	**1.39**	**1.43**	**1.41**

L.S.D (0.05) = 0.40

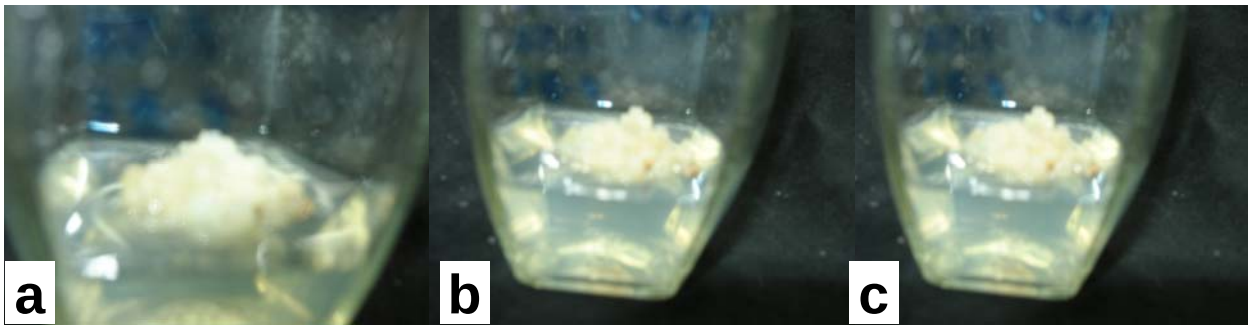

Fig. 2. Callus proliferation of different wheat genotypes under different phytohormone concentration a. 2,4-D 4mg/l +Kin 1.5mg/l+NAA 2mg/l, b. 2 .2,4-D 1mg/l +Kin 1mg/l+BAP 0.5mg/l, c. 2,4-D 1mg/l +IAA+ BAP0.52mg/l.

Regeneration: Regeneration started with the appearance of green dots on callus after 6 weeks of incubation on regeneration medium and generally produced normal stem and leaves. Sarsabz, showed best regeneration on M7 media (IAA 5.7mg/l + Kin 5mg/l) followed by M8 media (IAA 2mg/l + BAP2mg/l + IBA2mg/l) (Fig. 3). The minimum plantlet regeneration was recoded in M11 (Kin 2mg/l + 2ip 5mg/l) (Table 3). The maximum number of plantlet regeneration was observed in Sarsabz followed by Soghat-90 in media M7, while Soghat-90 showed minimum plantlets regeneration on media M11. Bhaskaran & Smith (1990) reported that in certain cases, media supplemented with 0.5mg/l BAP without auxin also showed shoot and root differentiation. Although, the role of cytokinins BAP in conferring competence to regenerate in cereals is not very clear. Saad *et al.*, (2004) reported the highest frequency of green spot formation and plant regeneration (84%) in Inquilab-91 and (52%) in Pavon at 0.5 and 0.1mg/l of BAP and IAA respectively. Gul *et al.*, (2006), observed maximum regeneration on medium containing only 2, 4-D in certain wheat lines, whereas, the variety Yilmaz exhibited best regeneration on media containing MS+ NAA. This confirms that genotype and media interaction plays pivotal role in *In vitro* manipulation of wheat.

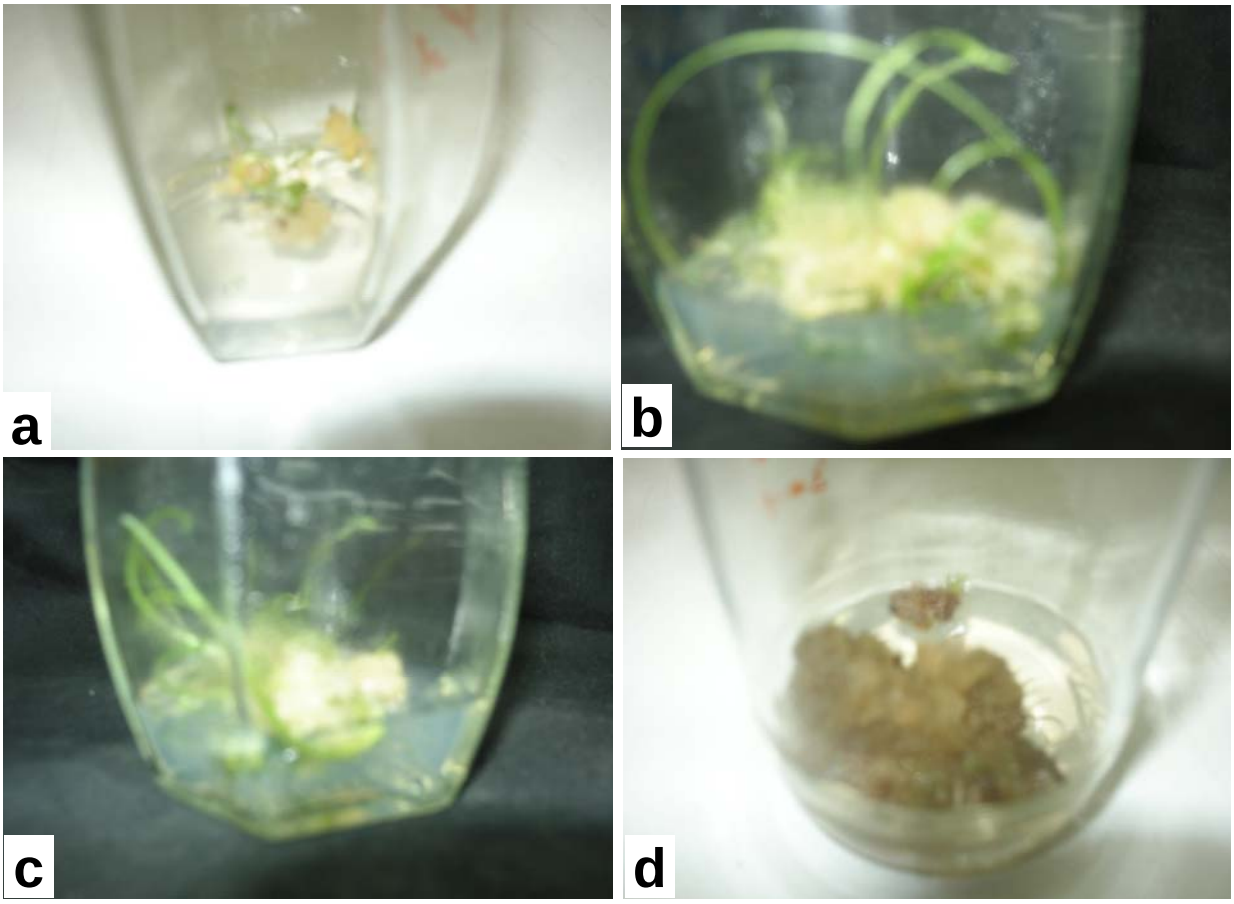

Fig. 3. Plantlet regeneration in *Triticum aestivum* a. Kin 1.5mg/l+NAA 2mg/l, b. IAA 2mg/l +BAP 2mg/l+IBA2mg/l, c. Kin 2mg/l 2ip 5mg/l d.IAA5.7mg/l+Kin 5mg/l.

Table 3. Plantlet regeneration *Triticum aestivum* callus derived from immature embryos of MS medium under different concentration of phytohormones.

Concentrations of auxins	Sarsabz	Soghat-90	Khriman	Mean of plantlet
IAA 5.7 mg/l+ Kin 5mg/l. (M7)	6.00 a	3.66 bc	2.00 cde	3.88
IAA 2mg/l+ BAP 2mg/l+IBA 2mg/l(M8)	4.00 b	3.00 bcd	1.33 de	2.77
Kin 1.5mg/l + NAA 2mg/l(M9)	3.00 bcd	2.33 bcde	2.33 bcde	2.55
IBA 2mg/l +IAA 2mg/l + Kin 2mg/l(M10)	1.33 de	1.66 de	1.33 de	1.44
Kin 2mg/l+ 2ip 5mg/l(M11)	1.33 de	1.00 e	2.00 cde	1.44
Mean of varieties	**3.13**	**2.33**	**1.79**	**2.41**

L.S.D (0.05) = 1.94

Chlorophyll deficiency or albinism is a standard marker in plant cytoplasmic genetics. Its stability is consistent with mutations in the plastid genome because nuclear mutation induces plastid ribosome deficiency (Smulders, 2005). A chlorophyll deficient phenotype can also be the result of recessive mutations such as *iojap* in maize and *albostrians* in barley (Hanzel *et al.*, 1985). Maximum number of chlorophyll mutants were observed in variety Soghat-90 (78.94%) and minimum (9.09%) was recorded in Sarsabz (Table 4). The presence of chlorophyll deficient plantlets confirmed the induction of genetic variability. Plants obtained through *in vitro* cultures gave phenotypic variability that was due to true genetic changes (Orton, 1980; Liu & Chen, 1978a, 1978b). Chaleff & Keil, (1982), reported that some phenotypic variability was the result of physiological changes during *In vitro* conditions; hence such plantlets normally revert to their parent type in field conditions.

Table 4. Number of chlorophyll mutants regenerated of *Triticum aestivum* callus derived from immature embryos on MS medium with different concentration of phytohormones.

Varieties	Total plantlets	Chlorophyll mutants	% of frequency of chlorophyll mutants
Sarsabz	110	10	9.09
Soghat-90	38	30	78.94
Khirman	62	20	32.25

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