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In Vitro Study of Callogenesis and Regeneration Potential of Elite Wheat (*Triticum aestivum* L.) Cultivars

Muhammad Iqbal[1*], Naveed Iqbal Raja[1], Saira Asif[1], Noshin Ilyas[1], Mubashir Hussain[1], Farhat Yasmeen[1], Muhammad Ejaz[1], Muhammad Asim Sultan[2], Sumaira Aslam[1], Hina Javed[1]

[1]Department of Botany, PMAS-Arid Agriculture University, Rawalpindi, Pakistan
[2]Department of Botany, University of Sargodha, Sargodha, Pakistan
Email: *mmiqballali@gmail.com

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Abstract

The potential for biotechnological applications in crop improvement programs requires identifying genotypes that allow cell/tissue culture with predictable plant regeneration. In the past, many genotypes of wheat (*Triticum aestivum* L.) have been examined for potential use in tissue culture studies. The present research work has also been designed to study *in vitro* callogenesis expression and regeneration potential of wheat cultivars under controlled laboratory conditions. Seeds of four elite commercial high yielding cultivars of wheat namely: NARC-2011, AAS-2011, PAK-2013 and GAL-2013, were collected from the Crop Science Institute National Agricultural Research Center (CSI-NARC) Islamabad, as the source of plant material for *in vitro* studies. The seeds were surface sterilized in 10% sodium hypochlorite solutions for 10 minutes with continuous shaking under laminar air flow hood. After that seeds were placed on MS (Murashige & Skoog, 1962) based callus induction and regeneration medium with various concentrations of 2, 4-D and BAP in separate test tubes. Maximum callus induction frequency of 90% for Pak-13 and AAS-11, followed by 87% and 83% for Gla-13 and NARC-11, respectively, was recorded at 4 mg/l and 6 mg/l of 2, 4-D. Similarly, maximum regeneration of 90% for AAS-11 and Pak-13, followed by 80% and 87% for NARC-11 and Gla-13 respectively, was recorded on MS basal medium containing 1.5 mg/l of BAP. An increasing trend in regeneration from 0.5 to 1.5 mg/l of BAP was observed but it gradually decreased with increasing concentration of BAP from 1.5 mg/l for all wheat cultivars. The callus formed under light was golden brown, dry nodule and smooth compact and less embryogenic while under dark conditions, it was white to yellowish white, dry nodule and compact and more embryogenic. Best results for callus induction and regeneration were obtained at temperature (24˚C ± 1˚C) for all wheat cultivars.

Keywords

Tissue Culture, MS Medium, 2, 4-D and BAP, Callus Induction, Regeneration, Wheat

1. Introduction

Wheat (*Triticum aestivum* L.) belonging to family *Poaceae* is the second most prominent grain crop in temperate countries and major staple food of the world [1] [2]. It is grown on more than 240 million hectares with 564.6 million tons production, an average of 2500 kg grain per hectare which is larger than any other crop in the world [3]. World trade in wheat is greater than for all the other combined crops and is the most favored staple food. Wheat provides more nourishment for humans than any other food source. It contains protein, minerals, vitamins and fats (lipids). A wheat-based meal is highly nutritious and is higher in fiber than a meat-based diet [4]. The population growth rate is increasing day by day so there exists a gap between wheat yield and its demand throughout the world [5]. Estimates projected to year 2025 suggest that an average yield of about 4 metric tons per year per hectare will be required to feed the human population expected to be around 8 billion [6] and this situation will become more critical in the year 2050 with projected world population of 9.5 billion from current population of 6.8 billion [7]. Achievement of desired goal of yield boost is becoming impracticable due to wide range of biotic and abiotic factors.

Pakistan is an agricultural country and wheat is its most important cereal crop grown on 36% of total crop area. Conventional breeding methods in Pakistan are being used for enhancing the production and quality improvement of wheat crop. However, its average yield is very low and uncertain due to limiting constraints like wide range of biotic and abiotic stresses (drought stress, salt affected areas, aluminum toxicity, pests, weeds, and diseases); conventional plant breeding strategies are slow and less selective; restricted gene pool availability and species barrier in addition to other biological limitations are involved in decreasing the per hectare yield of wheat [8] [9] [10]. This demands release of high yielding wheat cultivars with improved characteristics, particularly resistance to biotic and abiotic factors.

In recent years, biotechnology is playing important role in improving yield of many crops including wheat [11]. Adoption of novel techniques such as exploitation of *in vitro* tissue culture induced soma-clonal variation and recombinant DNA technology involving callus and regeneration phase may facilitate to increase grain production and nutritional values of cereal crops. Consequently, establishment of reliable tissue culture protocols for callus induction and regeneration is desired in order to improve wheat yield [12]. Several protocols have been developed for tissue culture based improvement of wheat depending upon genotype of wheat [13] [14], explants [15] [16], culture medium [12] [17] [18] and growth regulators [19]. Wheat has a large number of cultivars and genotypes. All genotypes have different tissue culture response that includes callus

induction, regeneration and transformation efficiency [20] [21] [22]. The success of genetic engineering in any crop is directly related to callogenesis and regeneration ability of the species [23] [24]. There is a dire need to screen the available wheat genotypes for tissue culture response. In the present study, it has been planned to look into the most responsive elite genotype of wheat in terms of callusing and regeneration expression with limited resources and time.

2. Materials and Methodology

2.1. Experimental Site

In vitro study pertaining to evaluate the efficient callusing and regeneration potentials of selected wheat cultivars was carried out in Plant Tissue Culture Laboratory, Department of Botany, Pir Mehir Ali Shah, Arid Agriculture University, Rawalpindi, Pakistan.

2.2. Plant Material

Seeds of four elite commercial high yielding cultivars of wheat namely, NARC-2011, AAS-2011, Pak-2013 and Gal-2013, were collected from Crop Science Institute National Agricultural Research Center (CSI-NARC) Islamabad as the source of plant material for *in vitro* studies.

2.3. Surface Sterilization of Explant

The seeds of wheat cultivars were brought into the lab and were washed with running tap water. Then seeds were surface sterilized with 10% sodium hypochlorite (NaOCl) solution for 10 minutes with continuous shaking under laminar air flow hood. After this seeds were washed six times with autoclaved distilled water to remove the traces of sodium hypochlorite solution and then transferred on autoclaved filter papers in sterilized Petri plates for drying [25].

2.4. Callus Induction and Regeneration

Ten seeds of uniform size of each cultivar were picked aseptically using forceps and placed on MS [26] based callus induction medium with various concentrations of phytohormones (MS + 30 g/l sucrose + 1, 2, 3, 4, 5 and 6 mg/l 2, 4-D) in separate test tubes. Each medium was solidified by adding agar (6 g/l). The pH of the medium was adjusted to 5.7 - 5.8 prior to autoclaving at 121˚C and 15lbs/inch^2 for twenty minutes. Explants were incubated in light and dark period at 20 ± 1 to 27 ± 1˚C temperature for three weeks. The number of calli induced from seeds was recorded. Then, calli were transferred to fresh maintenance medium (half of the best suited concentration of 2, 4-D for callus induction of respective cultivar) for callus growth and proliferation for a period of three weeks, refreshing callus maintenance medium after every 14 - 21 days. In the second study, seeds of each cultivar were raised with best suited regeneration medium to evaluate the regeneration potential (MS medium + various concentrations of BAP viz., 0.5, 1, 1.5, 2, 2.5, 3 mg/l) and incubated at temperature ranging from 20 ± 1 to 27 ± 1˚C with 16 hour light and 8 hour dark photoperiod. Percentage of calli regenerated

was calculated after 3 - 4 weeks of culturing the seeds on regeneration medium. Following protocols comprising various combinations of growth hormones were tested for callus induction and regeneration of the selected cultivars (Table 1 & Table 2).

2.5. Data Recording

The frequency of callus induction and regeneration were recorded according to following formulas:

$$\text{Callus induction frequency}(\%) = \frac{\text{No. of seeds producing calli}}{\text{No. of seeds cultured}} \times 100$$

$$\text{Plant regeneration}(\%) = \frac{\text{No. of calli producing plants}}{\text{No. of plants planted}} \times 100$$

2.6. Culture Environment

All *in vitro* culture were grown under ideal conditions of temperature, light and moisture.

Table 1. Callus Induction Frequency (%) of Wheat Cultivars in MS Medium at Various Levels of 2, 4-D.

Explant* = Seeds				Age of Cultures = 8 weeks			
Tr. No	Media	Conc. (mg/l)	No of explants* cultured	No. of explants showing callus induction			
				NARC-11	AAS-11	PAK-13	GLA-13
C_1		1	10	1.3 ± 0.6	2.0 ± 1.0	3.0 ± 1.0	3.3 ± 1.5
C_2		2	10	4.7 ± 1.5	4.0 ± 1.0	4.7 ± 1.2	5.0 ± 1.0
C_3	MS + 2, 4-D	3	10	5.7 ± 1.5	6.7 ± 1.5	7.0 ± 1.0	6.7 ± 1.5
C_4		4	10	7.0 ± 1.0	6.7 ± 0.6	9.0 ± 1.0	8.7 ± 0.6
C_4		5	10	6.3 ± 1.5	8.0 ± 1.0	7.7 ± 1.5	7.0 ± 1.0
C_4		6	10	8.3 ± 0.6	9.0 ± 1.0	8.7 ± 0.6	8.0 ± 1.0

Table 2. Effect of Various Levels of BAP in MS Medium on Regeneration Frequency of Wheat Cultivars.

Explant* = Seeds				Age of Cultures = 8 weeks			
Tr. No	Media	Conc. (mg/l)	No of explants* cultured	No. of explants showing callus induction			
				NARC-11	AAS-11	PAK-13	GLA-13
C_1	0	0.5	10	2.7 ± 0.6	2.0 ± 1.0	5.0 ± 1.0	4.3 ± 1.1
C_2	0	1	10	4.7 ± 1.5	4.0 ± 1.0	6.0 ± 1.0	5.7 ± 0.6
C_3	MS + BAP	1.5	10	8.0 ± 1.0	9.0 ± 1.0	9.0 ± 1.0	8.7 ± 0.6
C_4	0	2	10	7.0 ± 1.0	6.7 ± 0.6	7.0 ± 1.0	7.0 ± 1.0
C_4	0	2.5	10	5.7 ± 0.6	6.7 ± 1.5	6.7 ± 1.1	6.3 ± 1.5
C_4	0	3	10	5.0 ± 1.0	6.3 ± 1.5	6.0 ± 1.0	6.0 ± 1.0

2.6.1. Light Duration and Intensity

The inoculated cultures were grown at different light and dark conditions. The light intensity at photoperiod of 16 hours light and 8 hours dark varied from 2000 - 3000 lux were tested.

2.6.2. Temperature

The cultures were initially grown separately at 20˚C, 21˚C, 22˚C, 23˚C, 24˚C, 25˚C, 26˚C, and 27˚C in a growth cabinet (Hot pack) (Table 3). The growth of the explants either for callogenesis and regeneration was visually observed after every three days. The ideal temperature for *in vitro* callogenesis and regeneration was recorded.

2.7. Statistical Analysis

Each treatment was conducted with three replicates, and the results were presented as mean standard deviation. Data collected were analyzed statistically by using Student's t-test to find out the variation between and within the treatments, with respect to percentage of callus induction and regeneration.

3. Results and Discussion

3.1. Effect of Various Concentrations of 2, 4-D on Callus Induction

Various concentrations of 2, 4-D was used to check the callus induction in wheat cultivars. It was evident that the callus induction was triggered for all concentrations and among all cultivars, but the best results were observed on different concentrations in each cultivar (Table 1; Figure 1). Callus induction was initiated as white translucent tissue on the surface of scutellar region within 3 to 7 days of culturing under light having temperature 21˚C ± 1˚C. The data present in Table 1 indicates that cultivars differed significantly in their potential to produce callus under same medium and culture conditions. Maximum callus induction frequency 90% was recorded in wheat cultivars Pak-13 and AAS-11 at 4 mg/l and 6 mg/l of 2, 4-D. Similarly callus induction frequency 87% and 83% were observed in wheat cultivars Gla-13 and NARC-11 at 4 mg/l and 6 mg/l of 2, 4-D (Figure 2 & Figure 3). Our results reaffirm the findings of Mahmood *et al.* [14] who reported best callus induction responses at 4 mg/l of 2, 4-D in wheat cultivars.

Cultivars responded differently to concentrations of 2, 4-D in the media. All cultivars showed best callogenesis response at 4mg/l and 6 mg/l of 2, 4-D as compared to other concentrations (Table 1; Figure 1). This variability in callus formation frequency in response to different level of 2, 4-D may be due to *in vitro* cultural conditions provided

Table 3. Effect of Different Temperature Conditions on Callus Induction and Regeneration.

Explant	*Temperature* (˚C)							
	20 ± 1	21 ± 1	22 ± 1	23 ± 1	24 ± 1	25 ± 1	26 ± 1	27 ± 1
Seed	+	++	+ + +	+ + +	+ + + +	+ + +	+ + +	++

+ = Poor; + + = Fair; + + + = Good; + + + + = Excellent.

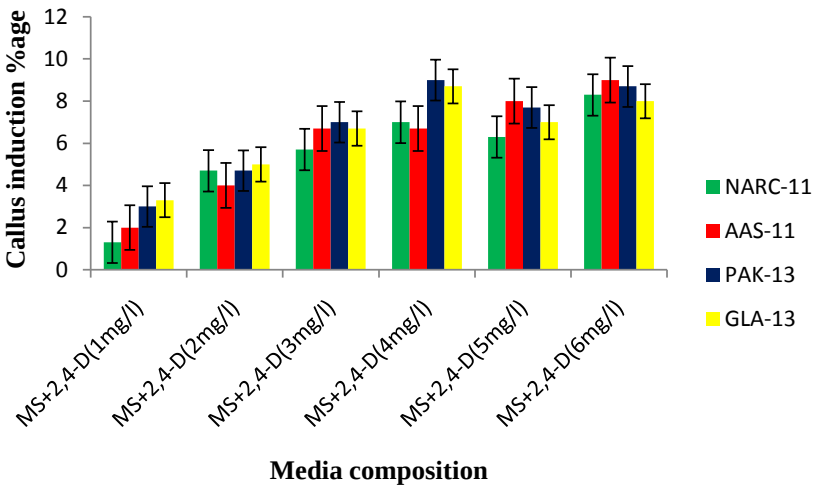

Figure 1. Callus Formation Percentage of Various Wheat Cultivars in Response to Varying Concentrations of 2, 4-D.

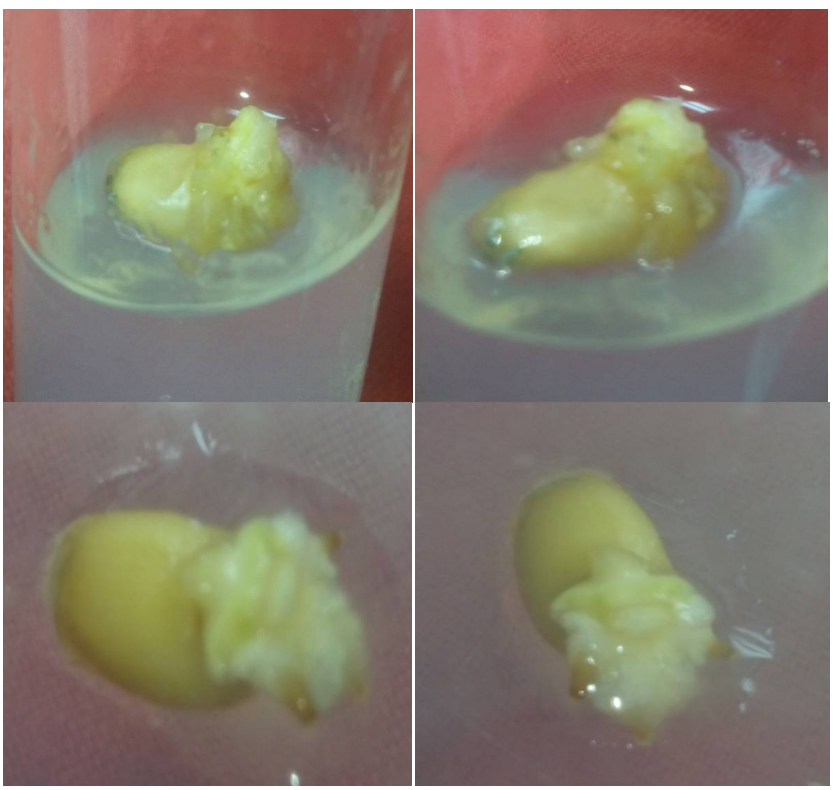

Figure 2. Golden brown callus formed in light on MS medium containing 4 mg/l 2, 4-Dafter 14 days of inoculation.

to different cultivars and differences in expression rate of callusing formation controlling genes. Yasmin *et al.* [22] also investigated the varying response of callogenesis of wheat cultivars related to genotype and media composition. It is evident that auxins induced callus formation is found to be genotype-dependent. Similar findings were ob-

served by Satyavathi *et al.* [27], Nasircilar *et al.* [21], Rashid *et al.* [28] and Mahmood *et al.* [14] in wheat cultivars. In present work the 4 mg/l and 6 mg/l concentration of 2, 4-D proved most suitable for best callus induction in all tested wheat cultivars. It was found that an increase or decrease in 2, 4-D concentration above optimum level may adversely affect the callus induction potential of wheat cultivars. Our results affirmed the findings of Kabir *et al.* [17]. Maximum callus induction was also reported by Rahman *et al.* [18] at 4 mg/l and 6 mg/l of 2, 4-D on MS basal medium in wheat cultivars.

3.2. Effect of Various Concentrations of BAP on Regeneration

MS medium with varying concentrations of BAP were tested to check their effect on *in vitro* regeneration of wheat cultivars (**Table 2**; **Figure 4**). BAP is considered as best cytokinin for regeneration of cereal crops [29]. Data presented in **Table 2** shows that regeneration percentage frequency of explants of wheat cultivars and mean values indicate that regeneration potential significantly varies with changing BAP concentrations.

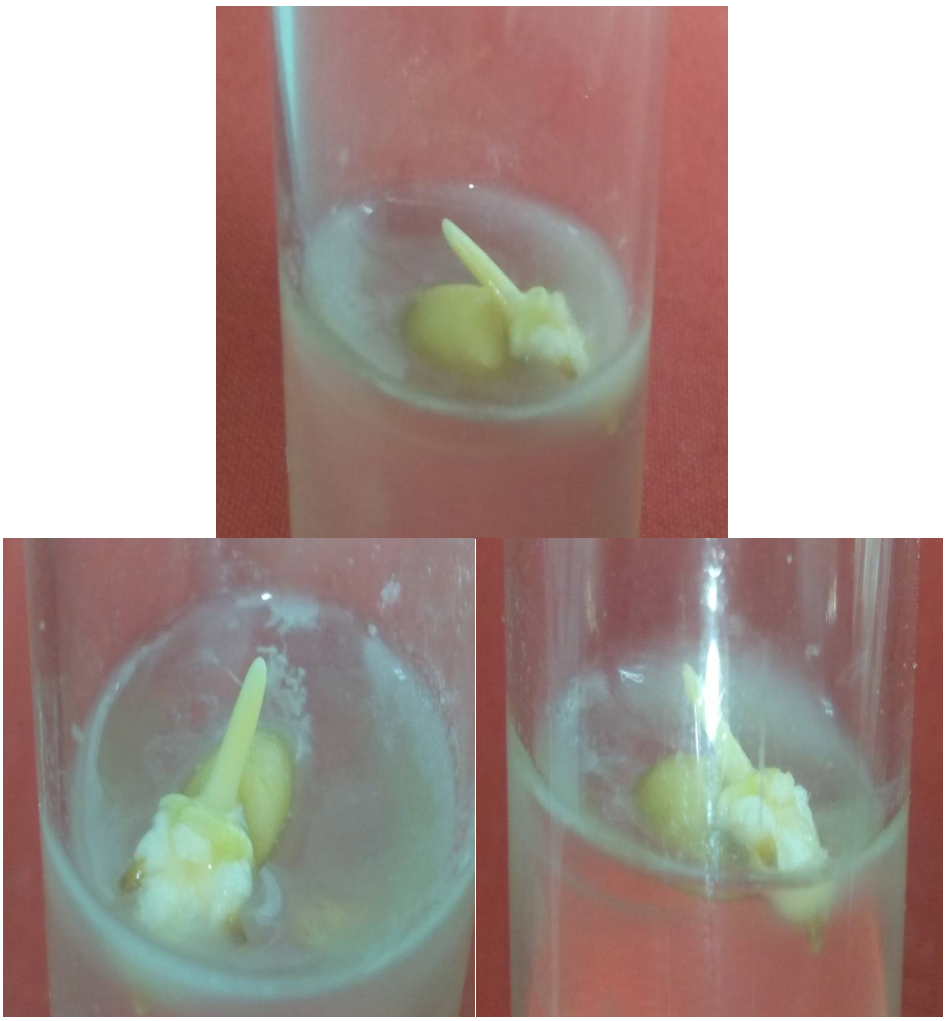

Figure 3. White to yellowish white callus formed in dark on MS medium containing 6 mg/l 2, 4-Dafter 14 days of inoculation.

Highest regeneration 90% was recorded on MS basal medium containing 1.5 mg/l of BAP in wheat cultivars AAS-11 and Pak-13, followed by 80% and 87% in NARC-11 and Gla-13, respectively (**Figure 4** & **Figure 5**). Mahmood *et al.* [14] reported best regeneration potential of wheat cultivars at 1.0 mg/l kinetin, followed by 1.5 mg/l kinetin.

An increasing trend in regeneration from 0.5 - 1.5 mg/l of BAP was observed but it gradually decreased with increasing concentration of BAP from 1.5 mg/l in all wheat cultivars (**Figure 4**). Similar findings were reported by Rashid *et al.* [28] and Yasmin *et al.* [22] who observed variable responses of various wheat genotypes to differing regeneration protocols. Significant differences were observed between interaction of concentrations of BAP and wheat cultivars (**Table 2**). All cultivars responded differently to MS medium applied with varying concentrations of BAP. The environment is one of the main causes altering internal hormonal balance of explant and can modify regeneration potential of callus [30]. Statistically no difference was found between regeneration frequency of cv. AAS-11 (90%) and Pak-13 (90%) at 1.5 mg/l of BAP. However, cv. NARC-11 and Gla-13 expressed significant differences in regeneration frequency at 1.5 mg/l of BAP where 80% and 87% regeneration frequency was recorded. In contrast, maximum regeneration was observed with MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l Kin [15]. Shah *et al.* [16] observed maximum regeneration on MS based medium comprising as high as 3.0 mg/l Kinetin. Additionally to this some other researchers reported cytokinins (*i.e.* BAP) as potent growth regulators for maximum regeneration [31] [32] [33] [34].

3.3. Effect of Light and Dark Regimes on Callus Type, Colour and Texture

Study of callus type, colour and texture is also important for identification of various responses of callus. The callus formed in light was golden brown in colour (**Figure 2**).

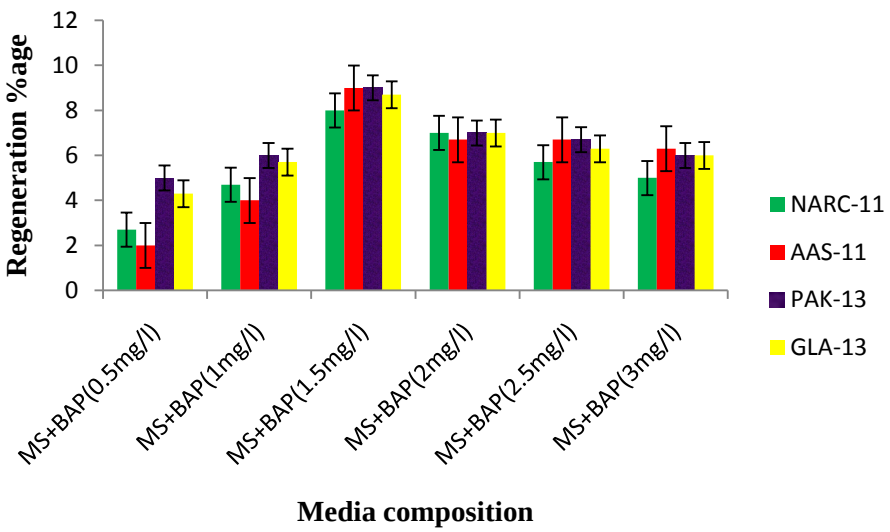

Figure 4. Regeneration (%) of Various Wheat Cultivars in Response to Various Concentrations of BAP.

On the other hand callus induced in dark was white to yellowish white in colour (Figure 3). It was found that not only the callus quantity but the quality of callus was also important. In case of dark conditions, mostly dry nodular and compact callus was formed which was morphogenic in nature. In light conditions, two types of callus were formed: dry nodular and compact which was morphogenic and smooth compact which was non morphogenic in nature (Table 4). Embryogenic callus with less proliferation tendency was formed in light conditions as compared to dark conditions where embryogenic callus with high proliferation tendency was produced.

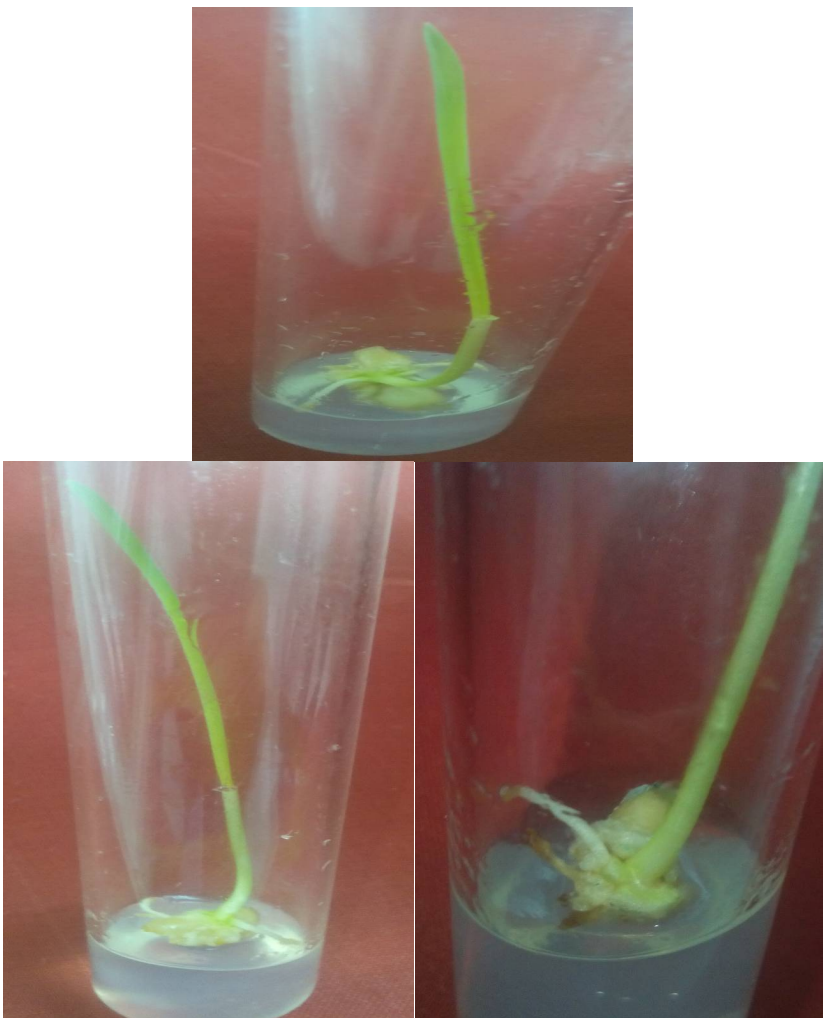

Figure 5. Plant regeneration from mature embryogenic callus after 8 weeks of inoculation on MS medium containing 1.5 mg/l BAP.

Table 4. Effect of Light and Dark Regimes on Callus Induction.

Condition	Explant	Colour of callus	Type of callus
Light	Seed	Golden brown	Nodular/Smooth compact
Dark	Seed	White to yellowish white	Nodular compact

3.4. Effect of Different Temperature Conditions on Callus Induction and Regeneration

Effect of different temperature levels ranging from 20˚C ± 1˚C to 27˚C ± 1˚C was studied for callus induction and regeneration (Table 3). Wheat cultivars responded differently to varying temperature conditions. The best results for callus induction and regeneration (++++) were obtained at 24˚C ± 1˚C for all tested wheat cultivars. By increasing or decreasing the temperature, the rate of callus induction and regeneration was decreased (Table 3). Above to temperature 27˚C ± 1˚C no callus formation or regeneration was observed. Similarly, temperatures lower than 20˚C ± 1˚C did not provide good results for callus induction and regeneration.

4. Conclusion

The present research work showed that high frequency callus induction and plantlet regeneration can be accomplished for selected wheat cultivars by using seeds as source of explants. The potential of callogenesis and regeneration in wheat cultivars is genotype and media dependent. Callusing and regeneration frequency of wheat cultivars can be enhanced to a considerable extent by using a combination of auxins and cytokinins in MS based medium. Optimization of these growth regulators, light and temperature conditions, is essential for maximum callus induction and plantlets regeneration in wheat cultivars. The results of present findings will be helpful for selecting the most tissue culture responsive wheat cultivars for further genetic transformation against different biotic and abiotic stresses as well as for improvement of important agronomic traits of wheat crop.

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