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Callus induction and efficient plant regeneration in wheat (*Triticum aestivum* L.) through mature embryo culture

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Abstract
Mature embryos of wheat were taken for callus development and efficient regeneration system through *in vitro* culture. Mature embryos in wheat have an added advantage that these are easily available round the year. In the present investigation we developed a protocol for sterilization of wheat seeds, callusing and regeneration. The technique developed used as a novel tool to assist plant breeders in crop improvements. Standardization of sterilization process was achieved successfully with ethanol (70%) for 30 second + $HgCl_2$ (0.1%) for 1 min + NaOCl (1.0%) for 5 minute in both the genotypes viz. HD 2967 and C 306 selected for this study.. The different concentrations and combinations of plant growth regulators (PGRs) such as ABA, 2,4-D and NAA were used for callusing and BAP, NAA and TDZ were used for regeneration of plants from calluses. In the present study we observed that 1.0 mg/l ABA + 1.0 mg/l 2-4D + 2.0 mg/l NAA was optimum for callus development in genotype C306 and 70.00 percent callus induction achieved with no precocious germination. Genotype HD2967 showed 76.6 percent callus induction with low precocious germinations with the same concentration combinations of PGRs. The highest regeneration percentage 93.33% was recorded with the concentration combinations of PGRs (1.0 mg/l BAP + 2.0 mg/l TDZ + 0.5 mg/l NAA) in both the genotypes. The concentration of 0.5 mg/L NAA + 0.1 mg/L IBA produced better roots in regenerated shoots in genotype HD2967 and for genotype C 306 the rooting media gave good rooting with PGRs (0.1 mg/L NAA + 0.5 mg/L IBA).

Keywords: Wheat, *In vitro* culture, regeneration, plant growth regulators, mature embryo

Introduction
Wheat (*Triticum aestivum* L.) is one of the most important staple food crops of the family *Poaceae.* It is the third largest cereal in terms of production in the world after rice and maize and the second most important in terms of calories after rice. Worldwide, it is cultivated on approximately 218 million hectares of land with a production of 771 million tons. In India, it is cultivated on approximately 30.60 million hectares of land with a production of 98.5 million tons (FAOSTAT 2017) [4]. A number of environmental factors such as temperature, moisture, soil and light intensity affect the growth and yield of wheat (Keresa *et al.,* 2000) [8]. The two main groups of commercial wheat are the durum (*Triticum durum* L.) and bread wheat (*Triticum aestivum* L.) with 28 and 42 chromosomes, respectively. The wild species are still a valuable source of useful agronomic traits for the continued improvement of cultivated wheat. Wide hybridization of wheat with grasses, coupled with cytogenetic manipulation of the hybrid material has been instrumental in the genetic improvement of wheat (Sramkova *et al*., 2009) [15]. Bread wheat is used in making bread, rolls, cakes, cookies, and pastries. Durum wheat is also used for making pasta products (Wiese *et al.,* 1987) [17]. If wheat have high amount of protein (13-16%), they can be used for bread making. Wheat having low protein content (8-11%) can be used for pastries, cookies, crackers, flat breads and oriental noodles (Cook *et al.,* 1993) [3]. For the improvement of wheat, a group of activities was focused on *in vitro* culture and regeneration as a tool for cereal breeding in the recent years. Since plant tissue culture technique is one of the potential techniques for the improvement of wheat (Kumar *et al.,* 2015) [9]. Wheat has been the last cereal to be genetically transformed partially due to its recalcitrant nature to *in vitro* plant regeneration, its complex hexaploid and large sized genome approximately 17 Gb with rich repetitive sequences (Malik *et al.,* 2017) [12].

Tissue culture is an Integral part of biotechnology breeding and provides an added advantage to crop improvement programme. The hindrance of different cereal crops to regeneration through callus is a major bottleneck in any crop improvement program including wheat (Kumar *et al.,* 2017) [10]. *In vitro* plant regeneration is mainly dependent upon three main factors: source of explants, media composition and genotype (Malik *et al.,* 2017) [12]. The high frequency of callus induction and regeneration from mature embryo in wheat depends on sterilization process, pH of media and concentration of growth hormones (Parmar *et al.,* 2012; Kumar *et al.,* 2017) [14, 10]. The different concentrations of plant growth regulators (PGRs) such as Auxins, Naphthalene acetic acid (NAA) and Dichlorophenoxyacetic acid (2,4-D) and two different cytokinins 6-Benzylaminopurine (BAP) and Kinetin (Kn) are highly affective for callus induction and plant regeneration using mature embryo of wheat (Jasdeep *et al*., 2019) [7]. The mature embryo of wheat genotypes showed significant difference with maximum frequency of callusing in concentration of plant growth regulators fortified in MS medium(Saha., 2017) [16]. The average 19 shoots per explants observed with a combination of thidiazuron (TDZ) and 2,4-D in MS medium (Parmar *et al.,* 2012) [14].
In the present study, we chosen two Indian agronomical cultivated genotypes of wheat Viz. C306 and HD2967 used for standardization of protocol for callus induction and efficient regeneration system using mature embryo cultures.

Materials and Method
Collection of germplasm
Mature seeds of two wheat (*Triticum aestivum* L.) genotypes i.e. C306 and HD2967 were procured from Department of Genetics and Plant Breeding, SVPUA&T, Meerut. *In vitro* culture experiments was conducted at Plant Tissue Culture Laboratory, Department of Agricultural Biotechnology, Sardar Vallabhbhai Patel University of Agriculture & Technology, Modipuram, Meerut, Uttar Pradesh.

Explants sterilization
The mature embryos of wheat seeds were used as explants in the present investigation. The wheat seeds were washed with running tab water thrice followed by washing of seeds with Tween-20 in sterilized distilled water. The seeds were then rinsed with 70% ethanol and dipped in 0.1% $HgCl_2$. Sterilization of seeds was done using 2% v/v Sodium hypochlorite for different duration followed by washing with sterile distilled water again. After sterilization, mature seeds were soaked in autoclaved distilled water in a jam bottle sealed with parafilm and incubated overnight at 10-12 ºC. The mature embryos from seeds were excised using a sterile needle under laminar air flow and were placed in culture media.

Callus induction
The explants (excised mature embryos) were used for culture establishment for callus induction. Explants were placed in reverse orientation on callus induction MS medium supplemented with different plant growth regulators (PGR) using different concentration combinations of NAA, 2 4-D and ABA ranging from 0.5 to 2.0 mg/l (Table 1). The cultures were then incubated at 22 ± 2°C in a dark environment for one week. The observations were recorded for growth pattern, type, colour and percentage of callus obtained from each type of explants after 28 days of culturing. The experiment was repeated thrice and callus induction was recorded after 10 days.

Shoot regeneration
The callus was cut aseptically and cultured on regeneration media containing the total eight different concentrations of BAP, TDZ and NAA (BAP 1.0, 1.5, and 2.0 mg/L, TDZ 1.0, 1.5, and 2.0 mg/L and NAA 0.5, and 1.0 mg/L) for shoot induction and placed in the slanted test tubes (25×150mm) having MS media (Table 1). Shoot regeneration callus subcultures were maintained under white fluorescent light with an intensity of 2400 lux for 16 hours at a temperature of 25± 2 °C. Each treatment is replicated thrice and numbers of shoot per callus were recorded after 28 days.

Root initiation
Well-developed shoots with leaves were rescued aseptically from the culture vessels and separated from each other and again cultured on freshly prepared half MS medium containing different concentrations of NAA or IBA for root induction. A total of four root initiation MS media were used. Root initiation MS media were maintained under white fluorescent light with an intensity of 2400 lux for 16 hours at a temperature of 25± 2 °C for the cultural room followed by three replications for each treatment and the number of shoot per callus were recorded after 14 days.

Table 1: The different concentrations of plant growth regulators on MS media used for callus induction, plant regeneration and rooting.

Medium name	Plant growth regulators (mg/l)
Callus induction on MS media (8 g/l agar and pH 5.6-5.8)	
MS0	2,4-D(0.0) + NAA (0.0)
MS1	2,4-D(0.5) + NAA (0.5)
MS2	2,4-D(0.5) + NAA (1.0)
MS3	2,4-D(0.5) + NAA (1.5)
MS4	2,4-D(0.5) + NAA (2.0)
MS5	2,4-D(1.0) + NAA (0.5)
MS6	2,4-D(1.0) + NAA (1.0)
MS7	2,4-D(1.0) + NAA (1.5)
MS8	2,4-D(1.0) + NAA (2.0)
MS9	2,4-D(1.5) + NAA (0.5)
MS10	2,4-D(1.5) + NAA (1.0)
MS11	2,4-D(1.5) + NAA (1.5)
MS12	2,4-D(1.5) + NAA (2.0)
MS13	2,4-D(2.0) + NAA (0.5)
MS14	2,4-D(2.0) + NAA (1.0)
MS15	2,4-D(2.0) + NAA (1.5)

MS16	2,4-D(2.0) + NAA (2.0)
Callus induction on MS media (8 g/l agar and pH 5.6-5.8) with ABA plant growth hormones	
MS1	2,4-D(1.0) + NAA (2.0) + ABA (0.5)
MS2	2,4-D(2.0) + NAA (0.5) + ABA (1.0)
MS3	2,4-D(2.0) + NAA (2.0) + ABA (2.0)
MS4	2,4-D(1.0) + NAA (2.0) + ABA (0.5)
MS5	2,4-D(2.0) + NAA (0.5) + ABA (1.0)
MS6	2,4-D(2.0) + NAA (2.0) + ABA (2.0)
Plant regeneration on MS media (8 g/l agar and pH 5.6-5.8)	
RM0	BAP (0.0) + TDZ (0.0) + NAA (0.0)
RM1	BAP (1.0) + TDZ (0.0) + NAA (0.5)
RM2	BAP (1.5) + TDZ (1.0) + NAA (0.5)
RM3	BAP (2.0) + TDZ (1.5) + NAA (0.5)
RM4	BAP (1.0) + TDZ (2.0) + NAA (0.5)
RM5	BAP (1.5) + TDZ (0.0) + NAA (1.0)
RM6	BAP (2.0) + TDZ (1.0) + NAA (1.0)
RM7	BAP (1.0) + TDZ (1.5) + NAA (1.0)
RM8	BAP (1.5) + TDZ (2.0) + NAA (1.0)
Rooting on half MS media (8 g/l agar and pH 5.6-5.8)	
RTM0	NAA (0.0) + IBA (0.0)
RTM1	NAA (0.1) + IBA (0.1)
RTM2	NAA (0.1) + IBA (0.5)
RTM3	NAA (0.5) + IBA (0.1)
RTM4	NAA (0.5) + IBA (0.5)

Acclimatization of regenerated plantlets

The wheat plantlets developed efficiently with 4-5 cm high shoots with several well ramified roots ready to transplant. Plantlets were removed from culture container and agar was gently washed from roots under running tap water. The developed plantlets were then dipped in a fungicide solution (Bavistin, 2g/l) prior to transplantation to the plastic pots to reduce the risk of any fungal attack. Individual plantlets were transplanted into different soil-peat mixture and water with 50 ml Hoagland solution e.g. Coco peat, Coco peat: Soil (1:1 v/v).

Statistical Analysis

The experiment was set up in a completely randomized design (CRD) with a minimum of three cultures per treatment. All the data were analyzed by running one way analysis of variance (ANOVA) using SPSS version 19 software. The means were compared using critical difference to find the difference at 5% ($P<0.05$) level. The results are expressed as a mean ± SE of five replications.

Results

Explants sterilization

The plant seeds from the environment are naturally contaminated on their surface with microorganisms such as various bacteria, viruses and fungus. In the present investigation, an exposure of surface sterilent was given to the explants (mature embryo) for different time period. Hundred percent survivals of wheat explants observed among the control with no contamination. Explants cultured on MS medium incubated at 25 + 2ºC at 16 hours photoperiod. The treatment MS 5; with ethanol (70%) for 30 seconds, $HgCl_2$ (0.1%) for 5 minutes and Sodium hypochlorite (2%) for 5 minutes (Data not shown) was found most effective for surface sterilization of wheat mature embryo explants.

Callus induction

A total of twenty two different combinations of MS media were used in two wheat genotype Viz. C306 and HD2967 for callus induction, they showed different percentage of precocious germinations. The result showed callus induction with concentration of plant growth hormone with MS media (MS-8) containing 1.0 mg/L 2-4D + 2.0 mg/L NAA, MS-13 containing 2.0 mg/L 2-4D + 0.5 mg/L NAA and MS-16 containing 2.0 mg/L 2-4D + 2.0 mg/L NAA (Table 1). An excellent callus growth was observed after 28 days of explants inoculation with low precocious germination. Three treatments i.e. MS-8, MS-13 and MS-16 used with different ABA concentration combinations of 0.5 to 2.0 mg/l plant growth regulator as a result of which genotype HD-2967 observed the highest percentage of callus induction (76.6 percent) with no precocious germination (Table 2). The highest callus induction (70.0 percent) in genotype C-306 was observed, while lowest (23.8 percent) precocious germination was observed. A compact white green color callus was observed with an excellent growth in both genotypes on MS-4 (1.0 mg/L ABA + 1.0 mg/L 2-4D + 2.0 mg/L NAA) after 28 days of explants inoculation. Most calluses were good to excellent in their growth. It was observed here that by adding a small amount of ABA, no precocious germination was observed. Callus growth was either good or excellent in all the treatments. All the calluses were creamy green (Fig 1).

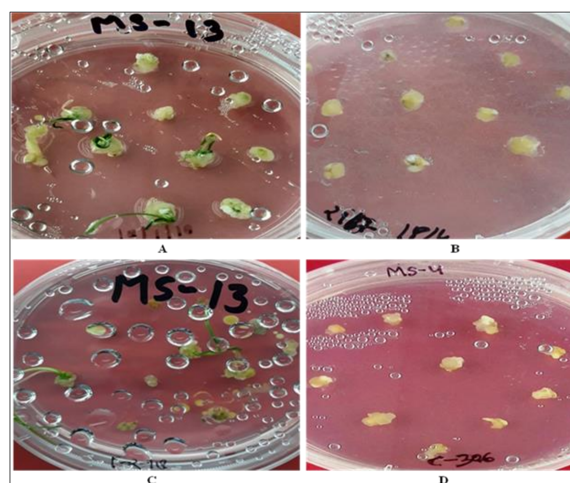

Fig 1: The effect of different concentration combinations of ABA in callus induction with NAA 2mg/l and 2,4-D 1mg/l (A- HD2967 without ABA, B- HD2967 with ABA, C- C306 without ABA, D- C306 with ABA)

Table 2: The callus induction and precocious germination of two Indian wheat genotypes in MS4 media containing 2,4-D (1.0 mg/l) + NAA (2.0 mg/l) + ABA (1.0 mg/l)

Genotype	Callus induction	Precocious germination	Color	Growth
C306	76.6c	0.0a	Creamy green	++++
HD2967	70.0c	23.3a	Creamy green	++++

Shoot regeneration

Twenty eight days old calluses were obtained from mature embryo cultures which subculture to eight different regeneration media on MS medium supplemented with different concentration combinations of plant growth regulators. Control treatment (RM-0, with no plant growth regulator) had 66.67 percent shoot regeneration in genotype C-306 while a 93.33 percent of shoot regeneration was observed in treatment RM-4 (containing 1.0 mg/L BAP + 2.0 mg/L TDZ + 0.5 mg/L NAA) with a shoot height 8.5 cm after 28 days. 3.3 shoots per callus were observed in treatment RM4, this is one of the best treatment observed for shoot regeneration using BAP, TDZ and NAA. In genotype HD-2967, 66.67 percent of shoot induction was observed in control treatment (RM-0) (with no plant growth regulator) and 93.33 percent of shoot induction observed in RM-4 treatment (1.0 mg/L BAP + 2.0 mg/L TDZ + 0.5 mg/L NAA) with 10.3 cm of shoot height and 4.2 shoots per callus (Table 3). In addition to regeneration we also observed the rooting in both treatments RM1 and RM4 in the genotype HD 2967, while no rooting was observed amongst rest of the treatments. Both the genotypes i.e. C306 and HD2967 showed a maximum amount of regeneration frequency using regeneration media RM-4 (1.0 mg/L BAP + 2.0 mg/L TDZ + 0.5 mg/L NAA) (Table 3). The growth regulator TDZ showed a higher concentration of shoot development in compare to BAP. We also observed that the growth was significantly higher in all the treatments with respect to control.

Table 3: The plant regeneration of two Indian wheat genotypes in MS4 media containing BAP (1.0 mg/l) + TDZ (2.0 mg/l) + NAA (0.5 mg/l)

Genotype	Regeneration	Plant height (in cm.)	Number of shoot/callus
C306	93.3c	8.5cd	3.3d
HD2967	93.3	10.3d	4.2c

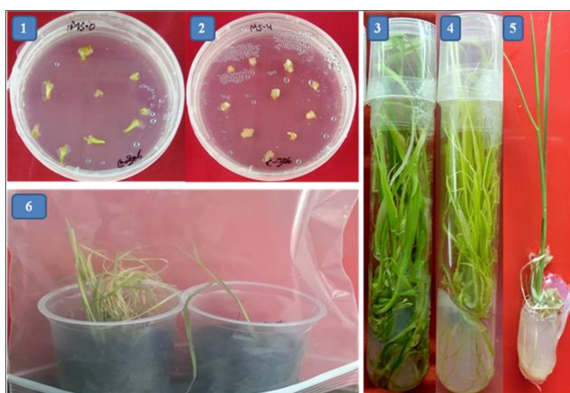

Fig 2: *In vitro* plant regeneration standardized protocol in mature embryos of wheat, 1. Precocious germination in MS-0 (without any plant growth regulator), 2. Callus induction in MS-4 media, 3. Shoot regeneration in RM-4 media, 4. root development in RTM-2 media, 5. Plantlets for acclimatization 6. Acclimatization of recovered plantlets in wheat.

Root initiation

All the regenerated plantlets transferred to four different concentrations of rooting medium designated as RTM. The genotype HD-2967 showed negligible amount of root emergence in control treatment RTM-0 (with no plant growth regulators added in the medium) while 4.0 roots per regenerated shoots observed in treatment RTM-3 (0.5 mg/L NAA + 0.1 mg/L IBA). In genotype C-306, control treatment RTM-0 (without any plant growth regulator) no rooting was observed while RTM-2 treatment (0.1 mg/L NAA + 0.5 mg/L IBA) showed 3.6 roots per regenerated shoots. The treatment RTM-3 (0.5 mg/L NAA + 0.1 mg/L IBA) and RTM-2 treatment (0.1 mg/L NAA + 0.5 mg/L IBA) showed the maximum number of roots (Table 4).

Table 4: The rooting of two Indian wheat genotypes in MS media containing different PGRs

Genotype	Medium	PGRs (mg/l)	Number of roots per plantlets
C306	RTM2	NAA (0.1) + IBA (0.5)	3.6c
HD2967	RTM3	NAA (0.5) + IBA (0.1)	4.0b

Acclimatization of rooted plantlets

The *in vitro* rooted plantlets (RTM-2 and RTM-3) were acclimatized in a growth chamber with different concentrations of soil-peat mixture and watered with 50 ml Hoagland solution on. Coco peat: Soil (1:1 v/v). The morphologically healthy plantlets were developed on concentration of Coco peat: Soil (1:1 v/v) (Fig2).

Discussion

Out of the two genotypes of wheat, Viz. C306 and HD 2967 genotype C306 were used as a positive control while HD 2967 is a high yielding variety and is being used by the farmers of western UP on a large scale in the present time. The MS media was used as a culture media fortified by different concentration combinations of three plant growth regulators 2,4-D, NAA and ABA for callus induction and development. For regeneration purpose BAP, TDZ and NAA were used. These PGRs were used because these are synthetic in nature, cheaper in cost and gave better results as per our past experiences. The sterilization of explants is the most important aspect of plant tissue culture. The mature embryo within the mature wheat seeds were difficult to surface sterilize as they are very much sensitive towards chemical treatment during sterilization process. Higher concentration of sterilizing agents such as sodium hypochlorite, mercuric chloride and ethanol causes damage to the explants which often lead to the death ultimately or reduced regeneration frequency in some cases. In the present investigation, explants sterilization procedure of Ethanol, Sodium hypochlorite leads to the hundred percent survival of explants followed by no contamination in cultures. The present study is similar as the result reported by Kumar *et al.* (2017) [10] where the mature embryos of wheat seeds were surface sterilized using HgCl2 and ethanol, surface sterilization with sodium hypochlorite (NaOCl) solution. Iqbal *et al.* (2016) and Hakam *et al.* (2015) [5] reported surface sterilization by washing in ethanol 70% (v/v) for 3 min, followed by using of 2.4% sodium hypochlorite plus a drop of Tween-20 for 15 minutes and was in good agreement with our study.

The plant growth regulators concentration in MS medium affects the callus induction by contributing as one of the most critical stage. In the present investigation, highest callus induction was observed among two wheat genotypes using

ABA and NAA with an creamy green colored callus growth. The high concentration combinations of NAA and low concentration in 2-4, D and ABA produced a good callus in wheat as similar as the results shown by Kumar *et al*. (2017) [10] and Malik *et al*. (2017) [12] with a maximum callus induction and low precocious germination using mature and immature embryos as explants. A good callus induction using wheat explants reported by Mahmood and Razzaq (2017) [11], they used higher concentration of 2-4 D**.** Nigar *et al.* (2017) [13] reported the high frequency of callus induction using 2, 4-D. Similarly, Ahmadpour *et al.* (2016) also reported a good callus induction using MS medium supplemented with a concentration of 2mg/L 2,4-D.

I*n vitro* culture involves a crucial step of shoot regeneration after callus induction. The twenty eight days old calluses were showed good regeneration in genotype C-306 with 3.33 shoots per callus and excellent plant heights while in the similar conditions genotype HD-2967 showed highest regeneration with 4.2 shoots per callus and 10.3 plant heights in compare to genotype C306. A high number of shoots observed among both the genotypes using the higher and lower concentration of TDZ and BAP, respectively, on MS media which are similar results reported by Kumar *et al*. (2017) [10] where healthy plantlets of wheat produced by using different concentration of shoot regeneration media i.e. BAP, kinetin and IAA. Likewise, Parmar *et al.* (2012) [14] reported the production of higher number of shoots using TDZ in combination with auxin 2-4,D. The combination of plant growth regulator BAP with NAA showed a higher shoot regeneration reported by Jasdeep *et al.* (2019) [7] and Baday (2018) [2] reported the highest number of shoots achieved with use of only BAP**.**

The developed shoots gave successful rooting on half MS medium supplemented with concentrations of NAA and IBA. Genotype HD- 2967 showed better rooting in compare to genotype C306. The similar result reported by Kumar *et al*. (2017) [10] which showed the maximum number of root development in wheat**.** Baday (2018) [2] also reported the highest development of roots using the higher concentrations of IBA**.** The healthy wheat plantlets were acclimatized well in Coco peat: Soil (1:1 v/v). The plant regeneration protocol was developed through mature embryos in wheat. The standardized callus induction and plant regeneration protocol involved the use of low concentration auxins and cytokinin showed good and fast results by producing healthy plantlets.

We developed a new protocol by choosing only two genotypes. The same protocol will be utilized to generate the changes using chemical and physical mutagens. Generated changes due to mutagen treatment may be used to generate beneficial agronomic traits.

Conclusions

In the present study, we tried to develop an efficient protocol for callusing and regeneration in wheat using mature embryo culture. Precocious germination is a major problem in wheat regeneration during *in vitro* culture; we also tried to minimize the precocious germination. The standardized medium and protocol may be effectively used in different wheat genotypes and may be utilized to development of new lines using different methods of gene engineering and mutagenesis for incorporation of different traits in future for speedy improvement in wheat.

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