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Callus induction and plant regeneration from immature embryos culture in spring wheat (*Triticum aestivum* L.) varieties

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Abstract
The experiment was carried out in Tissue Culture Laboratory, Plant Breeding and Genetics Division, Nuclear Institute of Agriculture Tando Jam. The Four wheat varieties viz. Amber, Khirman, Sarsabz and Soghat were used with different concentrations of phytohormones. The concentration of 2, 4-D, Picloram and NAA (2 mg l^{-1}) for callus induction, MS + indole-3-acetic acid (IAA), Kinetin (Kin) and indole-3-butyric acid (IBA) for regeneration of plantlets while, MS + IBA with different concentrations of sugar percent were used for root induction in wheat. The result showed that maximum proliferation and callus induction was achieved in Amber variety with 2, 4-D, maximum plant regeneration with concentration of MS + IAA 5.0 mg L^{-1} + 4.0 mg L^{-1} Kin+ 30 g sugar L^{-1} and more number of roots were developed under the concentration of MS + IBA 2.0 mg L^{-1} + 30 g sugar L^{-1} were achieved for immature embryo culture in wheat.

Keywords: wheat, callus induction, regeneration, 2, 4-D

Introduction

Wheat *(Triticum aestivum* L.) is one of the most important staple food crops of the family *Poaceae.* Among the food crops, wheat is a common source of energy and proteins for the world population. First requirement for the successful application of biotechnology in crop improvement is to have efficient plant regeneration from cultured cells and tissues. Immature embryos are the most responsive source to produce embryogenic callus and regenerate plantlets among other explants in culture (Kamil, 2002) [15]. Therefore, the success of cell and tissue culture research depends upon reliable callus culture and plant regeneration procedures. 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 (Ozgen *et al.,* 1998; Benkirane *et al.,* 2000) [21, 9]. It has been observed that wheat produces two types of calli viz., embryogenic and non-embryogenic (Benkirane *et al.,* 2000) [9]. Redway *et al.,* (1990) [25] 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) [21] reported that mature and immature embryos of 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 supplemented MS with 2 mg1^{-1} 2, 4-D produced more genetic variation as compared to solid media (Zheng and Konzak, 1999; Arun *et al.,* 1994) [27, 6].

Plant regeneration is one of the critical steps of plant transformation (Keresa *et al.,* 2000) [16]. 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 through tissue culture (Ahmad *et al.,* 2002; Ayes and Kenanturgut, 2006) [3]. During the initial period of excision and culture of explants in the presence of 2, 4-D, embryogenic competence is expressed a few cells. Some show, these cells are selected and preferred. The maintenance of adequate levels of 2, 4-D helps to perpetuate the embryogenic nature of culture by continued divisions in embryogenic cells and in active meristematic zones formed in proliferating tissues. In wheat species, different explants sources have been used for embryogenic callus formation and plant regeneration with mature and immature embryos (Özgen *et al.,* 1998; Özgen *et al.,* 1996) [21, 22], inflorescences (Redway *et al*., 1990; Benkirane *et al*., 2000) [25, 9], coleoptiles (Benkirane *et al*., 2000) [9], shoot apical meristems (Ahmad *et al.,* 2002) [3] and anthers (Asrmstrong *et al.,* 1987) [5].

Much attention is shifted towards crop improvement programs. One of such biotechnological techniques is the plant tissue culture (Parvin *et al*, 2012) [23]. Tissue culture techniques are becoming increasingly popular as an alternative means of plant vegetative propagation, mass production of chemicals, and genetic engineering (Shah *et al.,* 2009) [26]. Resent progress in genetic manipulation of plant cells has opened new potential in crop improvement. Callus culture is used as an *in vitro* technique for biochemical and physiological studies in response at the cellular level (Liu *et*

al., 2006) [18]. Many researchers have used the *in vitro* culture of cells on media supplemented with growth regulators and to utilize the somaclonal variation, as a source of variability for crop improvising (El-Shafey *et al.,* 2009) [12]. These tissues vary in their ability to regenerate whole plants (Delporte *et al*., 2001) [10]. Immature embryos and immature inflorescences gave the highest frequencies of regenerated plants *in vitro* (Benkirane *et al.,* 2000) [9]. Tissue culture responses which includes callus induction and regeneration capacity of wheat are influenced by the genotypes explants source, geographical origin, and physiological status of the donor plants, the culture medium and the interactions between them (Özgen *et al.,* 1996) [22]. In the present study showed that efficient *in vitro* plant regeneration system from immature embryos of wheat varieties. The effects of phytohormones on the calli induction and regeneration systems were compared. The present study was undertaken to develop efficient *in vitro* plant regeneration from immature wheat embryos of four varieties Amber, Khirman, Sarsabz and Soghat-90. In this study favorable media, which gave good results both in the callus induction and in the regeneration of plantlets, were identified and were be used for callus induction. 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.

Materials and Methods

The research was carried out as joint venture among tissue culture laboratory, Plant Breeding and Genetics Division, Nuclear Institute of Agriculture Tando Jam and Department of Biotechnology, Sindh Agriculture University, Tando Jam. Four wheat genotype viz. Amber, Khirman, Sarsabz and Soghat were used throughout this study. The basal culture media consisted of the mineral salts of Murashige and Skoog (1962) [20] with different concentrations with four wheat varieties viz. Amber, Khriman, Sarsabz and Soghat-90 of (*Triticum aestivum* L.) were conducted under *in vitro condition*. The spikes were surface sterilized with 70% alcohol for one minute. Spikes were taken from wheat trials of Experimental Farm, Nuclear Institute of Agriculture, Tando Jam, Pakistan. Spikes were sterilized with alcohol for one minute, followed by dip in sodium hypochlorite (10 %) for twenty minutes and finally washed three times with sterilized distilled water. The immature embryos were aseptically removed from the imbibed seeds. Fifteen embryos were cultured per bottle on modified Murashige and Skoog media (Murashige & Skoog, 1962) [20] containing different concentrations of phytohormones.

Following media were used

Proliferation and callus induction

MS + 2, 4-D (2 mg L^{-1}) + 30 g sugar L^{-1}
MS + Picloram (2 mg L^{-1}) + 30 g sugar L^{-1}
MS + NAA (2 mg L^{-1}) + 30 g sugar L^{-1}

Plant regeneration

MS (Control)
MS + IAA 6.0 mg L^{-1} + 6.0 mg L^{-1} Kin + 30 g sugar L^{-1}
MS + IAA 5.0 mg L^{-1} + 4.0 mg L^{-1} Kin + 30 g sugar L^{-1}
MS + IAA 2.0 mg L^{-1}, IBA 2.0 mg L^{-1} + 2.0 mg L^{-1} BAP + 30 g sugar L^{-1}
MS + IAA 2.5 mg L^{-1}, IBA 2.5 mg L^{-1} + 2.5 mg L^{-1} BAP + 30 g sugar L^{-1}

Root induction

MS + IBA 2.0 mg L^{-1} + 5 g sugar L^{-1},
MS + IBA 2.0 mg L^{-1} + 10 g sugar L^{-1},
MS + IBA 2.0 mg L^{-1} + 20 g sugar L^{-1},
MS + IBA 2.0 mg L^{-1} + 30 g sugar L^{-1}
MS + IBA 2.0 mg L^{-1} + 40 g sugar L^{-1}

The experiment was conducted randomized complete block design with three replications. For callus induction, immature embryos were taken from anther with a scalpel. For callus induction, the effects of different media were used. Immature embryos were placed with the scutellum upwards on a solid agar medium in sterile bottles and cultured 25±2°C under a 16 h photoperiod. The was adjusted to pH 5.7, solidified with 8 g L^{-1} agar and autoclaved at 121 ^{o}C for 20 minutes under 1.1 kg cm^{2} pressures (Ayse *et al*, 2006) [7]. The weight proliferation of callus $bottle^{-1}$, weight of callus $bottle^{-1}$, number of plantlet $bottle^{-1}$and number of roots $plant^{-1}$ were recorded. The experimental data were recorded and statistically analyzed through Statistix 8.1 computer software. Least significant (LSD) test was applied to test level of significance among different combination means by Gomez and Gomez (1984).

Results and Discussions

Proliferation

These results of proliferation indicated that varieties and concentration were higher significant, while their interactions were non-significant, data are presented in Appendix-I Table 1. These result of varieties showed that maximum proliferation were observed in Amber (0.99 g), followed by Soghat (0.82 g), Khirman (0.77 g) and minimum in Sarsabz (0.69 g) with MS + 2 mg L^{-1} 2, 4-D. The maximum proliferation were observed in (1.12) under 2 mg L^{-1} 2, 4-D, followed (0.97 g) with MS + 2 mg L^{-1} Picloram and minimum proliferation was noted (0.77 g) with MS + 2 mg L^{-1} NAA. The results of their interactions indicated that maximum proliferation was observed in Amber (1.35 g), followed by Soghat (1.15 g), Khirman (1.02 g), under concentration of MS + 2 mg L^{-1} 2, 4-D and minimum proliferation was observed in control. The results showed that 2, 4-D produced more proliferation as compared with Picloram and NAA. The results agreed with Khalid *et al.* (2013) that callus induction and proliferation for the improvement of six varieties in wheat by using MS and N6 medium supplemented with different concentrations of 2, 4-D (2, 4-Dichlorophenoxyacetic acid) and found that best proliferation at 3.5 mg l^{-1} of 2, 4-D respectively.

Table 1: Proliferation of callus (g) in *Triticum aestivum* of immature embryos on MS medium supplemented with different concentrations of phytohormones

Concentrations + 30 g sugar L^{-1}	Varieties				Mean
	Amber	Khirman	Sarsabz	Soghat	
MS (Control)	0.60 i	0.38 j	0.30 j	0.37 j	0.41 d
MS + 2 mg L^{-1} 2,4-D	1.35 a	1.02 b-d	0.97 de	1.15 b	1.12 a
MS + 2 mg L^{-1} Picloram	1.12 bc	0.92 d-f	0.84 e-g	1.00 cd	0.97 b
MS + 2 mg L^{-1} NAA	0.88 d-g	0.78 f-h	0.67 hi	0.75 gh	0.77 c
Mean	0.99 a	0.77 b	0.69 c	0.82 b	
Varieties SE (0.0352) LSD (5%) (0.0718), Concentrations SE (0.0352) LSD (5%) (0.0718), V x C SE (0.0703) LSD (5%)(0.1436)					

Callus

The results showed that varieties, concentrations and their interactions were highly were significant and data are presented in Appendix-I, Table 2. There result of varieties showed that maximum callus in Amber (1.43 g); followed by Soghat (1.28 g), Khirman (1.17) and minimum in Sarsabz (0.95 g) were observed. The maximum callus weight (1.63 g) with MS + 2 mg L^{-1} 2, 4-D, followed (1.44 g) under concentration of MS + 2 mg L^{-1} picloram and (1.18 g) with MS + 2 mg L^{-1} NAA and minimum (0.63 g) were observed under control. The results of their interactions indicated that maximum callus proliferation was observed in Amber (1.87 g), followed by Soghat (1.73 g), Khirman (1.59 g), Sarsabz (1.32 g) with concentration of MS + 2 mg L^{-1} 2, 4-D and minimum callus was recorded under control. The results supported that 2, 4-D produced good callus proliferation as compared with Picloram and NAA respectively. The result supported by Abdallah *et al.* (2012) [1] that Murashige and Skoog medium supplemented with different levels (1.5 to 4.0 mg l^{-1}) of 2, 4-dichlorophenoxy, indole-3- butyric acid and indole-3- acetic acid and observed that more callus weight was achieved on MS medium supplemented with 2.0 mg L^{-1} of 2, 4 -D. The results also agreed with Rahman *et al.* (2008) [24] that studied for *in vitro* regeneration in wheat plants was carried out from immature embryos in different of spring wheat. Three concentrations of 2, 4-D were used for callus induction. Maximum number of callus were produced on MS medium supplemented with 6.0 mg L^{-1} of 2, 4-D, while the genotype DH-10 performed better for callus induction. Ahloowalia, (1982) [2], Elena and Ginzo (1988) [11] that five media are based on MS medium (Murashige and Skoog, (1962) [20] and are supplemented with 2, 4-D, which is commonly used as growth regulator in wheat tissue culture. The results also agreed with Bartok and Sagi (1990) [8] that higher amount of callus was obtained at low concentration of 2, 4-D (2mg l-1) respectively. The results also fully supported by Benkirane *et al*. (2000) [9] that maximum proliferation of callus can be obtained from 2, 4-D as compared of Picloram and NAA under (2 mg l^{-1}) respectively.

Table 2: Callus induction (g) in *Triticum aestivum* of immature embryos on MS medium supplemented with different concentrations of phytohormones

Concentrations + 30 g sugar L^{-1}	Varieties				Mean
	Amber	Khirman	Sarsabz	Soghat	
MS (Control)	0.78 k	0.54 m	0.42 n	0.66 l	0.60 d
MS + 2 mg L^{-1} 2,4-D	1.87 a	1.59 cd	1.32 g	1.73 b	1.63 a
MS + 2 mg L^{-1} Picloram	1.67 bc	1.45 ef	1.12 i	1.52 de	1.44 b
MS + 2 mg L^{-1} NAA	1.41 f	1.12 i	0.97 j	1.22 h	1.18 c
Mean	1.43 a	1.17 c	0.95 d	1.28 b	
Varieties SE (0.0206) LSD (5%) (0.0421), Concentrations SE (0.0206) LSD (5%) (0.0421), V x C SE (0.0413) LSD (5%) (0.0842)					

Plant regeneration

The results showed that varieties, concentrations and their interactions were highly were significant and data are presented in Appendix-I, Table 3. The result of varieties showed that maximum plantlets were regenerated in Amber (3.66), followed by Khirman (3.59), Sarsabz (3.39) and minimum in Soghat (2.80). The interaction of varieties x concentrations indicated that maximum plantlets were generated MS + IAA 6.0 mg L^{-1} + 6.0 mg L^{-1} Kin+ 30 g sugar L^{-1}, Amber (5.29), Khirman (4.98), Sarsabz (4.09) and Soghat (3.69), followed by MS + IAA 5.0 mg L^{-1} + 4.0 mg L^{-1} Kin+ 30 g sugar L^{-1}, Amber (4.05), Khirman (4.53), Sarsabz (3.83) and Soghat (3.21) respectively and minimum plantlets were regenerated under control. The results fully supported by Rahman *et al.* (2008) [24] conducted an experiment for *in vitro* regeneration in wheat plants was carried out from immature embryos in different of spring wheat varieties. The maximum numbers of plants were regenerated on the MS medium supplemented with 1.0 mg L^{-1} of kinetin which was followed by 2.0 mg L^{-1} of kinetin in the medium. Keresa *et al.,* (2000) [16] reported that plant regeneration is one of the critical steps of plant transformation. 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 (Ahmad *et al.,* 2002) [3]. The results supported by Mohammad (1993) [19] that 2, 4-D produced more callus proliferation as compared with Picloram and NAA for good callus formation from immatured embryos of spring wheat varieties. The results also supported by Benkirane *et al*. (2000) [9] that maximum callus can be obtained from 2, 4-D as compared of Picloram and NAA under (2 mg l^{-1}) respectively.

Table 3: Regeneration of plantlets in *Triticum aestivum* of immature embryos on MS medium supplemented with different concentrations of phytohormones

Concentrations	Varieties				Mean
	Amber	Khirman	Sarsabz	Soghat	
MS (Control) + 30 g sugar L^{-1}	2.42 m	2.10 n	2.03 n	1.80 o	2.09 e
MS + IAA 6.0 mg L^{-1} + 6.0 mg L^{-1} Kin+ 30 g sugar L^{-1}	5.29 a	4.98 b	4.09 d	3.69 f	4.51 a
MS + IAA 5.0 mg L^{-1} + 4.0 mg L^{-1} Kin+ 30 g sugar L^{-1}	4.05 d	4.53 c	3.83 e	3.21 ij	3.90 b
MS + IAA 2.0 mg L^{-1}, IBA 2.0 mg L^{-1} + 2.0 mg L^{-1} BAP+ 30 g sugar L^{-1}	3.39 gh	3.30 hi	3.47 g	2.77 l	3.23 c
MS + IAA 2.5 mg L^{-1}, IBA 2.5 mg L^{-1} + 2.5 mg L^{-1} BAP + 30 g sugar L^{-1}	3.17 jk	3.06 k	3.14 jk	2.52 m	2.97 d
Mean	3.66 a	3.59 b	3.31 c	2.80 d	
Varieties SE (0.0238) LSD (5%) (0.0483), Concentrations SE (0.0267) LSD (5%) (0.0540), V x C SE (0.0533) LSD (5%) (0.1080)					

Root induction

The results showed that varieties, concentrations and their interactions were highly were significant and data are presented in Appendix-I, Table 4. The results of varieties showed that maximum numbers of roots were observed in Khirman (3.36), followed by Amber (3.02), and in Soghat and Sarsabz (2.29 and 2.28) were at par. The concentrations indicated that maximum number of roots (4.07) with MS + IBA 2.0 mg L^{-1} + 30 g sugar L^{-1}, followed by (3.40) under concentration of MS + IBA 2.0 mg L^{-1} + 20 g sugar L^{-1}, however, minimum number of roots (1.19) was achieved under concentration of MS + IBA 2.0 mg L^{-1} + 5 g sugar L^{-1} respectively. The indole-3-butyric acid with the different concentrations of sugar were used in media and to achieve improvement through tissue culture in wheat. Irfan *et al.*, (2012) [14] that rooting medium was observed at MS + IBA 2.0 mg L^{-1} + 30 g L^{-1} respectively. I am also satisfying from Irfan *et al.*, (2012) [14] that MS + IBA 2.0 mg L^{-1} + 30 g sugar L^{-1} was found suitable in root induction.

Table 4: Rooting induction in *Triticum aestivum* of immature embryos on MS medium supplemented with different concentrations of phytohormones

Concentrations	Varieties				Mean
	Amber	Khirman	Sarsabz	Soghat	
MS + IBA 2.0 mg L^{-1} + 5 g sugar L^{-1}	1.40 n	1.23 o	1.13 p	1.02 q	1.19 e
MS + IBA 2.0 mg L^{-1} + 10 g sugar L^{-1}	3.27 g	3.46 f	2.02 m	2.34 j	2.77 c
MS + IBA 2.0 mg L^{-1} + 20 g sugar L^{-1}	3.72 c	4.89 a	2.40 i	2.61 h	3.40 b
MS + IBA 2.0 mg L^{-1} + 30 g sugar L^{-1}	4.59 b	4.57 b	3.61 d	3.52 e	4.07 a
MS + IBA 2.0 mg L^{-1} + 40 g sugar L^{-1}	2.12 l	2.65 h	2.23 k	1.98 m	2.24 d
Mean	3.02 b	3.36 a	2.28 c	2.29 c	
Varieties SE (0.0122) LSD (5%) (0.0247), Concentrations SE (0.0136) LSD (5%) (0.0276), V x C SE (0.0273) LSD (5%) (0.0552)					

Appendix-I Mean squares of proliferation, callus, plant regeneration and callus induction in *Triticum aestivum* of immature embryos on MS medium supplemented with different concentrations of phytohormones

Source	DF	Proliferation	Callus	Plant regeneration	Root induction
Replications	2	0.00386	0.09388	0. 0690	0.1017
Varieties	3	0.18255**	0.48061**	2.3227**	4.3562**
Concentrations	3	1.11945**	2.39215**	10.1901**	14.5614**
V x C	9	0.00698ns	0.00737*	0.2603**	0.6005**
Error	30	0.00741	0.00255	0.0043	0.0011

Conclusion

It was concluded that proliferation and callus were founded best with the concentration MS + 2 mg L^{-1} 2, 4-D as compared to picloram and NAA. The results showed best medium MS + IAA 6.0 mg L^{-1} + 6.0 mg L^{-1} Kin+ 30 g sugar L^{-1} for plant regeneration, while MS + IBA 2.0 mg L^{-1} + 30 g sugar L^{-1}swhowed best root induction. It is recommended that wheat varieties should be sown under *in vitro* condition for immature embryo in wheat under MS + 2 mg L^{-1} (2, 4-D) for proliferation and callus induction, while MS + IAA 6.0 mg L^{-1} + 6.0 mg L^{-1} Kin+ 30 g sugar L^{-1} for plant regeneration, however MS + IBA 2.0 mg L^{-1} + 30 g sugar L^{-1} for root induction are the best combinations.

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