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CONTENTS

In vitro plant regeneration from mature embryo using different plant growth regulators in wheat genotype HD 3059

SWATI SHARMA[1], ASHWANI KUMAR[1], ANIL SIROHI[2], R. S. SENGAR[1], KAMAL KHILARI[3], MUKESH KUMAR[4] and MANOJ K. YADAV[1]

[1]Department of Agricultural Biotechnology, [2]Department of Molecular Biology and Genetic Engineering,[3]Department of Plant Pathology, [4]Department of Genetics and Plant Breeding, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut (Uttar Pradesh)

ABSTRACT: Genetic transformation and many other genome engineering processes in wheat still lags behind due to non availability of a better callus induction and regeneration systems. In the present study, an efficient embryogenic based regeneration protocol is using different combinations of plant growth hormones. The combinations of commercial bleach along with mercuric chloride reported for effective sterilization of the explants (mature embryos) used for *in vitro* culture. The combinations of two auxin *viz*., 2, 4-D and NAA were taken for an efficient callus culture for callus induction, while cytokinin *viz*., kinetin and BAP were used to achieve the efficient regeneration capacity of the mature explants in wheat genotypes HD3059. A significant callus induction rate was reported among the callus cultured on MS media supplemented with concentration combinations of 4.0 mg L^{-1} 2, 4-D, 1.0 mg L^{-1} NAA and 1.0 mg L^{-1} ABA. Similarly, the concentration combination of 2.0 mg L^{-1} BAP; 1.0 mg L^{-1} NAA gave the maximum regeneration capacity along with maximum number of shoots. Further, MS media supplemented with 1.5 mg L^{-1} NAA in combination with 1.0 mg L^{-1} IAA produced better roots in regenerated shoots. The present study would be useful in ameliorating Indian wheat cultivars and would probably get over the problems regarding developing a new plant with a modification of a targeted gene using various recent technologies such CRISPR-Cas9 and gene engineering approaches. Therefore, efficient regeneration system has been achieved in the wheat genotype HD 3059.

Key words: CRISPR-Cas9, mature embryo, plant growth hormone, regeneration, transgenic plant, wheat

ABBREVIATIONS

2, 4-D (2, 4-Dichlorophenoxyacetic acid), NAA (Napthalene acetic acid), ABA (Abscisic acid), IAA (Indole-3-acetic acid), BAP (6-Benzylaminopurine).

India is the second largest wheat producing country after China (Wani *et al*., 2017). The production of wheat during 2019-20 is pegged at 106.21 million tonnes. Though it is higher by 2.61 million tonnes in compare to the production during 2018-19 and is higher by 11.60 million tonnes than the average wheat production of 94.61 million tonnes (Anonymous, 2020). It was observed that the decrease in production continuously during the last few years due to climate change intensified by various biotic as well as abiotic factors is alarming (Parmar *et al*., 2012; Krishnaveni *et al.,* 2019). Due to the low frequency of meiotic recombination in the crop leading to unwanted linkage drag, conventional breeding did not achieved much success, especially against different types of rust and drought conditions (Adero *et al*., 2019). Genetic transformation as a result of genetic engineering is a more precise and efficient method to improve the biotic and abiotic resistance among wheat genotypes. The method depends upon the callus induction and plant regeneration system influenced by genotype, explants source, geographical origin, culture medium and the interactions between them (Dargahlou *et al*., 2017). Various parts of the plant could be used as explants for tissue culture of wheat plant such as shoot tips, embryos, leaf basis, anthers, microspores and inflorescences. The use of mature embryos is recommended because of its low cost and round the year easy availability. It has been observed that the plant regeneration frequency using mature embryos is relatively lower than immature embryos. Therefore, some limiting factors interfere with the use of immature embryos such as the need to grow donor plants for round the year to supply the explants continuously, therefore, requiring greenhouse space as well as demanding extra labour works and cost. Further, finding the most suitable developmental stage of immature embryos for successful culture has been observed as another limiting factor (Murin *et al.,* 2012). In the present study, wheat genotype HD3059 is chosen in order to standardize the protocol for callus induction and *in vitro* regeneration that would be useful to study the developmental behaviour of the somatic embryos and its different developmental stages while somatic embryogenesis. In addition, the protocol would be useful in development of improved wheat genotypes in future.

MATERIALS AND METHODS

The seeds of high yielding wheat genotype HD3059 were used for the present study. Seeds were procured from the Krishi Vigyan Kendra, Nagina, Uttar Pradesh. The assessment of *in vitro* callus induction and plant regeneration capacity was done on mature embryos culture, using different plant growth regulators with different concentration combinations.

Explants sterilization and *in vitro* germination

Mature embryos of the seeds were used as an explants source. The seeds were washed in running tap water first and sterilized with Tween20 detergent followed by Bavistin solution for 5-8 minutes. The seeds were then soaked in 4% (v/v) sodium hypochlorite solution with continuous shaking for 20 minutes. Thereafter, disinfection of seeds was done using 0.1% solution of mercuric chloride for 30 seconds. The seeds were submerged in distilled water and allowed to incubate overnight in a 250 ml beaker. Mature embryos were excised from the imbibed seeds carefully with the help of blade and forceps. Mature embryos were wounded with the help of a sterile needle and put it on the MS culture medium (Murashige and Skoog, 1962) with scutellum in contact with the medium to start initiation of callus formation. All the operations were done strictly aseptically under horizontal laminar air flow hood.

Callus induction

Murashige and Skoog (1962) medium was supplemented with 33mg L^{-1} sucrose (Yadav, 2003) and used for different media preparations along with different concentration combinations of plant growth regulators and solidified by adding agar (HI-MEDIA^{R}) (8 mgL^{-1}). The pH of the media was set at 5.6±1. About 10 excised embryos were placed on each petri-dish for initiation of embryogenic callus induction using different concentration combinations of auxin (2, 4-D and NAA) with ABA (1.0 mgL^{-1} to 4.0 mgL^{-1}) for regulation of precocious germination among the explants (Table 1). The culture was incubated for 30 days at 24±2^{0}C followed by covering the parafilm to avoid any contamination. The experiment was repeated thrice and the callus induction was recorded on weekly basis.

In vitro regeneration and root initiation

The induced callus was sub-cultured on regeneration medium consisting of eight different concentration combinations of auxin (NAA) along with cytokinin (Kinetin and BAP) ranging from 1.0mgL^{-1} to 3.0 mg L^{-1} (Table 1) along with control were placed in the slanted test tubes. The experiment was repeated thrice and the shoot regeneration percentage and the number of shoots developed per plant were recorded. Similar pattern was applied to test the root initiation efficiency of the explants. The well-developed shoots were separated aseptically and cultured on separate MS media supplemented with six different concentration of auxin (NAA) and a control. The cultures were kept at 24±2 °C and relative humidity of 60–70% under fluorescent light intensity of 24µmol $m^{-2}s^{-1}$ with 16-h photoperiod for 30 days.

Hardening of regenerated plantlets

The *in vitro* generated plantlets are generally difficult to acclimatize as their stems and leaves got succulent due to the high humidity in the culture vessel (Grout and Aston, 1978). The reason of concern is the difference of nutrition in the culture medium and the soil environment. The *in vitro* rooted shoot plants are removed from the culture medium and washed with double distilled water to separate the traces of agar from it. Plants are transferred to the perforated plastic containers containing 1:1 (v/v) of coco-peat: vermi-compost at 25^{0}C under a 16/8 h. Then plantlets were given 50 ml Hoagland solution and covered with the polythene bag with three or four holes in it in order to maintain the humidity. After two weeks of continuous observation, the covers were removed and plants transferred to the greenhouse and were allowed to grow for few more weeks.

Data analysis

A completely randomized design (CRD) with three replications per treatment was used to determine the actual efficiency of callus induction as well as *in vitro* regeneration. The efficiency of callus induction is calculated as number of explants generated callus divided by total number of explants cultured multiplied by hundred. The statistical analysis was done using one way analysis of variance (ANOVA) using SPSS software version 19.

RESULTS AND DISCUSSION

Explants sterilization

A reproducible and independent regeneration system is pre-requisite for wheat improvement for development of successful transgenic crops. The present investigation was designed in order to overcome the problem by suitably generating an *in vitro* plant regeneration system in wheat genotype HD 3059, which is also known as *Pusa Pachheti* . This genotype was released by Indian Agricultural Research Institute; New Delhi is being used widely by

farmers of Western Uttar Pradesh and very much popular among the farmers. It was chosen due to its higher achieved production, resistance to yellow and brown rust and its superior quality parameters like protein content (13.6%), high sedimentation value (52 ml), best Glu-1 Score (10/10) and good extraction rate (70.1%) (Singh *et al*., 2014). The present study emphasizes the effect of plant growth regulators on callus induction and *in vitro* plant regeneration to determine the optimum concentration of efficient plant growth regulators. The dose and different duration of sterilizing agents has a direct influence on the viability and regeneration efficiency of explants. The standardization of seed sterilization and disinfection method was done at first in the present investigation by using 0.1% of mercuric chloride for 30 s and 4% v/v sodium hypochlorite for 20 minutes as the optimum concentration. The blackening of the seed coat cover occurred on exceeding the dose of mercuric chloride for longer durations such as for 60 seconds, 120 seconds and 180 seconds and the explants when cultured was observed to lose their viability resulting into no growth and division (Data not shown) However, seed sterilization is the common process to sterilize the explants but only embryo sterilization is also possible, in case where explants source is embryo, as reported by Parmar *et al*., 2012 by using 0.1% $HgCl_2$ and 70% ethanol for 20 seconds followed by washing with sterile distilled water. Our study also indicate that the mature embryos obtained from the imbibed seeds

Table 1: Composition of different MS medium used for callus induction, regeneration and root induction in wheat genotype HD 3059

Callus Induction medium	**PGRs (**mg L^{-1} **)**
(33mgL^{-1} sucrose+8mgL^{-1} Agar+pH 5.6±1)	
CI0	(control)CI medium (without PGRs)
CI1	2.5 mg L^{-1} 2, 4-D+1.0 mg L^{-1} NAA+0.5 mg L^{-1} ABA
CI2	3.0 mg L^{-1} 2, 4-D+0.5 mg L^{-1} NAA+0.5 mg L^{-1} ABA
CI3	4.0 mg L^{-1} 2, 4-D+0.5 mg L^{-1} NAA+0.5 mg L^{-1} ABA
CI4	4.0 mg L^{-1} 2, 4-D+1.0 mg L^{-1} NAA+1.0 mg L^{-1} ABA
CI5	4.0 mg L^{-1} 2, 4-D+1.5 mg L^{-1} NAA+1.5 mg L^{-1} ABA
CI6	2.0 mg L^{-1} 2, 4-D+1.0 mg L^{-1} NAA
CI7	3.0 mg L^{-1} 2, 4-D+1.0 mg L^{-1} NAA
CI8	4.0 mg L^{-1} 2, 4-D+1.0 mg L^{-1} NAA
CI9	4.0 mg L^{-1} 2, 4-D+1.0 mg L^{-1} NAA
Regeneration Medium	**PGRs (**mg L^{-1} **)**
(30 mgL^{-1} sucrose+8 mgL^{-1} Agar+pH 5.6±1)	
RM0	RM (without any PGRs)
RM1	1.0 mg L^{-1} Kin+0.5 mg L^{-1} NAA
RM2	1.5 mg L^{-1} Kin+0.5 mg L^{-1} NAA
RM3	2.0 mg L^{-1} Kin+1.0 mg L^{-1} NAA
RM4	2.5 mg L^{-1} Kin+1.0 mg L^{-1} NAA
RM7	2.0 mg L^{-1} BAP+1.0 mg L^{-1} NAA
RM9	3.0 mg L^{-1} BAP+ 1.5 mg L^{-1} NAA
Rooting Medium (30 mg L^{-1} sucrose+8 mg L^{-1}Agar+pH 5.6±1)	
RTM0	RTM 0 (without any PGRs)
RTM1	0.5 mg L^{-1} 2,4-D+1.0 mg L^{-1} NAA
RTM2	1.0 mg L^{-1} 2,4-D+2.0 mg L^{-1} NAA
RTM3	1.0 mg L^{-1} 2,4-D+2.5 mg L^{-1} NAA
RTM4	1.0 mg L^{-1} 2,4-D+3.0 mg L^{-1} NAA
RTM-7	1.5 mg L^{-1} IAA+1.5 mg L^{-1} NAA
RTM-8	1.0 mg L^{-1} IAA+2.0 mg L^{-1} NAA
RTM-9	1.5 mg L^{-1} IAA+2.0 mg L^{-1} NAA

Table 2: Callus induction response in genotype HD 3059 using different plant growth regulators at second, third and fourth week.

Treatments (Callus Induction)	Callus Induction (per cent)		
	After 14 days	After 21 days	After 28 days
0	0.00^{a}	0.00^{a}	0.00^{a}
1	90.00^{ef}	90.00^{c}	93.33^{c}
2	80.00^{def}	90.00^{c}	93.33^{c}
3	83.33^{ef}	83.33^{c}	83.33^{c}
4	96.67^{f}	96.67^{c}	96.67^{c}
5	73.33^{cde}	80.00^{c}	83.33^{c}
6	80.00^{def}	83.33^{c}	83.33^{c}
7	60.00^{bc}	63.33^{b}	63.33^{b}
8	63.33^{bcd}	63.33^{b}	63.33^{b}
9	53.33^{b}	60.00^{b}	60.00^{b}

Percentage callus induction = Calli with visible mass of tissue/ total embryos cultured x 100

Table 3: Precocious germination response by using plant growth regulators with or without ABA

Treatments	Precocious germination percentage		
CI	After 14 days	After 21 days	After 28 days
0	100.00^{f}	100.00^{d}	100.00^{d}
1	13.33^{cd}	20.00^{b}	20.00^{a}
2	3.33^{ab}	3.33^{a}	16.67^{a}
3	0.00^{a}	10.00^{ab}	13.33^{a}
4	0.00^{a}	00.00^{ab}	10.00^{a}
5	10.00^{bc}	10.00^{ab}	6.67^{a}
6	16.67^{cd}	10.00^{ab}	10.00^{a}
7	33.33^{e}	43.33^{e}	43.33^{bc}
8	46.67^{f}	50.00^{e}	50.00^{c}
9	20.00^{d}	20.00^{b}	23.33^{ab}

Table 4: Effect of different PGRs (auxin and cytokinin) on regeneration percentage in 30 days old culture

Treatment (RM)	Regeneration percentage	Plant height (cm)	No. of shoots
0	30.55^{a}	1.00^{a}	1.33^{a}
1	41.66^{a}	5.50^{ab}	1.00^{a}
2	38.88^{a}	3.16^{ab}	1.33^{a}
3	41.66^{a}	5.40^{ab}	1.67^{a}
4	66.66^{ab}	8.50^{b}	2.00^{a}
7	88.88^{b}	10.33^{b}	2.67^{a}
9	38.88^{a}	4.96^{ab}	3.00^{a}

Percentage regeneration = Numbers of compact calli with green spots /total number of calli×100.

Table 5: Effect of different PGRs (auxin) on root initiation in two weeks old culture

Treatment(RTM)	No. of roots developed per plant
0	1.33[a]
1	2.00[a]
2	2.00[a]
3	1.33[a]
4	3.33[a]
7	3.67[a]
9	1.00[a]

Data of each trait represents the average values of three replications of each respective treatment.

in a petri-plate for 16 hours were easy excised from the seeds in compare to seeds that seeds were submerged completely overnight. The sterilized mature seeds were wounded and excised by using a sterilized needle blade necessary for plant regeneration. The effect of different sterilizing agents like calcium hypochlorite, sodium hypochlorite, mercuric chloride, ethanol has been studied to determine the regeneration efficiency of the explants by Filippov *et al.* (2006), Ahmadpour *et al*., (2016). Tissue damage for plant regeneration is called *de novo* organogenesis where shoots and roots are regenerated from the wound locations and detached organs (Bidabadi and Jain, 2020).

Callus induction

In the present study, MS medium (Murashige and Skoog, 1962) enriched with sucrose 33gL^{-1} (Yadav, 2003) as a carbon source and 8gL^{-1} agar used as a gelling agent with pH 5.6±1 supplemented with different concentrations of auxin, cytokinin and ABA has been used to culture the explants for initiating formation of callus induction (Bhushan *et al*., 2017). In the present investigation, the maximum callus induction response was achieved (96.67[f]) with callus induction medium 4 (CI4) after 28 days when supplemented with 4.0 mg L^{-1} 2,4-D; 1.0 mg L^{-1} NAA and 1.0 mg L^{-1}ABA in the medium with almost no precocious germination, while callus induction medium, CI1 and CI2 gave the second best response for callus induction when compared to control at different time intervals (Table 2, Fig. 1a-c), Many other researchers (Xhulaj, 2019), reported that the combination of BAP (2.0 mg L^{-1}) and 2, 4-D (0.6 mg L^{-1}) was the best auxin/cytokinin ratio for callus induction and development. Dicamba is also an auxin that has been reported in several studies known to provide better callus induction results but not used in the present study. Therefore, it is clear that for callus induction response in wheat genotype HD 3059 required high concentration of 2,4-D (4 mgL$^{-1)}$ and optimum amount of NAA and ABA (1 mgL^{-1}). Although, sugar (33 gL^{-1}) (Table 1) also played a key role as a carbon sources used mostly in wheat tissue culture and acts as an important factor in callus induction and development (Jabeen *et al*., 2016). It was reported by Kowalska and Arsenuik, 2016 that maltose as compared to sucrose gave better results in embryonic callogenesis. Sucrose when used as a carbon source in MS media is correlated with the rooting percentage and number of roots per plant (Khan *et al*., 2015). ABA concentration (1.0 mgL^{-1}) when supplemented with 2, 4-D and NAA in CI4 reduced the precocious germination up to 90 percent (Only 10 percent precocious germination was reported with this concentration combination) (Table 3). In the present study, we also observed that ABA which is a stress hormone played an important role to check the precocious germination. Precocious germination is a major hurdle in the wheat tissue culture and it affects the regeneration process greatly during *in vitro* culture of wheat.

In vitro regeneration and root initiation

A total number of six growth hormone concentration combinations were assessed for regeneration of explants including NAA, kinetin and BAP. In fact, the ratio between auxin and cytokinins plays an important role regeneration of shoots during *in vitro* culture. By the previous studies 2,4-D and NAA used for callus induction, are pervasive in nature and ABA is used to regulate precocious germination, as discussed in the previous section, while cytokinin *viz.*, BAP and kinetin with auxin is generally used for regeneration of shoots (Caraballo *et al*., 2010) . In the present study, we used kinetin and NAA in different concentration combinations for regeneration purpose. We

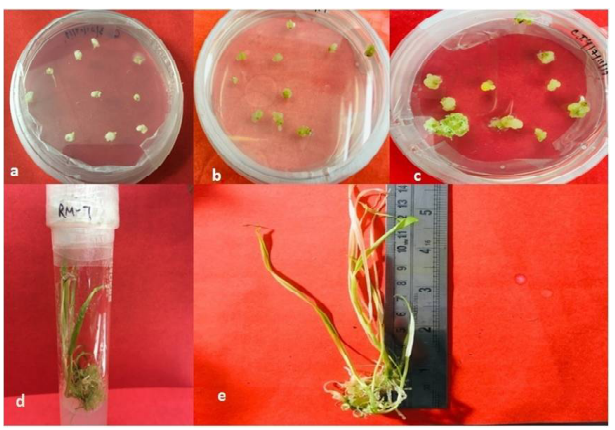

Figure 1: Schematic representation of the standardized protocol for, (a) callus induction in treatment CI4 with no precocious germination after (a) second, (b) third and (c) fourth week, (d) regeneration shown by RM 7 with maximum plant height (in cm) and number of shoots after 30 days and (e) rooting after two weeks of sub-culturing

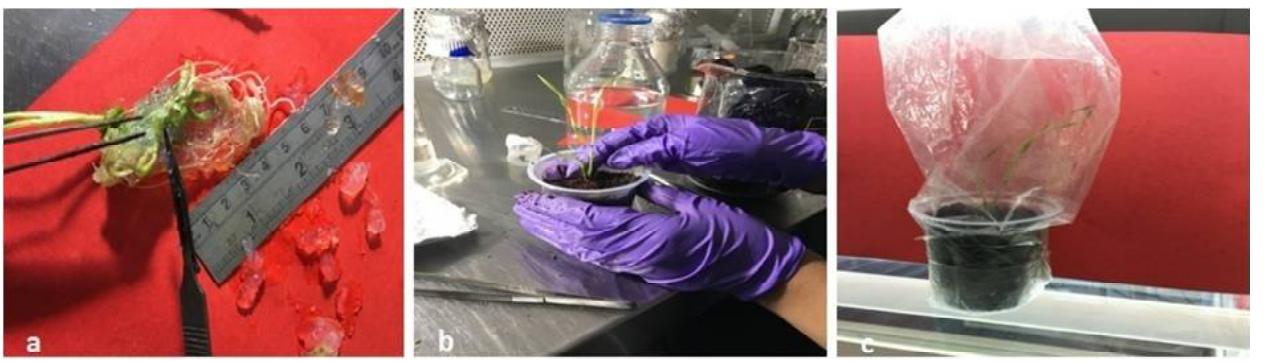

Figure 2: Removal of *in vitro* grown wheat genotype HD 3059 plantlets with (a) well developed rooting system for acclimatization purpose, (b) transferred in a small plastic cup containing cocopeat and soil mixture and (c) maintaining the humidity for hardening process

observed that out of all treatments used in the present study, RM7 (2.0 mg L^{-1} BAP with 1.0 mg L^{-1} NAA) showed the highest regeneration percentage 88.88^{b} and produced the highest average number of shoots per plant viz., 3.00^{a} (Table 4; Fig 1d). It was also reported from the present study that more plantlets were regenerated with this concentration combination and all the plantlets were healthy when compared to the other ones. Moreover, the plantlets recovered from the RM1 medium (1.0 mgL^{-1} kin+0.5 mg L^{-1} NAA) showed the least number of shoots per plant (Table 4). Hence, it is assumed that kinetin and NAA was the optimum combination to obtain the best regeneration capacity in the selected genotype during *in vitro* culture. Dargahlou *et al*., 2017 also did the similar study in wheat where they observed that 2,4-D is not desirable for regeneration, while it is good to generate somaclonal variations. The developed shoots gave successful rooting on half MS medium supplemented with concentrations of NAA and IBA (Kumar *et al*., 2019). The higher auxin to cytokinin ratio promotes rooting and higher cytokinin to auxin ratio promotes shooting in the plant (Kurepa *et al*., 2019). The similar study was proposed by Jasdeep *et al.,* 2019 and Kumar *et al*., 2017. Further, Hesami *et al*., 2018 reported that the MS medium comprising of 0.5 mg L^{-1} 2,4-D and 0.05 mg L^{-1} BAP (10:1) was the best treatment for callus induction. Iqbal *et al*., 2016 reported an excellent (90 %) quality of callus induction using 4.0 mg L^{-1} and 6.0 mg L^{-1} of 2,4-D and higher regeneration among genotypes by using 1.5mg L^{-1} BAP. Saha *et al*., 2017 found that MS media supplemented with 2.0 mg L^{-1} BAP + 1.0 mg L^{-1} IAA considered the best concentration for plant regeneration by the wheat genotype Pavon 76.

The optimized concentration of sterilization, callus induction and regeneration observed in the present study is a suitable observation toward development of a reproducible protocol for callus induction and better regeneration capacity with less precocious germination. The regenerated shoots were sub-cultured on rooting MS media comprising of different auxin concentration combinations. Good rooting was achieved in the rooting media RTM7 which was supplemented with IAA (0.5 mg L^{-1}) and NAA (1.5mg L^{-1}) i.e. 3.67^{a} (average number of roots per plants (Table 5; Fig. 1e). The treatment RTM 5, RTM6 and RTM 8 got contamination during optimum laboratory conditions (Data not shown).

Hardening and acclimatization

The rooted plantlets were hardened using 50 ml of Hoagland solution. The plants recovered from the culture media were grown in controlled conditions. The morphologically healthy plantlets with well developed roots were transferred into sterilized coco-peat: soil (1:1) mixture (Fig 2 a-c). About 70 per cent of plants survived well after acclimatization.

CONCLUSION

In the present study, it is clear that the interaction between the genotype source, concentration of carbon source and gelling agent, different growth hormones are crucial for a callus induction and regeneration in plants. An appropriate explants sterilization protocol is standardized on priority to avoid any type of contamination in further experiments. The concentration of carbon source also affects the rate of callus induction. It was observed that 30 mg L^{-1} of sucrose was found effective in embryogenic callus induction. Among all the tested concentrations of auxin and cytokinin used in the present study, we optimised the 2, 4-D; NAA and ABA concentrations for callus induction when MS media supplemented with 2.0 mgL^{-1} BAP + 1.0 mgL^{-1} NAA and maximum plant height along with maximum number of shoots per plant while, 1.5 mgL^{-1} IAA + 1.5 mgL^{-1} proved good for regeneration capacity

(88%). NAA has been found appropriate concentration to generate good rooting in *in vitro* regenerated plantlets in the present study. The standardized concentration may help to decide the further concentrations to study the somatic embryogenesis using mature embryos of wheat plant as an explants source. Further, the present protocol would help in developing transgenic and CRISPR-Cas9 based approach to develop improved genotypes with desired traits.

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