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Pakistani Wheat Cultivars Depict Genotype Dependent Callus and Regeneration Response

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Abstract

Wheat (*Triticum aestivum* L.) is considered as one of the major cash and food crop throughout the world. Tissue culture methods are the major focus of the scientists to explore the genetic modification of this crop. Selection of responsive genotypes and best culture media is imperative for wheat *in vitro* investigation. Mature embryos of ten wheat genotypes were evaluated for tissue culture response. Varying concentrations of 2-4-Dichlorophenoxyacetic acid, Indole Acetic Acid and Kinetin were tested to induce callus and regeneration. Callus induction response ranged between 21% to 94% in Chenab 2000 and Atta Habib. The best optimized concentrations recorded for callus induction were found to be as on 2 and 1 mg/L of 2, 4D. Whereas, the best genotypes that responded well towards callus inducing response were Siran, Atta Habib, Inqalab 2000, Marvi 2000 and Iqbal 2000. On the other hand maximum regeneration response was recorded as 35% in Atta Habib at 0.1- 0.4 mg/L (IAA- Kinetin) in Atta Habib followed by Siran (30%). Best responsive cultivars screened in this study for *in vitro* culturing and economic and efficient methods can be used in wheat improvement programs.

Keywords: Atta Habib, Callus, 2, 4D, Kinetin, Regeneration, Siran
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Introduction

Triticum aestivum is regarded as the imperative food crops. In the scenario of rapid gain in world population demand for food is also expected to be too high in the coming 40 years (Godfray et al., 2010). Consequently, high wheat yield with enhanced quality traits in also required (Gao et al., 2013). Different conventional plant breeding methods have been adopted to meet the demands for good quality wheat grains. Although the wheat production has risen in 2019- 2020 upto 184.5 million tones (www.fao.org/faostat/worldfoodsituation). In spite of this progress of wheat production, still there is a huge gap in production and demand (Uauy, 2017) because the conventional methods are hampered due to the complex wheat genome and genotype dependency (Shah et al., 2009; Jones, 2015).

Modern biotechnological tools like tissue culturing and genetic transformation methods offer a great advantage as they can cope with the complexity of wheat genome. (Bhalla 2006). These methods also offer the variations in the wheat genome through tissue culture and genetic transformation for improved and enhanced characters (Abdallah et al., 2012; Miroshnichenko et al., 2016). Wheat is recalcitrant towards modern genetic transformation system as it has a complex large genome, high repeat sequence of DNA and low regeneration ability (Bhalla, 2006; Moore et al., 1995; Khan et al., 2015). Therefore, major challenge for modern biotechnological applications is the lack of a robust tissue culture system (Delporte et al., 2014; Kapil et al., 2014). *In vitro* culturing techniques are mainly controlled by the media composition, genotype and explant (Benkirane et al., 2014; Hafeez et al., 2012).

Various explants like inflorescence stem sections (Benkirane et al., 2014), isolated anthers (Konieczny et al., 2003), nodes (Lu et al., 1988), coleoptiles (Benkirane et al., 2014), shoot apical meristems (Haliloglu, 2006), leaf base (Wang & Wei, 2004; Yu et al., 2012) mature and immature embryos (Wang et al., 2009; Parmar et al., 2012; Abdollah et al., 2014; Ahmadpour et al., 2018) are in use for wheat regeneration. It is well established fact that different explants show varying responses towards their regeneration potential, immature embryos depicted good response towards calli formation and regeneration (Sarker & Biswas, 2002), however its availability only in specific time period do pose limitations as well (Abdallah et al., 2012).

While mature embryos also remained a good choice for the researchers as they are available round the year due to their storage ease (Kapil et al., 2014; Xia et al., 2012). Apart from explant choice, a balanced hormonal combination and their optimum concentration play a pivotal role for efficient *in vitro* wheat culture. Among plant growth hormones auxins are known to have a great impact in callus formation when they are combined with cytokinin they promote organogenesis.

Current work was aimed to analyze and screen the favorable wheat cultivars that give efficient response to callus induction and regeneration using economic protocol, which might be used for the advanced wheat improvement methods like genetic transformation and genome editing.

The current study aimed the analysis of callus induction and regeneration ability of different plant growth hormones using their varying combinations to find the optimum combinations and concentrations.

Materials and Methods

Plant material

Mature embryos of ten wheat cultivars were obtained from the Institute of Biotechnology and Genetic Engineering (IBGE), Agricultural University, Peshawar, Pakistan. The seeds were: Siran, Atta Habib, Bhakhar 2001, Chenab 2000, Inqalab 91, Iqbal 2000, Marvi 2000, Marwat 01, MH-97and Wafaq 2000.

Seed sterilization

Explants were sterilized using 70% (v/v) ethanol and 0.1% $HgCl_2$ (w/v). At first, they were dipped into the 70% ethanol for five to six minutes and then rinsed with distilled water for four times. Explants were then treated with 0.1% $HgCl_2$ for five minutes and then cleaned with distilled water for five times. After sterilization seeds were placed in water for imbibition till next morning.

Callus induction media and explant preparation

Basic nutrient MS media (Murashig & Skoog, 1962) was used with 30g sucrose and 6g agar under 5.8. pH. Media was sterilized using autoclaved for 20 min 2,4D was used as auxin source with varying concentrations (1-5mg/L). Embryos were detached from the endosperms using sharp needle and were introduced to the culture media. Embryos were placed in dark at room temperature for next fourteen to twenty days.

Regeneration

Healthy and embryogenic calli were selected for regeneration. The calli were transferred to the media composed of MS medium containing different concentrations of auxin (IAA) and cytokinins (Kin). To examine the regeneration potential in wheat, various amounts of IAA (0.05 to 0.35 mg/L) and Kin (0.5 to 0.6 mg/ L) were used.The cultures for regeneration were placed in a growth room at a room temperature with 16/8 hours light/dark photoperiod. Subculturing was monitored at every1.5 week.

Statistical analysis

Each experimental unit was considered a completely randomized design. All the experiments involved in callus formation or regeneration was repeated three times. ANOVA (Analysis of Variance) was tested to evaluate any variations in hormonal exposures their interactions, and genotypic response of cultivars towards callus and regeneration percentage. Means values were compared using Duncan's Multiple Range Test at $P \leq 0.05$ level.

Results

Callus morphology

Callus formation started as outer portion of scutellum turned into whitish mass (Fig. 1A). At initial stage all the cultivars under observation showed the signs of callus formation, but this callus was flat and no nodules were observed at this stage. Color of callus appeared to be either creamy or even colorless. It was observed that texture and color got changes as time proceed till third week when some nodules appeared color became darker, and some shoots development was also seen (Fig. 1B & 1C) at this stage embryogenic calli got distinguished from non-embryogenic calli. Darker, whitish, compact structure loaded with nodules and relatively dry callus was considered as embryogenic (Fig. 1B). On the other hand, non-embryogenic callus appeared to be brownish or yellow colored loosely arranged, irregular shaped, and watery (Fig. 1C).

A combination of both embryogenic and non-embryogenic callus was also found. At this stage embryogenic callus was distinguished while non-embryogenic calli was removed from the culture during sub culturing. For further experiments only embryogenic callus was considered.

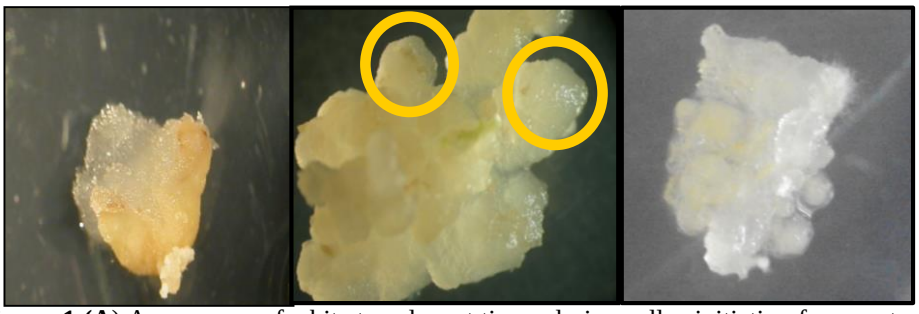
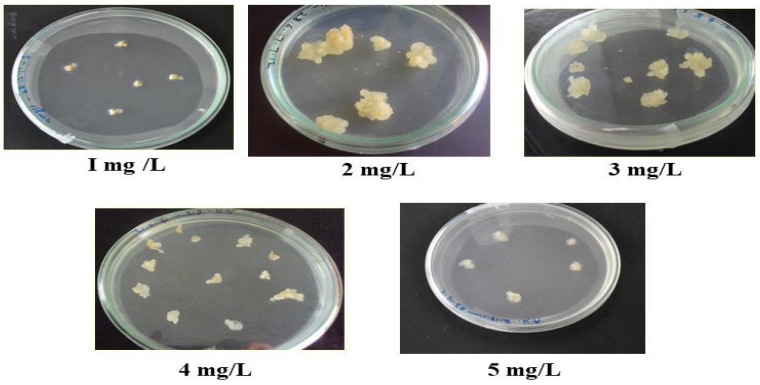


Figure-1 (A) Appearance of white translucent tissue during callus initiation from mature embryo (**B)** Embryogenic callus with nodules (encircled) **(C)** non-embryogenic callus showing watery appearance.

It was found that callus types and textured were greatly defined by plant growth hormone auxin type and its strength in the media. In all the hormonal concentrations that were used during experiment, 3 and 2mg/L concentrations that gave optimum embryogenic callus quantity.

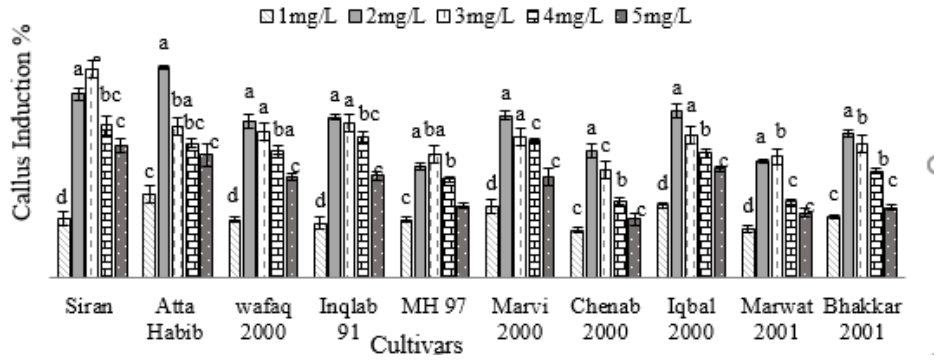
Differential callus induction responses

Though the callus induction response was found dependent upon the concentrations of 2, 4D applied where 3 and 2 mg/L of 2, 4D were found optimum for the maximum callus formation (Fig. 2A & B). However, genotype dependency was another factor to be considered, very genotype behaved differently on the same hormonal concentration. Significantly highest callus induction (94%) was obtained in cv. Atta Habib at 2 mg/L as compared to other tested cultivars (Fig. 2B)



A

Figure-2 A. Callus induction at varying 2-4-D conc. (1 to 5 mg/L) after four weeks



B. Comparison of callus induction frequency at different 2-4-D concentration ranging from 1-5 mg/L. The vertical bars indicate the calli induction frequency at each concentration. Different alphabets on the bars represent the significant difference in the callus induction of wheat cultivars at 5 different 2-4-D concentration

Siran produced a maximum of 93% on 3 mg/L a reduced trend of callus induction was observed at 4 mg/L. Minimum induction of callus was seen at 5 and 1 mg/L. Maximum of 37% callus induction was observed in Atta Habib while least was found in the 26% in Chenab 2000 where at 1 mg/L callus induction maximum of 37% callus induction was observed in Atta Habib and least was seen in the Chenab 2000 (21%) (Fig. 2B).

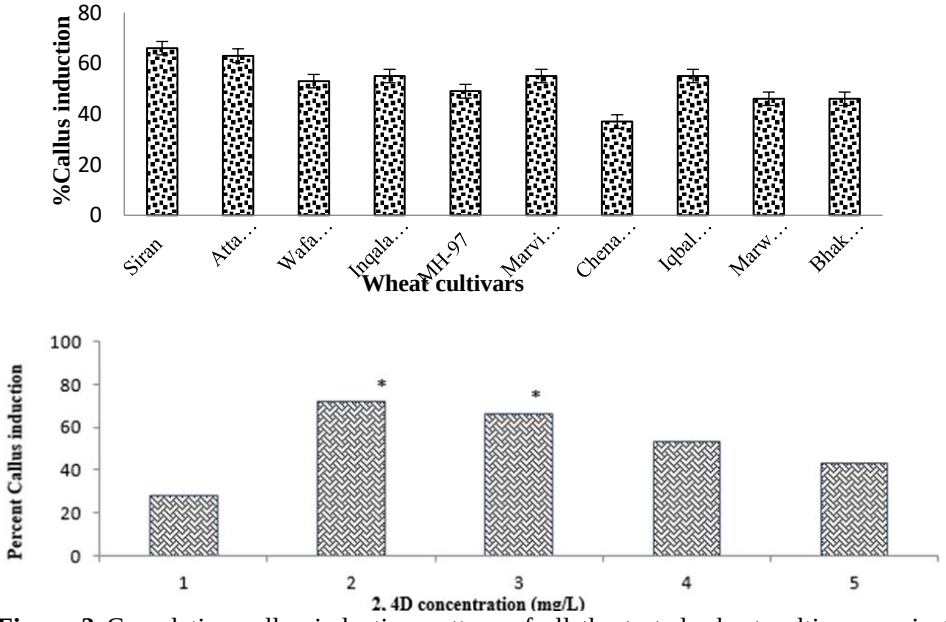


Figure-3 Cumulative callus induction pattern of all the tested wheat cultivars against varying 2-4-D concentrations. The vertical bars indicate the callus frequency, bars with asterisk signs show significant differences among the cultivars

On comparison of mean values for callus formation for each concentration also showed 3 and 2mg L^{-1} of 2-4-D found to be the optimum concentrations giving significant higher callus response in wheat (Fig. 3A), but genotype response had also a great role. For screening the best genotype having higher potential for callus formation every genotype was investigated individually it was found that Siran retained the highest callusing frequency of 66% followed by the cv. Atta Habib with 63% whereas Chenab 2001 was found to produce least of 37% callus induction (Fig. 3B). Significant differences were found at each experimental concentration while when the interaction of cultivars with concentration was measured it was also found significant (Table. 1).

Table 1. Analysis of variance showing the interaction between cultivars and 2-4-D concentrations

Source	Callus induction Frequency				
	Degree of Freedom	**Sum of Squares**	**Means Squares**	**F Value**	**Probability**
Cultivars (a)	9	9249.3	1027.70	78.57*	0.000
2-4-D con. (b)	4	37841.6	9460.41	723.27*	0.000
a*b	36	3415.3	94.87	7.25*	0.000
Error	100	1308.0	13.08		
Total	149	51814.3			

*Means are highly significant at 0.05 level

Regeneration

Highly embryogenic fully-grown and friable callus was selected for regeneration of plants (Fig. 4A) as non -embryogenic callus has least ability to regenerate. Regenerating callus appeared with the green spots that turned to the green shoots later (Fig. 4B), that was further separated for the growth (Fig. 4C) and after acclimatization were shifted to soil (Fig. 4D).

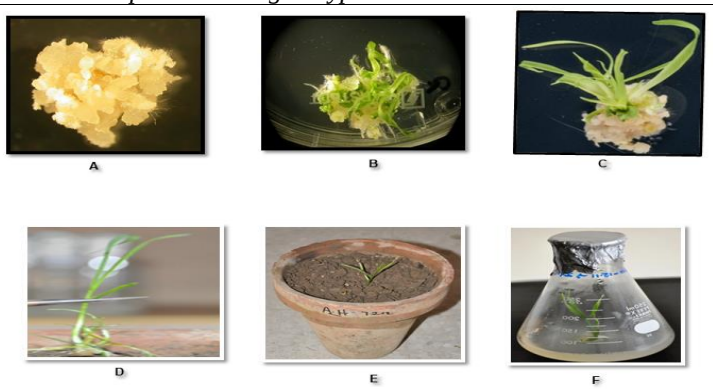


Figure-4. *In vitro* plant regeneration from the mature embryo of wheat cultivar Atta Habib A Four week old callus B Regeneration of plantlets on regeneration medium C Regenerated plant D Transferred of a regenerated plant to the soil in a pot

Among tested concentrations of IAA and Kinetin, IK.2 combination produced highest regeneration response in Atta Habib (35%) (Fig. 5). Using IK.1 (0.05, 0.3 mg/L) high regeneration frequency was achieved in Siran. On the other hand, IK.3 (0.2, 0.5 mg/L) and IK.4 (0.3, 0.6 mg/L) produced 24 and 16 % as highest. A mutual regeneration response pattern depicted IK.2 as the best hormonal combination that gave optimum regeneration response (Fig. 6A) the cultivar with maximum regeneration potential was found to be as Atta Habib according to the values of means (Fig. 6B) with significant variations between the hormonal strengths and genotypic response (Table 2).

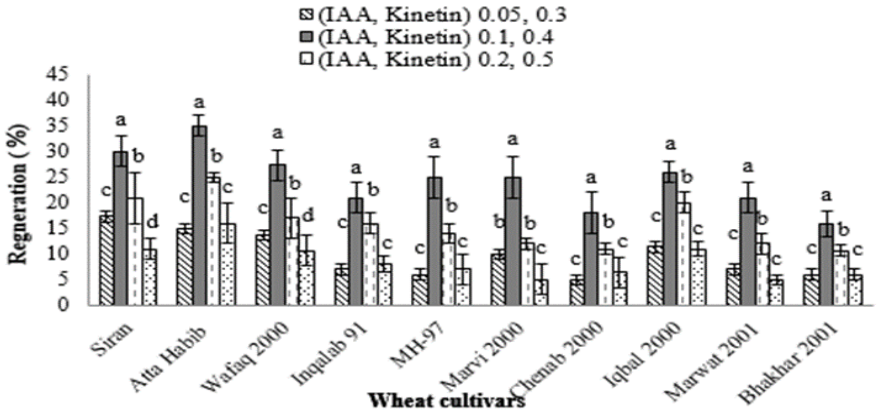


Figure 5. Comparison of regeneration frequency at different concentration of IAA and Kinetin. The vertical bars indicate the regeneration frequency at each concentration. The vertical lines on each bar are the error bars showing the deviation from the mean within each replication. Different alphabets on the bars represents the significant difference within varying IAA and kinetin concentrations for each wheat cultivar

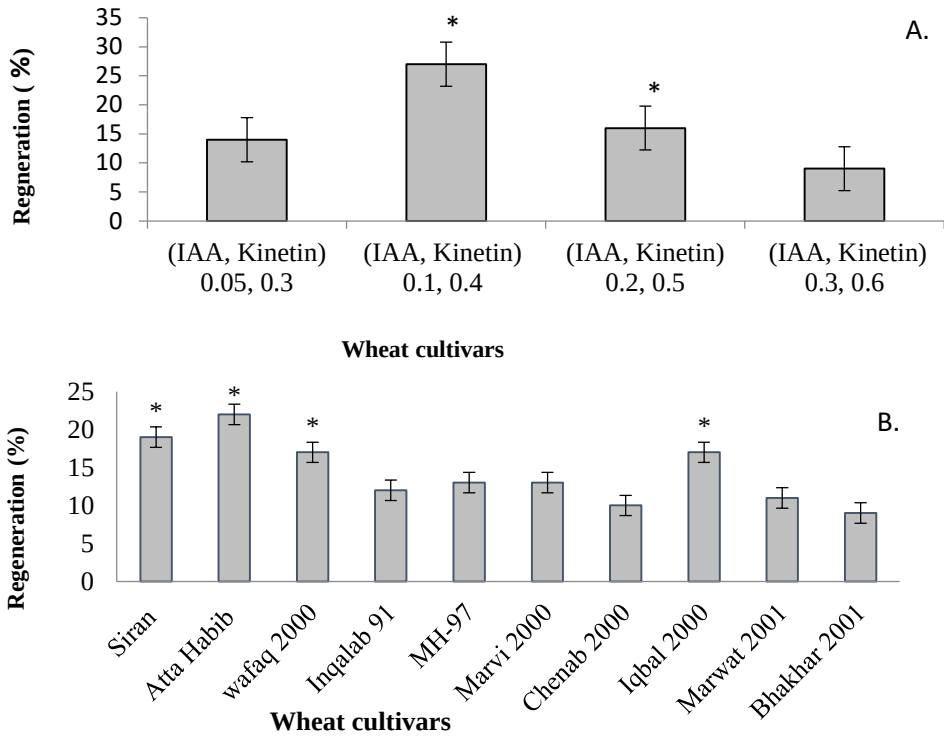


Figure 6. A Cumulative regeneration pattern obtained at selected hormonal concentrations (IAA and Kinetin) against Wheat cultivars. The vertical bars indicate the regeneration frequency, Bars with asterisk signs show significant differences among the other concentrations **B** Cumulative Regneration pattern of all the tested wheat cultivars against varying setsof regeneration media

Table 2. Analysis of variance showing the interaction between wheat cultivars and varying concentrations of IAA and Kinetin

Source	Reference Frequency				
	Degree of Freedom	**Sum of Squares**	**Means Squares**	**F Value**	**Probability**
Cultivars(a)	9	349.91	38.8	16.84*	0.000
IAA and Kinetin(b)	3	5636.16	1878.72	813.89*	0.000
a*b	27	3415.3	23.73	10.28*	0.000
Error	80	184.67	2.31	80	
Total	119	6811.32			

*Means are highly significant at 0.05 level

Discussion

Among plant growth hormones auxin has a major role for the gene activation controlling mitosis and the phenomenon of de differentiation (Erkoyuncu et al., 2017). Among other auxin, 2-4-D is believed to control the formation of shoots and differentiation of somatic embryogenesis during wheat tissue culture (Miroshnichenko et al., 2016; Shafi et al., 2010; Mahalakshmi et al., 2003) .

Callus induction efficiency was found depended upon 2-4-D strengths, genotypic response and their mutual interaction. Minimum callus formation was observed at 1 mg/L of 2-4-D with cumulative callus 28% during experiment (Fig. 2A &B). Low callus induction at minimum hormonal strength is because of the reason that callus and embryogenic of wheat cultivars potential get effected at lower hormonal concentrations (Ahmadpour et al., 2023; Vasil & Vasil, 1981). However previously in some cultivars of wheat gave optimum callus induction under low concentrations of 2, 4D. (Tinak et al., 2013; Munazir et al., 2010).

The highest callus formation was witnessed at 3 and 2 mg/L of 2-4-D, in contrast to other concentrations tested (Fig. 2A &B). The similar observations regarding callus initiation were also reported by the (Ahmadpour et al., 2023; Tinak et al., 2013; Satyavathi et al., 2004 Mahmood et al., 2012). Findings of (Haliloglu, 2006; Satyavathi , 2004) report the similar findings of best callus induction with 2 mg/L 2-4-D. At these concentrations optimum calli formation were reported in different wheat genotypes (Jones, 2015, Abdallah et al., 2012; Yu et al., 2008; Baday 2018). While in some other

reports 3 mg/L of 2-4-D, maximum callus induction was found (Shah et al., 2003; Rashid et al., 2009) which is also consistent with our findings.

Though some genotypes responded at their maximum for callus formation on 3 and 2 mg/L of 2, 4D but when these were subject to some higher concentrations like 4 to 5 mg/L callus formation declined (Fig. 2A & B) This change attributed to the fact that strong hormonal concentrations leads to deterioration effects on the *in vitro* response in wheat (Kabir et al., 2008; Malik et al., 2017). In addition, at high concentration of 2, 4D soma clonal variation are caused (Miroshnichenko et al., 2016; Ahmadpour et al., 2018; Satyavathi et al., 2004; Sun et al., 2013) . Callus formation at higher concentrations promote the formation of non-embryogenic calli (Qin et al., 2013; Sales and Butardo 2014). Therefore, lowest possible auxin strength is always suggested to induce callus (Malik et al., 2017).

Callus type i.e embryogenic or non-embryogenic is always the focus for researchers during wheat tissue culture. Embryogenic callus retains the maximum ability for regeneration in comparison with non-embryogenic callus that though contains the proliferation ability but failed to regenerate (Benkirane et al., 2000; Wang & Wei, 2004; Qin et al., 2013). *Triticum aestivum* contains the ability to have both type calli (Shah et al., 2009; Benkirane et al., 2000; Shafi et al., 2010). In this report we also witnessed both types of callus **(Fig. 1B &C).** While non embryogenic type of callus was observed to be converted to the embryogenic callus on approach of 20th day of culture such changes were also found in previous reports (Mahmood et al., 2012; Li et al., 2009). At 3 and 2 mg/L nodular, creamy and compact callus was formed that is prominent sign for embryogenic nature of callus. **(Fig. 1B)** (Tinak et al., 2013; Mahmood et al., 2012; Hassan et al., 2009) have also found same morphological features of callus at low concentrations of plant hormones.

In addition to the hormonal level tissue culture response in wheat was also found dependent upon genotype, but all the other cultivars responded to the different 2-4-D levels according to their own callus induction potential. In previous report it is evident that genotype dependency have a greater share in callus induction (Baday, 2018; Rashid et al., 2009). It is believed that genotype behavior towards the callus induction is co related with the gene function controlling the *in vitro* response in wheat. Level of gene expression controls the calli induction response in the explants (Rashid et al., 2009).

Findings of this study supports the previous results (Dağüstü, 2008) where genotype differences were found on the varying hormonal level. Based upon the findings and previous records it can be deduced that maximum callus induction can be achieved by manipulating the hormonal concentrations, length of culture period and genotype selection (Arzani and Mirodjagh, 1999) . In this study maximum callus induction was achieved using lowest possible concentration of 2-4-D i.e. 2 mg/L that is not only economical but also favors the embryogenic calli production and efficient regeneration response. This study suggests that genotypic factor seems to be operating in response of particular concentrations of growth regulators.

In this study the regeneration potential was evaluated using the embryogenic callus induced the lowest possible concentration that gave maximum callus induction percentage i.e. 2mg/L. The callus induced at higher concentrations affects the regeneration rate negatively (Fazeli-nasab et al., 2012) therefore, callus induced at lower

concentration is always recommended for enhanced regeneration (Abdallah et al., 2012; Rashid et al., 2009; Barro et al., 1998).

Length of culture was also given a special attention for the evaluation of regeneration response. The cultures that retained on the media for longer then four weeks were not considered for the experimentation as (Fig. 4A), the culture that retained on the media with the addition of auxin source for prolonged time causes stops the process of de- differentiation in the explants (Fischer-Iglesias et al., 2001; Schulze, 2007) . It is also reported that excess quantity and long period time exposure of hormones to culture period alters the function of exogenous plant hormones (Gong and Pua, 2004; Özgen et al., 2017).

Regeneration potential strongly co related with the hormonal concentrations, cultivars, and their mutual interactions. Different cultivars behaved differently to varying hormonal concentrations, which suggest that regeneration response is dependent upon the media and genotype. The similar trend of varying genotypic response of wheat cultivars under different regeneration media was also noted by (Hassan et al., 2009; Mitić et al., 2006; Alikina et al., 2016)

In order to establish an optimum regeneration protocol, it is very important to consider plant growth hormones auxin and cytokinin their mutual interaction controls the vital processes of cell division and somatic embryogenesis (Nikolić et al., 2006; Feher et al., 2003). The callus response towards regeneration is mainly dependent upon the combination and ratio between the auxin and cytokinins and genotype (Kabir et al., 2008). Generally, a low auxin and higher cytokinins concentration is required for regeneration (Elena and Ginzo, 1988) because high concentration of 2, 4D or IAA retards the morphogenic process of embryos that ultimately ceases the regeneration ability of the explant.

In this study lower and higher combinations of IAA and Kinetin were used in order establish an efficient regeneration system ins wheat **(Fig. 4)**. Mean values indicated that IK.1 (0.1, 0.4 mg/L) combination of the regeneration produced the maximum regeneration response where auxin to cytokinins ratio was found to be in 1:2. Delporte et al (2001) also established the efficient regeneration under experimental under 1:2 (2-4-D and Zeatin) auxin to cytokinins ratio. With the same hormonal ratios (Rashid et al., 2009) maximum regeneration rate was achieved in the past (Rashid et al., 2009; Afzal et al., 2010) obtained maximum of 65% regeneration in the callus induced from mature embryos, that is in line with current study.

We observed that at higher cytokinins and auxin concentration a reduced regeneration rate. Similarly (Noor et al., 2009) reported regeneration rate was slowed down with the rise in hormonal concentration. On contrary (Shah et al., 2003) observed maximum regeneration at higher concentration of cytokinins. This divergence in findings can be due to difference in the genetic background of explant, different *in vitro* conditions and environmental conditions. In our study we were successful in optimizing the regeneration frequency with minimal use of growth hormone that is not only economic but is also reduces the chances of soma clonal variations and chromosomal re arrangements. The current study screened Atta Habib, Iqbal 2000, Siran and Wafaq 2000 as the better cultivars having more regeneration ability therefore, these cultivars can be recommended for the wheat transformation and gene editing tools.

Conclusion

Results of the current study indicated that *in vitro* culture in wheat is strongly dependent upon the hormonal concentration and the genotypic response. However we were able to screen some most responsive genotypes and an optimum tissue culture protocol of wheat by maintaining a balance between auxin and cytokinin. In addition, we optimization of regeneration establishment with the mature embryos may compensate the dearth of immature embryos and fragility of other explants.

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