Research Article

# Standardizing *In-vitro* Embryogenic Callus Induction and Plant Regeneration Protocol for the Indigenous Wheat Varieties

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**Abstract** | Plant regeneration through *in-vitro* culture is an important step in genetic transformation and wheat amenability to *in-vitro* propagation is influenced by genotypes and phytohormones. In present study, an efficient plant regeneration system was developed by evaluating the effects of genotype, seed soaking, and plant growth regulators on callus induction, shoot formation, and rooting. Three indigenous wheat genotypes (Atta-Habib, Ghanimat-e-IBGE, and Siran) were tested under various concentrations of 2,4-D, BAP, NAA, and IAA. Significant differences were observed among genotypes and treatments for most parameters. Results declared that Atta-Habib was observed with the highest callus induction frequency, maximum seed producing calli, and the least days to callus induction. Shoot induction was significantly influenced by BAP and NAA, with 3 mg/L BAP and 0.1 mg/L NAA based on less induction time, increased shoot number, and higher shoot length. Similarly, root induction was mostly affected by 3 mg/L IAA with highest number of roots, root length, and root induction percentage. It was concluded that wheat genotype Atta-Habib is the most amenable to callus induction and regeneration and can be used in *in-vitro* studies to refine the techniques of genetic engineering. However, certain other factors like carbon sources, amino acids and gelling agents shall be investigated to achieve a higher regeneration capacity.

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## Introduction

Wheat (*Triticum* spp.) belongs to grass family Poaceae and it is cultivated worldwide. It is self-pollinated annual plant and is the most widely grown cereal crop in the world (Zhou *et al*., 2003). Considerable efforts are being made to improve its productivity by using biotechnology (Rashid *et al*., 2009). The cultivation of wheat (*Triticum* spp.) reaches far back into history. Wheat can be cultivated over a wide range of soils and can be successfully grown over large portions of the world, ranging in altitude from sea level to over 3,050 metres (10,000 feet). Annual rainfall of 254 mm (10 inches) is generally considered the minimum, and the soil should be sufficiently fertile. Barley and rye can be

grown in soil less fertile than that required for wheat. Soils with a good humus content (partially decayed organic matter) and chemical fertilizers generally are necessary (Kent-Jones, 2016).

Over the past three decades, increased agricultural productivity in Pakistan occurred largely due to the deployment of high-yielding cultivars, increased fertilizer use and greater availability of irrigation water. By the mid-1980s, semi dwarf wheat cultivars had been adopted on almost all irrigated land and over 100 kg/ha on average of fertilizer was being applied to wheat. Pakistan produces aver-aged 16.1 million tonnes on 8.2 million ha each year during the period 1993-1995. Rice-wheat, berseem-wheat and cotton-wheat are major systems of intense cropping in Pakistan (Aslam *et al*., 1989). Although the crop is most successful between the latitudes of 30˚ and 60˚N and 27˚ and 40˚S (Nuttonson, 1955), wheat can be grown beyond these limits, from within the Arctic Circle to higher elevations near the equator. Development research by the International Maize and Wheat Improvement Center (CIMMYT) during the past two decades (Saunders and Hettel, 1994) has shown that wheat production in much warmer areas is technologically feasible. In altitude, the crop is grown from sea level to more than 3 000 masl, and it has been reported at 4 570 masl in Tibet (Percival, 1921). About two third of the world populations feed on wheat grain and is the second largest cereal crop after maize. The present wheat grown are known for being intermediaries of modern and wild varieties (Hafeez *et al*., 2012). However, the yield of wheat is known of being affected by number of environmental factors such as temperature, moisture, soil and light intensity. They usually exhibit variation for qualitative and quantitative traits as well as biotic and abiotic stress resistance, owing to their heterogeneity (Afzal *et al*., 2010).

Biotechnology in recent years have provided such tools and methods that have enhance research in various sectors, on the basis of both, quantitative and qualitative outcome. Some of the basic approaches of plant biotechnology include genetic altering and controlling techniques, which enable control of plant development and performance (Patnaik and Khurana, 2001). These technique including the transformation of genes within cereal crops have pave the way of researcher for developing powerful research tool for gene discovery and functioning, as well as become key element in the process of improved plant varieties (Jones *et al*., 2005). Among the major crops, wheat was the last crop to which these techniques were applied (Vasil *et al*., 1992), because of its highly genotype dependency and lower efficiency of foreign gene (Shewry and Jones, 2005; Bhalla *et al*., 2006; Joyia and Khan, 2013). Plant tissue culture phenomena include the development of whole plant from a single cell or tissue. In such cases, both mature and immature embryos have been exploited extensively, however mature embryos were found to be a superior choice in contrast to immature embryos for development of whole plant (Özgen *et al*., 1998). Immature embryos are enhanced explant source, plant material that develops into whole plant, once when its restored and as well as with optimum growth regulators (Zale *et al*., 2004). On the other hand, mature embryos either be dissected or used directly in developing plant consisting desirable traits (Özgen *et al*., 1998; Yu et al., 2008). Consequently, the development of such desirable trait plant requires define approach that excites explants in developing into callus followed by rooting and shooting of the plant. Various explant sources, such as immature embryos, immature leaves, immature inflorescences, mature embryos, mesocotyls, seeds as well as apical meristems have been used for callus culture in wheat (Özgen *et al*., 1998).

Tissue culture is an important tool for last few decades for various crop improvements. Many scientists used novel genes by many techniques to create genetic variability for crop genetic improvement. Mendoza and Kaeppler (2002) described the tissue culture as very fine technique for cereals to be genetically engineered. The genetic engineering of these cereals is fully dependent on the techniques of tissue culture. Cilar *et al*. (2006) utilized mature form of the embryo among 5 different species of the *Triticum*, that give rise to two mutually different media of MS like Murashige and Skoog (MS) media that is Naphthaleneacetic acid (NAA) free or 2, 4-D. The in vitro development of a whole plant from a single cell (e.g. microspore or somatic cells) is a characteristic feature of plants. The amenability of a plant to in vitro culture is influenced by the genotype, which is thus of major importance in the plant tissue culture response (Henry *et al*., 1994). Taking all this into consideration, the present experiment was designed to establish an effective plant regeneration system based on shoot organogenesis from the seeds explants of the

indigenous wheat genotypes developed at Institute of Biotechnology and Genetic Engineering (IBGE), Faculty of Crop Production Sciences, The University of Agriculture, Peshawar 25000, Pakistan, and to study the effect of seed treatment and phytohormones on the callus induction and organogenesis of different wheat genotypes.

## Materials and Methods

### *Plant material*

Mature seeds of wheat (*Triticum aestivum* L), Atta-Habib, Siran and Ghanimat-e-IBGE were used throughout this study. Seeds of these wheat genotypes were obtained from the Institute of Biotechnology and Genetic Engineering (IBGE), University of Agriculture Peshawar.

### *Seed sterilization*

For seed sterilization of the explants, mature seeds were taken and washed with running tap water for 15- 20 minutes and then rinsed with distilled water, along with the addition of fungicide. These seeds were soaked in 70% ethanol for 1 to 2 minutes. Then they were sterilized with 50% Bleach, along with 1-2 drops of Tween-20, for 20 minutes by means of regular shaking. For further cleansing of seeds, they were washed with autoclaved distilled water three times at regular interval of 5 minutes. All this procedure took place in Leminar Flow Hood (LFH). Then they were stored at 4°C in a refrigerator.

### *Culture media*

Murashige and Skoog (1962) (MS) media was used for this study. Stock solutions of MS salts and vitamins were prepared and kept in the refrigerator for this experiment. Agar based media was used, which was prepared by autoclaving the nutrient solutions like; sucrose, myo-inositol, MS media, iron source and agar. Then it was poured in sterile 90mm Petri plates. After sometimes when it got cool, four to five seeds of wheat were cultured on the same Petri plates. These Petri plates were fully covered by parafilm, in order to block the air leakage. The cultures were incubated in growth room. The MS media was enhanced by different attentiveness of auxins and cytokenins to make specialized media for callus shoot and root induction.

### *Callus induction*

The callus induction medium (CIM) comprised of the MS salts containing vitamins, 3 % (w/v) sucrose and solidified with 0.8 % (w/v) agar. The basic MS medium was supplemented with 0, 1.0 and 2.0 $mg.L^{-1}$ of 2,4-dichlorophenoxy acetic acid (2,4-D). The pH of the medium was set 5.8 prior to autoclaving. The media was poured into petri plates after autoclaving and was allowed to solidify at room temperature. Furthermore, the mature seeds were soaked in autoclaved distilled water for 0, 1, 2 and 3 days prior to placement on CIM. Seeds of the three wheat genotypes were used for inducing callus in each treatment. Callus induction frequency was calculated by dividing the amount of seeds generating calli with overall quantity of seeds refined. Cultures by means of considerable callus induction as well as development were transferred to shoot induction medium in a LFH. The inducted calli were proliferated on MS media having the most favorable attentiveness of 2,4-D.

### *Shoot induction*

For the shoot induction from one month old callus, the MS medium in the company of vitamins, 3% (w/v) sucrose and 0.8 % (w/v) agar was enhanced through 0, 1, 2 as well as 3 $mg.L^{-1}$ of Benzyl adenine phosphate (BAP) with or without 0.1 $mg.L^{-1}$ of Naphthalene acetic acid (NAA). The calli for differentiation into shoots were incubated in a growth chamber. Data was recorded on the occurrence of days to shoot induction, number of shoots induced, shoot length and shoot percentage.

### *Root induction*

For the root induction, the regenerated shoots were excised from the undifferentiated calli and incubated in MS medium in the company of vitamins, 3% (w/v) sucrose and 0.8 % (w/v) agar that were enhanced by way of 0, 1, 2 as well as 3 $mg.L^{-1}$ of Indole acetic acid (IAA), with or without supplementation of 0.2 $mg.L^{-1}$ Naphthalene acetic acid (NAA). The material for *in vitro* root differentiation was incubated in a growth chamber. Data was recorded on the frequency of days to root induction, number of roots induced, root length and root percentage.

### *Statistical analysis*

Data was examined by analysis of variance (ANOVA), in an absolute randomized plan, by means of statistical software Statistix 8.1.

## Results

### *Callus induction frequency*

Data regarding the callus induction frequency of wheat genotypes under different concentration of 2,4-D and seed soaking revealed that a significant variation was noted in the callus induction frequency of the wheat genotypes. Among the three different genotypes, Atta-Habib showed maximum callus induction frequency (50.2%) followed by Ghanimat-e-IBGE (47.9%), while minimum callus induction was recorded in Siran (42.8%). The supplementation of 2,4-D also had statistically significant effect on the callus induction frequency of wheat. Consequently, the maximum callus induction was recorded on CIM media containing 2 mg.L$^{-1}$ 2,4-D (55.9%), followed by CIM containing 1 mg.L$^{-1}$ 2,4-D (46.0%), whereas minimum callus induction frequency was recorded for CIM medium without any supplementation of 2,4-D (38.9%). Seed soaking prior to placement on CIM also had a significant effect on the callus induction frequency. Maximum callus induction frequency was recorded when seeds were soaked for 3 days (51.2%), followed by seed soaking for 2 days (47.7%). In contrast, minimum callus induction frequency was noted when seeds were directly placed on CIM medium without any pre-soaking (43.6%) (Table 1). However, The different interaction treatments including genotype x 2,4-D, genotype x seed soaking, 2,4-D x seed soaking as well as genotypes x 2,4-D x seed soaking do not have a significant effect on the callus induction frequency of the wheat genotypes (Supplementary Table S1 and S1a).

**Table 1:** *Callus induction frequency, days to callus induction, and seeds producing calli of the wheat genotypes under different conditions.*

| Factors | Levels | Callus induction frequency | Days to callus induction | Seeds producing calli |
|---|---|---|---|---|
| 2,4-D (mg.L$^{-1}$) | 0 | 38.9 C | 7.2 A | 19.5 C |
| | 1 | 46.0 B | 6.5 B | 23.0 B |
| | 2 | 55.9 A | 6.2 C | 28.0 A |
| Soaking (Days) | 0 | 43.6 D | 8.2 A | 21.8 D |
| | 1 | 45.3 C | 7.0 B | 22.6 C |
| | 2 | 47.7 B | 5.9 C | 23.8 B |
| | 3 | 51.2 A | 5.4 D | 25.6 A |
| Genotypes | Siran | 42.8 C | 7.1 A | 21.4 C |
| | Atta-Habib | 50.2 A | 6.2 C | 25.1 A |
| | Ghanimat-e-IBGE | 47.9 B | 6.6 B | 23.9 B |

### *Days to callus induction*

It can be inferred from the data that genotypes, days of seed soaking and 2,4-D concentration had significantly affected the days to callus induction. Data of days to callus induction showed that wheat genotype Atta-Habib took least days (6.2) to produce callus followed by Ghanimat-e-IBGE (6.6) where as Siran took maximum number of days to produce callus (7.1). The 2,4-D supplement had shown statistically significant effect on days to callus induction. The highest days to callus induction were observed in CIM containing no supplementation of 2,4-D (7.2 days), which was followed by supplement of 2,4-D at 1 mg.L$^{-1}$ (6.5 days). The minimum days to callus induction was recorded in CIM with highest level of supplementation 2,4-D at 2 mg/L (6.2 days). The seeds soaking prior to placement on CIM also had significant effect on days to callus induction. The days to callus induction was recorded maximum when seeds were directly placed on CIM without any soaking (8.2 days) followed by seed soaking for 1 day (7 days) and 2 days seed soaking (5.9 days). The least days to callus induction was observed with the 3 days seed soaking prior to placement on CIM (5.4 days) (Table 1). Whereas, the different interactions among the genotypes, seed soaking and plant growth hormones i.e., genotypes x hormones, genotypes x seed soaking, seed soaking x hormones and the genotypes x hormones x seed soaking have been statistically observed with no significant effect on days to callus induction among the wheat genotypes (Supplementary Table S2 and S2a).

### *Seed producing calli*

The seeds producing calli frequency significantly varied among the wheat genotypes. The maximum number of seeds producing calli was noted for in Atta-Habib (25.1), followed by Ghanimat-e-IBGE (23.9), while the minimum number of seeds producing calli was observed in Siran (21.4). The effect application of 2,4-D had been recorded statistically significant on number of seeds producing calli. Consequently, the maximum number of seeds producing calli was recorded on CIM media containing 2 mg/L 2,4-D (28) followed by CIM 1 mg/L 2,4-D (23), whereas minimum number of seeds producing calli was recorded for CIM medium without any supplementation of 2,4-D (19.5). The seed soaking for 3 days prior to placement had been observed with the maximum seeds producing calli (25.6) followed by seed soaking for 2 days (23.8) and 1 day (22.6). The

least number of seeds producing calli was observed with no seed soaking (21.8) (Table 1). However, the interaction analysis among the various experimental factors i.e., genotypes x hormones, genotypes x treatment, hormone x treatment and genotypes x hormones x treatment had been observed statistically non-significant in effecting the seeds producing calli of the wheat genotypes (Supplementary Table S3 and S3a).

*Days to shoot induction*

Results of days to shooting of different wheat genotypes i.e., Siran, Atta-Habib and Ghanimat-e-IBGE under different concentration of BAP and NAA showed significant variation in days to shoot induction of the wheat genotypes. The highest days to shoot induction were observed in Siran (7.71 days), which was followed by Ghanimat-e-IBGE (7.13 days), and the least days to shoot induction was observed in Atta-Habib (6.50 days). The plant growth hormones i.e., NAA, was analyzed, determining the effect of these hormones on days to shoot induction being statistically significant. The hormone NAA effect was observed maximum for regeneration induction medium (RIM) with 0 supplement of NAA (7.89 days), which was followed by fewer days to shoot induction (6.51) to be recorded with 0.1 mg/L supplementation in the medium. The days to shoot induction statistically had been derived with significant effect due to BAP on RIM. The RIM with no supplement of BAP had been observed with the maximum days of shoot induction (9.53 days) followed by BAP supplement in RIM at 1 mg/L (8.09 days). The least days to shoot induction was observed for RIM to which BAP supplement was applied with 3 mg/L (4.68 days) followed by supplement of BAP at 2 mg/L (6.51 days) (Table 2). However, the days to shoot induction of the tested wheat genotypes had been statistically observed non-significant for the interaction of all the factors within themselves i.e., BAP x NAA, BAP x genotypes, genotypes x NAA and BAP x NAA x genotypes (Supplementary Table S4).

*Number of shoots*

Results of the data regarding the number of shoots of wheat genotypes under different concentration of BAP and NAA revealed that a significant variation was noted in the number of shoots of the wheat genotypes i.e., Siran, Atta-Habib and Ghanimat-e-IBGE. Results showed that Atta-Habib was observed with the highest number of shoots (3.00), followed by Ghanimat-e-IBGE (2.56) and the least number of shoots were observed in Siran (2.47). The supplementation of NAA also had statistically significant effect on the number of shoots of wheat. Number of shoots had been observed to increase with increase in hormones i.e., NAA. The highest number of shoots was observed for RIM to which 0.1 mg/L of NAA was added (3.01). The other RIM without the NAA had been recorded with the least number of shoots (2.34). The plant growth hormone BAP had significant effect on number of shoots of the various wheat genotypes. Maximum number of shoots was recorded for BAP with supplement of 3 mg/L in medium (4.21), followed by BAP at 2 mg/L (3.47). In contrast, minimum number of shoots was noted for RIM with no supplement of BAP (1.25), which was followed by BAP supplement at 1 mg/L (1.78) (Table 2).

**Table 2:** *Days to shoot induction, number of shoots, shoot length, and shoot induction percentage of the wheat genotypes under different conditions.*

| Factors | Levels | Days to shoot induction | Number of shoots | Shoot length | Shoot induction percentage |
|---|---|---|---|---|---|
| BAP ($mg.L^{-1}$) | 0 | 9.53A | 1.25D | 2.39D | 31.17D |
| | 1 | 8.09B | 1.78C | 3.23C | 39.22C |
| | 2 | 6.51C | 3.47B | 4.44B | 48.86B |
| | 3 | 4.68D | 4.21A | 5.15A | 58.36A |
| NAA ($mg.L^{-1}$) | 0 | 7.89A | 2.34B | 3.68 | 34.57B |
| | 0.1 | 6.51B | 3.01A | 3.92 | 54.24A |
| Genotypes | Siran | 7.71A | 2.47B | 3.60C | 40.36 |
| | Atta-Habib | 6.5B | 3.00A | 4.09A | 47.95 |
| | Ghanimat-e-IBGE | 7.4A | 2.56B | 3.71B | 44.9 |

Consequently, the number of shoots produced from the various wheat genotypes effected by different tested factors i.e., BAP, NAA and the genotypes interactions had shown that except for interaction of BAP x NAA, the others interaction were determine with non-significant effect on number of shoots. The number of shoots produced from the various wheat genotypes effected by different tested factors i.e., BAP, NAA and the genotypes interactions had shown that except for interaction of BAP x NAA, the others interaction were determine with non-significant effect on number of shoots. The number of shoots among the genotypes to which 0.1 mg/L

NAA supplement was applied showed the maximum shoots number in Atta-Habib followed by Siran and Ghanimat-e-IBGE with resultant 3.37, 2.86 and 2.81 numbers of shoots, respectively. The medium of genotypes to which no NAA supplement was applied had shown the least number of shoots in all the three genotypes i.e., Atta-Habib, Ghanimat-e-IBGE and Siran (2.63, 2.31 and 2.07 number of shoots). The number of shoots was maximum for overall genotypes that were incubated on growth medium with supplement of BAP at 3 mg/L and NAA at 0.1 mg/L (4.49). These results were followed by BAP at 3 mg/L in medium and that no NAA as supplement was applied (3.93). The least number of shoots was observed for the mean of genotypes incubated on medium with no plant growth hormones i.e., BAP and NAA (1.11) followed by genotypes incubation medium with only NAA supplement at 0.1 mg/L (1.39) (Supplementary Table S5 and S5a).

*Shoot length*

The resultant significant variation was observed in shoot length among the wheat genotypes i.e., Siran, Atta-Habib and Ghanimat-e-IBGE under different concentration of BAP and NAA. The maximum shoot length was observed in Atta-Habib (4.09) which was followed by Ghanimat-e-IBGE (3.71) and the minimum shoot length to be observed in Siran (3.60). The NAA supplement had statistically significant effect on shoot length. Consequently, the maximum shoot length was recorded on RIM containing 0.1 $mg.L^{-1}$ NAA (3.92), followed by RIM without any supplementation of NAA (3.68). The BAP supplement on shooting medium also had significant effect on shoot length. The shoot length was recorded maximum for the shooting medium with highest concentration of BAP supplement at 3 mg/L (5.15), followed by 2 mg/L BAP supplement (4.44). The least shoot length was recorded for medium to which no BAP supplement was applied (2.39), followed by the set of medium to which BAP supplement was applied at 1 mg/L (3.23) (Table 2).

Consequently, the interaction among the tested factors BAP, NAA and the genotypes had been observed statistically significant for shoot length only in BAP x NAA, NAA x genotype, BAP x genotypes and BAP x NAA x genotypes had shown significant effect Whereas, the interaction among the tested factors BAP, NAA and the genotypes had been observed statistically significant for shoot length only in BAP x NAA. The shoot length had been observed maximum for Atta-Habib (4.12) on medium with supplement of NAA at 0.1 mg/L, followed by Ghanimat-e-IBGE (3.88) at this supplement. The minimum shoot length was recorded for Siran (3.44) on RIM with no supplement of NAA, followed by Ghanimat-e-IBGE (3.54). The mean of shoot length for overall genotypes was recorded maximum for medium containing the growth hormones BAP and NAA at maximum concentration i.e., 3 mg/L and 0.1 mg/L (5.27). This result was followed by the genotype's seeds incubated on medium with only BAP supplement at 3 mg/L (5.02). The least shoot length mean of overall genotypes was recorded for medium to which seeds were incubated with no addition of plant growth hormones i.e., BAP and NAA at 0 mg/L (2.42). This result was followed by the medium with 0.1 mg/L of NAA supplement, with mean genotypes shoot length of 2.35. The interaction of BAP, NAA and genotypes results showed that the maximum shoot length was observed for all the genotypes to which level of BAP and NAA was applied with the highest concentration i.e., 3 and 0.1 mg/L. Among the genotypes the maximum shoot length was observed in Atta-Habib genotype, followed by Ghanimat-e-IBGE and then Siran with resultant 5.69, 5.21 and 4.91 shoot lengths, respectively. The minimum shoot length was observed for Siran (2.15) that was incubated on medium with supplement of NAA at 0.1 mg/L, followed by similar genotype (2.21) that was incubated on medium with no hormones applied i.e., BAP and NAA (Supplementary Table S6 and S6a).

*Shoot induction percentage*

The shoot induction percentage non-significantly varied among the wheat genotypes while the hormone NAA effect was observed significantly maximum for RIM with 0.1 mg/L supplement of NAA (54.24 %), which was followed by low shoot induction percentage with 0 mg/L (34.57 %) supplementation in the medium. The shoot induction percentage had been determined statistically significant with supplement of BAP. The shooting medium to which supplement of BAP at 3 mg/L was applied showed maximum shoot induction percentage with resultant 58.36 % of shoot induction percentage, followed by 2 mg/L and 3 mg/L BAP supplement with 48.86 and 39.22 % shoot induction percentage. The minimum shoot induction percentage was recorded for the medium to which no supplement of BAP was applied i.e., 31.17 % (Table 2). However, the tested factors i.e., BAP,

NAA and different genotypes interaction effect on shoot induction percentage had been observed non-significant (Supplementary Table S7 and S7a).

*Days to root induction*

Results of days to root induction of different wheat genotypes i.e., Siran, Atta-Habib and Ghanimat-e-IBGE under different concentrations of IAA and NAA showed significant variation in days to root induction of the wheat genotypes. Results showed that the maximum days to root induction were observed in Siran wheat genotype (9.16 days), followed by Ghanimat-e-IBGE (8.05 days) and the minimum days to rooting in Atta-Habib wheat genotype (7.74 days). The plant growth hormones i.e., NAA was analyzed which showed significant effect of these hormones on days to root induction. The days to root induction was observed highest with RIM to which no NAA supplement (9.16 days) was provided, whereas the least days to root induction was observed in NAA supplement at 0.2 mg/L (7.47 days). The plant growth hormone IAA on RIM also had significant effect on days to root induction of wheat genotype. The minimum days to root induction was recorded for IAA with supplement of 3 mg/L in medium (6.13 days), followed by IAA at 2 mg/L (7.70 days). In contrast, maximum days for root induction were noted for rooting medium with no supplement of IAA (10.42 days), followed by IAA supplement at 1 mg/L (9.02 days) (Table 3). However, the interaction among the factors i.e., IAA x NAA, IAA x genotypes, NAA x genotypes and IAA x NAA x genotypes, had been statistically determined with non-significant effect on days to root induction (Supplementary Table S8 and S8a).

*Number of roots*

The number of roots of three wheat genotypes i.e., Siran, Atta-Habib and Ghanimat-e-IBGE under different concentration of NAA had statistically been observed with non-significant effect on the number of wheat roots. While the number of roots had been determining to be effected significantly with supplementation of IAA. The IAA supplement with highest concentration i.e., 3 mg/L in medium had shown the maximum number of roots (5.82) which was followed by IAA supplement applied 2 mg/L (3.49). The least number of roots was observed for the medium to which IAA supplement was applied at 1 mg/L (2.27) which was followed by the medium with no IAA supplement (2.50) (Table 3). Similarly, the number of roots produced from the various wheat genotypes were effected by different tested factors i.e., IAA, NAA and the genotypes interaction levels had shown non-significant effect on number of roots. These interactions include IAA x NAA, IAA x genotypes, NAA x genotypes and IAA x NAA x genotypes (Supplementary Table S9 and S9a).

*Root length*

The resultant non-significant variation was observed in root length among the wheat genotypes i.e., Siran, Atta-Habib and Ghanimat-e-IBGE under different concentrations of IAA and NAA. While the IAA supplement on rooting medium had been observed with significant effect on root length. The IAA supplement at 3 mg/L had shown the genotypes with the maximum root length (5.38), which was followed by medium with IAA supplement at 2 mg/L (3.10). The least root length was recorded for the medium to which 1 mg/L IAA supplement was applied (1.96) followed by the medium to which no supplement of IAA was applied (2.27) (Table 3). Similarly, the different interactions among the factors i.e., IAA x NAA, IAA x genotypes, NAA x genotypes and IAA x NAA x genotypes, had also shown no significant effect on root length of the wheat genotypes (Supplementary Table S10 and S10a).

**Table 3:** *Days to root induction, number of roots, root length, and root percentage of the wheat genotypes under different conditions.*

| Factors | Levels | Days to Root induction | N. of roots | Root length | Root percentage |
|---|---|---|---|---|---|
| IAA ($mg.L^{-1}$) | 0 | 10.42 A | 2.50 C | 2.27 B | 16.94 |
| | 1 | 9.02 B | 2.27 C | 1.96 B | 25.28 |
| | 2 | 7.70 C | 3.49 B | 3.10 B | 36.62 |
| | 3 | 6.13 D | 5.82 A | 5.38 A | 61.6 |
| NAA ($mg.L^{-1}$) | 0 | 9.16 A | 3.34 | 3.29 | 33.74 |
| | 0.1 | 7.47 B | 3.69 | 3.07 | 36.48 |
| Genotypes | Siran | 9.16 A | 3.2 | 2.69 | 31.36 B |
| | Atta-Habib | 7.74 B | 3.8 | 3.52 | 39.25 A |
| | Ghanimat-e-IBGE | 8.05 B | 3.56 | 3.32 | 34.72 AB |

*Root induction percentage*

Results regarding the root induction percentage of wheat genotypes under different concentration of IAA and NAA showed that a significant variation was

noted in the root induction percentage of the wheat genotypes i.e., Siran, Atta-Habib and Ghanimat-e-IBGE. Consequently, the supplementation of NAA had statistically been observed with non-significant effect on the root induction percentage of wheat while the IAA supplement had been determined with statistically significant effect on the different wheat genotype root induction percentage. The highest root induction percentage produced from the seeds were observed in Atta-Habib (39.25 %), followed by Ghanimat-e-IBGE (34.72%), while the minimum root induction percentage was observed in Siran (31.36%). Subsequently, the root induction percentage was recorded maximum for the wheat genotypes on medium with highest concentration of IAA supplement applied i.e., 3 mg/L (61.60 %), followed by 2 mg/L of IAA supplement (36.62 %). The least root induction percentage was recorded for the medium to which no IAA supplement was applied (16.94 %), followed by the set of medium to which IAA supplement was applied at 1 mg/L (25.28 %) (Table 3). Furthermore, the root induction percentage was affected due to interaction of tested factors including IAA, NAA and genotypes had been determined with statistically non-significant effect (Supplementary Table S11 and S11a).

## Discussion

As for the increase demand for wheat production, such tissue culture based methods are required that can influencial increase overall regeneration system. The inital steps of plant tissue culture involve the sterilization of the explants by reagent such as sodium hypochlorite, calcium hypochlorite, ethanol and mercuric chloride. However, these reagents have shown to reduce the regeneration efficiency. The callus induction using 2,4-D have been claimed to have optimum effect in wheat (Yu *et al.*, 2008). The shoot regeneration of wheat using BAP and kinetin have extensively reported in literature (Kumar *et al.*, 2017).

The present study results had shown that the Atta-Habib (50.2) variety had shown to induce highest frequency of callus compare to Ghanimat-e-IBGE and Siran (47.9 and 42.8) (Figure 1). The Atta-Habib had also shown better callus induction with the least days i.e., 6.2, followed by Ghanimat-e-IBGE and the highest days to callus induction was recorded in Siran. The influenced of genotype to callus induction rate and callus induction days have been observed greatly by Benderradji *et al.* (2012), who had study, the callus induction, proliferation and plantlets regeneration of

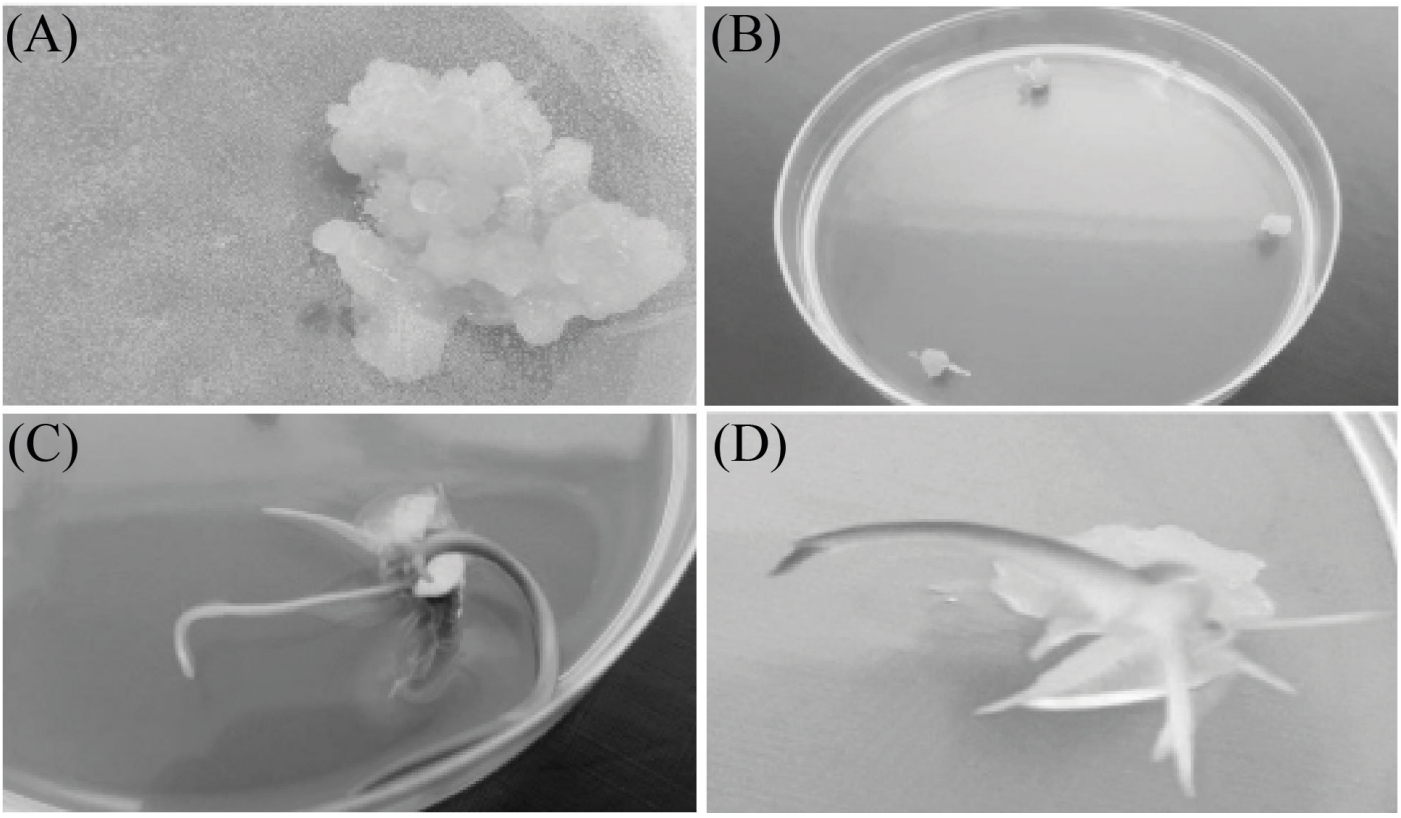


**Figure 1:** *The 4 weeks old calli of wheat genotypes. Atta-Habib growing on CIM (A), SIM (B) and the in vitro regenerated wheat plantlet on RIM (c and D).*

two bread wheat i.e., Mahon-Demias and Hidhab. The present research results were also in line with findings of Nasircilar *et al.* (2006), who had reported that the callus induction and seedling regeneration frequencies among the wheat genotype ranged from 12 to 100 %. Such variation among the genotypes of wheat is because of the many factors of that variety including the nature of genotype, the source of explant and the medium composition. Ozgen *et al.* (1998) in their study had shown that immature embryos were recorded with the highest callus induction, whereas the mature embryo plants had shown the least frequency of callus induction. Though alike the present study, the 2, 4-D synthetic auxin have shown higher callus induction in previous studies, as its higher concentration have shown aberration in inducing chromosome which alternately results in soma-clonal variation.

The calli in the present study had shown that Atta-Habib had the highest callus producing (25.1) from seeds followed by Ghanimat-e-IBGE and the least by Siran (23.9 and 21.4), respectively. Saha *et al.* (2017) had reported that 2, 4-D application for mature seeds were observed around 50 % whereas for immature seed the callus induction was recorded around 60% in MS medium. Kowalska and Arseniu (2016) have reported that 2,4-D, diamba (3,6-dichloro-2-methoxybenzoic acid), NAA and the carbon source maltose and sucrose have shown effectiveness in producing winter wheat cultivars from embryos. Malik *et al.* (2017) evaluated efficiency of wheat cultivars by addition of zeatin and dicamba at 1mg/L and 0.1 mg/l, respectively, which had shown highest regeneration capacity of callus into shoots. As for carbon sources, maltose had shown the highest callus induction efficiency for the tested cultivars i.e., HD 2967, PBW 343, WH 1105, RAJ 3765 and C 306.

In our study, BAP at 3 mg/l and NAA at 0.1 mg/l had shown the best shoot regeneration effeciency, whereas other combination of hormones had also shown similar results. The NAA and BAP combination have also shown efficient shoot regeneration in other plants family such as Brassicaceae. Saha *et al.* (2017) have reported that BAP with combination of IAA have shown successful regeneration of the wheat plant from mature seeds calli. According to Salama *et al.* (2013), who evaluated callus induction and regeneration of wheat cultivars in Egypt. From their study it was concluded that callus induction was observed best for 2,4-D at 2 mg/l, whereas the regeneration was perfect for medium with supplement of BAP at 3 mg/L and NAA at 0.1 mg/l.

Consequently, the root regeneration was optimized with supplement of IAA alone as well as in combination of NAA. These results were also in line with the findings of Xiaohong *et al.* (1999), who had study, the improvement of wheat plant regeneratin from callus using various plant hormones. From the study Xiaohong *et al.* (1999) it was concluded that supplement with IAA or NAA in medium for wheat rooting will strengthen formation of the root. Yurkova *et al.* (1981) had studied 9 different spring and winter wheat cultivars organogentic ability. With combination of IAA or NAA with kinetin had shown the highest frequency of plant regeneration, as well as increase in calli age was observed with declined organogenetic ability.

## Conclusion

In present study three different wheat genotypes Siran, Atta-Habib, and Ghanimat-e-IBGE were experimented for callus induction and regeneration frequency. The excellence of calli was accessed on visual observation and the data was recorded on the basis of days to callus induction, callus induction frequency, number of seeds producing calli, shoot induction percentage, days to shoot induction, shoot length, number of shoots, root induction percentage, days to root induction, root length and number of roots. The Attanhabib wheat cultivar had the highest callus frequency, callus induction from seed, days to shooting, shoot length, root percentage and number of root. Siran cultivar had the highest root length whereas Ghanimat cultivar was observed with the highest numbr of shoot and shoot percent. The least days to callus induction and rooting was of Attanhabib, whereas the least days to shooting was recorded of Siran. The phytohormone 2,4-D had been highly efficient for callus induction frequency, days to callus induction and seeds producing calli. Phytohormones BAP @ 3 mg/l and NAA @ 0.1 mg/l were highly efficient for days to shooting, number of shoot, shoot length and shoot percent. Phytohormones IAA @ 3 mg/l and NAA @ 0.2 mg/l were highly efficient for days to rooting and root percentage. The root length and number of root was maximum for phytohormone IAA @ 3 mg/l followed by the combination of NAA @ 0.2 mg/l.

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## Novelty Statement

The developed system is recommended for in vitro culture of wheat and other related advance research in plant tissue culture. Various other factors associated with wheat regeneration such as carbon sources, gelling agents and other growth regulators are further recommended to be investigate in these cultivars as well as presently other cultivars in the country.

## Author's Contribution

Conceptualization, methodology, formal analysis: Jawaria Urooj and Aqib Iqbal, Writing-Original draft: Jawaria Urooj, Data curation and validation: Fareeha Sharif and Muhammad Junaid, Supervision, Writing-Review and editing: Aqib Iqbal.

*Supplementary material*

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*Generative AI and AI-assisted technology statement*

During the preparation of this work, no generative artificial intelligence (AI) or AI-assisted technologies were used in the writing, editing, data analysis, or figure generation. All content was produced entirely by the authors.

*Conflict of interest*

The authors have declared no conflict of interest.

## References

Afzal, A., H. Rashid, M.H. Khan, Z. Chaudhry and S.A. Malik. 2010. High frequency regeneration system optimization for wheat cultivar Inqilab-91. Pak. J. Bot., 42(3): 1857-1862.

Aslam, M., A. Majid, P.R. Hobbs, N.I. Hashmi and D. Byerlee. 1989. Wheat in the rice-wheat cropping system of the Punjab: A synthesis of On-farm research results 1984-1988.

Benderradji, Laid, F. Brini, K. Kellou, N. Ykhlef, A. Djekoun, K. Masmoudi, and H. Bouzerzour. 2012. Callus Induction, Proliferation, and Plantlets Regeneration of Two Bread Wheat (*Triticum Aestivum* L.) Genotypes under Saline and Heat Stress Conditions. ISRN Agronomy 2012:1–8. doi:10.5402/2012/367851.

Bhalla, P.L., H.H. Ottenhof and M.B. Singh. 2006. Wheat transformation–an update of recent progress. Euphytica, 149(3): 353-366. https://doi.org/10.1007/s10681-006-9087-6

Cilar A., K. Turgutand and K. Fiskin. 2006. Callus induction and plant regeneration from mature embryos of different wheat genotypes. Pak. J. Bot. 38(2): 637–645.

Kent-Jones, D.W., 2016. Cereal farming. Encyclopedia Britannica.

Gerszberg, Aneta, K. Hnatuszko-Konka, and T. Kowalczyk. 2015. In Vitro Regeneration of Eight Cultivars of Brassica Oleracea Var. Capitata. In Vitro Cellular & Developmental Biology - Plant 51(1):80–87. doi:10.1007/s11627-014-9648-7.

Hafeez, I., B. Sadia, N.A. Sadaqat, R.A. Kainth, M.Z. Iqbal and I.A. Khan. 2012. Establishment of efficient in vitro culture protocol for wheat land races of Pakistan. Afr. J. Biotechnol., 11(11): 2782-2790. https://doi.org/10.5897/AJB11.2126

Henry, Y., P. Vain and J. De Buyser. 1994. Genetic analysis of in vitro plant tissue culture responses and regeneration capacities. Euphytica, 79(1): 45-58. https://doi.org/10.1007/BF00023575

Jones, H.D., A. Doherty and H. Wu. 2005. Review of methodologies and a protocol for the Agrobacterium-mediated transformation of wheat. Plant Methods, 1(1): 1-5. https://doi.org/10.1186/1746-4811-1-5

Joyia, F.A. and M.S. Khan. 2013. Scutellum-derived callus-based efficient and reproducible regeneration system for elite varieties of indica rice in Pakistan. Int. J. Agric. Biol., 15: 27-33.

Kowalska, L., and Arseniu, E. (2016). The effect of medium composition on callus induction and plant regeneration frequencies from mature embryos of wheat cultivars with various resistance to Parastagonospora nodorum. Indian research journal of genetic and biotechnology, 8(3), 183-189. www.ihar.edu.pl/download.

php?id=2879

Kumar, Rakesh, H.M. Mamrutha, A. Kaur, K. Venkatesh, A. Grewal, R. Kumar and V. Tiwari. 2017. Development of an Efficient and Reproducible Regeneration System in Wheat (*Triticum Aestivum* L.). Physiology and Molecular Biology of Plants 23(4):94554. doi:10.1007/s12298-017-0463-6.

Malik, Kapil, D. Birla, H. Yadav, M. Sainger, D. Chaudhary and P.K. Jaiwal. 2017. Evaluation of Carbon Sources, Gelling Agents, Growth Hormones and Additives for Efficient Callus Induction and Plant Regeneration in Indian Wheat (*Triticum Aestivum* L.) Genotypes Using Mature Embryos. Journal of Crop Science and Biotechnology 20(3):185–92. doi:10.1007/s12892-017-0046-0

Mendoza, M.G. and H.F. Kaeppler. 2002. Auxin and sugar effects on callus induction and plant regeneration frequencies from mature embryos of wheat (*Triticum aestivum* L.). *In Vitro* Cell. Dev. Biol. Plant, 38(1): 39-45. https://doi.org/10.1079/IVP2001250

Murashige, Toshio, and Folke Skoog. 1962. A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiologia Plantarum 15(3):473. doi:10.1111/j.1399-3054.1962.tb08052.x

Nasircilar, Gul, K. Turgut and K. Fiskin. 2006. Callus Induction and Plant Regeneration from Mature Embryos of Different Wheat Genotypes. Pakistan Journal of Botany 38.

Nuttonson, M.Y., 1955. Wheat-climatic relationships and the use of phenology in ascertaining the thermal and photothermal requirements of wheat. Washington, DC, American Institute of Crop Ecology. Crop Sci., (17): 388.

Özgen, M., M. Türet, S. Altınok and C. Sancak. 1998. Efficient callus induction and plant regeneration from mature embryo culture of winter wheat (*Triticum aestivum* L.) genotypes. Plant Cell Rep., 18(3): 331-335. https://doi.org/10.1007/s002990050581

Patnaik, D. and P. Khurana. 2001. Wheat biotechnology: A minireview. Electron. J. Biotechnol., 4(2). https://doi.org/10.2225/vol4-issue2-fulltext-4

Percival, J., 1921. The wheat plant. A monograph. New York, NY, USA, E.P. Dutton and Company. https://doi.org/10.5962/bhl.title.25683

Rashid, U., S. Ali, G.M. Ali, N. Ayub and M.S. Masood. 2009. Establishment of an efficient callus induction and plant regeneration system in Pakistani wheat (*Triticum aestivum*) cultivars. Electron. J. Biotechnol., 12(3): 0717-3458. https://doi.org/10.2225/vol12-issue3-fulltext-1

Saha, Supria, Z. Islam, S. Islam, M.F. Hassan, M.S. Hossain and S.M.S. Islam. 2017. Enhancement of Somatic Embryogenesis by Mature and Immature Seeds in Wheat (*Triticum Aestivum* L.). Journal of Biology and Life Science 8(2):20. doi:10.5296/jbls.v8i2.11529.

Salama, E. A., A. I. A. Abido, A. E. Khaled, and N. R. Abdelsalam. 2013. Embryo Callus Induction and Regeneration of Some Egyptian Wheat Cultivars. https://www.cabidigitallibrary.org/doi/full/10.5555/20143012278.

Saunders, D.A. and G.P. Hettel. 1994. Wheat in heatstressed environments: irrigated, dry areas and rice-wheat farming systems. Mexico, DF, CIMMYT.

Shewry, P.R. and H.D. Jones. 2005. Review Paper. Transgenic wheat: Where do we stand after the first 12 years. Ann. Appl. Biol., 147: 1. https://doi.org/10.1111/j.1744-7348.2005.00009.x

Vasil, V., A. Castillo, M. Fromm and I. Vasil. 1992. Herbicide resistant fertile transgenic wheat plants obtained by micro-projectile bombardment of regenerable embryogenic callus. Biotechnology, 10: 667-673. https://doi.org/10.1038/nbt0692-667

Xiaohong, Yu, Z. Zhen and A. Lijia. 1999. Improvement in Plant Regeneration from Callus in Wheat. https://agris.fao.org/search/en/providers/122431/records/6472327ae17b74d2224e8f9b.

Yu, Y., J. Wang, M. L. Zhu, and Z. M. Wei. 2008. Optimization of Mature Embryo-Based High Frequency Callus Induction and Plant Regeneration from Elite Wheat Cultivars Grown in China. Plant Breeding 127(3):249–55. doi:10.1111/j.1439-0523.2007.01461.x

Yurkova, Galina N., Boris A. Levenko, and Oleg V. Novozhilov. 1981. Induction of Plant Regeneration in Wheat Tissue Culture." Biochemie Und Physiologie Der Pflanzen 176(3): 236–43.

Zale, J.M., H. Borchardt-Wier, K.K. Kidwell and C.M. Steber. 2004. Callus induction and plant regeneration from mature embryos of a diverse

set of wheat genotypes. Plant Cell, Tissue Organ Culture, 76(3): 277-281. https://doi.org/10.1023/B:TICU.0000009248.32457.4c

Zhou, H., J.D. Berg, S.E. Blank, C.A. Chay, G. Chen, S.R. ESkelsen, J.E. Fry, S. Hoi, T. Hu, P.J. Isakson, M.B. Lawton, S.G. Metz, C.B. Rempel, D.K. Ryerson, A.P. Sansone, A.L. Shook, R.J. Starke, J.M. Tichota and S.A. Valenti. 2003. Field efficacy assessment of transgenic Roundup Ready wheat. Crop Sci., 43(3): 1072-1075. https://doi.org/10.2135/cropsci2003.1072