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RESEARCH ARTICLE

An improved *in-vitro* regeneration protocol using scutellum of mature and immature embryos of wheat (*Triticum aestivum* L.)

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Abstract

Generally, conventional practices take a long time to improve agronomic traits in common wheat (*Triticum aestivum* L.). However, biotechnological tools provide the best opportunity to enhance crop potential within a limited time. A simple and repeatable protocol for wheat regeneration is the priority for transforming the genetic makeup of elite wheat cultivars. Among 10 types of callus induction media (CIM), CIM4 with supplemented Murashige and Skoog (MS) media+ dicamba (2 mg/l) has 98.22 and 97.33% callus induction efficiency (CIE) in immature and mature scutellum, respectively. Precocious germination, considered a key barrier during callus induction, was fully controlled by excising the embryonic axis. Among 29 different shoot induction media (SIM) assessed in the study, SIM 29 containing zeatin 5 mg/l, 6-benzylaminopurine (BAP) 5 mg/l, 2,4-dichlorophenoxyacetic acid (2,4-D) 0.25 mg/l,1-naphthaleneacetic acid (NAA) 0.25 mg/l, and copper sulfate ($CuSO_4$) 20 mg/l was found most responsive for shoot induction. The combined effect of cytokinin and $CuSO_4$ resulted in an improved wheat regeneration response in two popular Indian wheat varieties, namely HD2967 and HD3171. Rooting was observed at 100% using IAA and IBA @ 0.2 mg/l concentration in root induction media (RIM). The whole in-vitro regeneration protocol from embryo to transfer in the greenhouse took 8 to 9 weeks. The *in-vitro* regeneration protocol developed in the present study could further be utilized to improve the genetic makeup of elite wheat cultivars utilizing genetic engineering tools.

Keywords: Common wheat, mature and immature embryos, scutellum, callus, regeneration,

Introduction

Wheat is one of the most important cereal crops, utilized for a variety of applications, including food and feed, as well as processing sectors. It constitutes about one-fourth of the total cereal production. The bulk of the world's population relies on wheat to meet their calorie needs (FAO 2022). Moreover, due to the ever-increasing human population, demand for wheat is going to increase in the future. Traditional crop improvement and marker-assisted breeding techniques have been utilized to develop crop plants with useful agronomic traits. However, these techniques take longer and rely on the already available limited gene pool among related species to develop new varieties (Shivakumar et al. 2018; Ahmar et al. 2020). The combination of genetic engineering with plant tissue culture (PTC) techniques has emerged as an alternative to traditional crop improvement methods that are more precise and less time-consuming (Giri et al. 2004).These new techniques require subject specialization and proper handling during PTC operations. Expensive growth regulators and culture media further enhance the cost of operation. Nonetheless, combining these new-generation techniques with traditional and marker-assisted breeding could aid in the development of superior genotypes in a shorter period. These techniques have been used in the identification of new genes along with their function. Useful transgenes have been modified and introduced using genetic engineering and plant

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transformation tools (Gasser and Fraley 1989; Schaart et al. 2016). However, efficient callus induction and regeneration protocols are essential for successful gene transformation. The choice of explant decides the fate of callus induction and regeneration (Patel et al. 2022). Tissue culture has been exploited to create genetic variability to increase the number of desirable germplasms available to the plant breeder. These methods have enabled the production of completely homozygous breeding lines from gamete cells. Various genes were found to be associated with callus induction and regeneration responses. In several crops, quantitative trait loci associated with regeneration-related genes have been identified and mapped. Several regeneration-related genes were found to be differentially expressed during somatic embryogenesis. Some genes like *Os22A* and *OsNiR* in *Oryza sativa* L. were found to be associated with high regeneration ability (Nishimura et al. 2005). In wheat, a regeneration-related gene tailless complex polypeptide 1 (TaTCP-1) was found highly upregulated in embryogenic calli during callus induction. *TaTCP-1* was reported to promote wheat regeneration and regulate the somatic embryogenesis of wheat (Li et al. 2020). Expression of another GRF4–GIF1 fusion protein in wheat, rice and triticale significantly increased the speed and efficiency of regeneration in the absence of exogenous cytokinins (Debernardi et al. 2020). Plant tissue culture is difficult in wheat due to the lack of a robust protocol. However, *in-vitro* plant regeneration is extremely important for different purposes, of which production of double haploid through androgenesis (microspore culture/anther culture) and embryo culture are relevant for the wheat breeding program (Santra et al. 2017; Stewart 1981). To date, a few wheat regeneration protocols have been standardized (Patnaik and Khurana 2003; Pérez-Piñeiro et al. 2012). Most of these reports were on two cultivars, namely Fielder and Bobwhite because of their better response to regeneration and genetic transformation. However, these cultivars lack superior qualities and yield compared to the elite (Jones et al. 2005). The available literature points towards the fact that *in-vitro* wheat regeneration protocols differ with wheat genotypes, media composition and explant used for callus induction and regeneration (Parmar et al. 2012). Many available wheat regeneration protocols utilized apical meristems (Ahmad et al. 2002), anthers (Machii et al. 1998), mature embryos (Medvecká and Harwood 2015), immature embryos (Nizamani et al. 2016), immature inflorescence (Amoah et al. 2001; Yadava and Chawla 2002), and leaf tissues (Haliloglu 2006; Yu et al. 2012) as explants, and different type of explants show variable responses in callus induction and regeneration. Immature and mature embryos are regarded as better explants for *in-vitro* regeneration as their results are convincing (Murín et al. 2012). The appropriate time for using immature embryos is 12 to 20 days after anthesis (Howells et al. 2018). But, in the natural environment, the availability of immature embryos is restricted to a limited window of about 7 to 10 days. Alternatively, growth chambers and greenhouses with controlled temperature and light could be used to produce immature embryos year-round in tropical conditions, but the requirement of infrastructure and cost of producing immature embryos becomes very high. Mature embryos are good substitutes for immature embryos, but their regeneration efficiency was reported lower (Ahmadpour et al. 2018; Sarker and Biswas 2002). In the present study, an experiment was designed to optimize major steps of regeneration of wheat explants, including explant sterilization, media composition and growth hormone concentration (conc.) in callus induction media (CIM), shoot induction media (SIM) and root induction media (RIM) considering bread wheat (*Triticum aestvum* L.) cultivars, HD2967 and HD3171 along with Bobwhite for determining their callus induction and regeneration response in different combinations of media. An attempt has been made to develop a simplified and improved bread wheat *in-vitro* regeneration protocol which may be of great help for wheat transformation as well as embryo rescue.

Materials and methods

Plant material

Three hexaploid wheat (*Triticum aestivum* L.) cultivars Bobwhite, HD2967 and HD3171 were used to determine CIM, SIM and RIM components. Seeds of Bobwhite were obtained from Punjab Agricultural University, Ludhiana and seeds of HD2967 and HD3171 elite wheat cultivars were taken from the Division of Genetics, Indian Agricultural Research Institute, New Delhi. Spikes with immature seeds (12-20 days after anthesis) were harvested from the experimental fields of IARI, New Delhi, India.

Sterilization and excision of mature/immature seeds

Matured seeds were used to obtain mature embryos. Seeds were washed twice with single distilled water (SDW) and treated with tween 20 surfactant/detergent for 30 minutes with continuous shaking at 200 rpm followed by 4-6 washings using SDW. Autoclaved SDW (ASDW) was added to the seeds which were incubated overnight at room temperature (RT) (28°C). Further steps were carried out in a laminar air flow (LAF) hood, where 70% ethanol was added to the incubated seeds and left at RT for 90 seconds with intermittent shaking. The seeds were washed again with ASDW 3-4 times to remove the ethanol completely. The treatment of sodium hypochlorite (2% w/v) was given to seeds 10 minutes at RT with continuous shaking. The seeds were washed with ASDW 4-5 times to completely remove sodium hypochlorite. Now completely sterilized seeds were used directly for embryo excision under the stereo zoom microscope in LAF.

Around 10 spikes at milk ripe to the initial soft dough stage were selected. to obtain immature embryos. Grains

were taken from the middle of the spike to obtain uniform-sized embryos. The overnight incubation step as discussed above was skipped. The rest of the procedure of seed sterilization was the same as for mature seeds. Mature/immature embryos were excised under a stereo zoom microscope working under LAF as shown in Fig. 1. Rasco-Gaunt et al. (2001) reported the most appropriate size of an immature embryo was approximately 0.5-1.5 mm (Rasco-Gaunt et al. 2001).

As described by Sparks et al. (2014) the embryonic axis was gently excised (plumule and radicle) from the embryo using a sterile scalpel blade (LA770 HiMedia) under a stereo zoom microscope (Sparks et al. 2014). The excised scutellum was put on CIM, keeping the cut side in contact with the media. The only left scutellum was used as starting material.

Callus induction

Murashige and Skoog's (MS) media was used in all types of tissue culture media assessed in the experiment. Supplemented MS media for callus induction contains MS media (Himedia (PT021-1L) 4.4 g/litre supplemented with 700 mg/l glutamine, 150 mg/l proline, 100 mg/l asparagine and 3% sucrose as a carbon source. All the components were mixed well and the pH 5.7–5.8 was set with 0.5 N NaOH. Phytagel (P8169 sigma) 0.4% was added to the media as a solidifying agent. The media was autoclaved and cooled to 50°C before adding growth hormones. Ten different callus induction media CIM1-CIM10 (Table 1) were prepared to determine their callus induction efficiency (CIE) by adding different concentrations of auxins, dicamba (1–2 mg/l), 2,4-D (1–2 mg/l), and picloram (1–2 mg/l). The media was poured into sterile disposable petri plates (Himedia PW001) of dimensions 90–15 mm. The media was allowed to incubate at RT for two days to exclude contamination due to handling during media preparation. The excised mature/immature scutella were placed onto CIM and kept in the dark for 21 days at 22 ± 2°C. Around 25 scutella were placed on each plate and examined on a routine basis for further precocious germination and any fungal/bacterial contamination. With in 4 days the scutella started bulging and growing to calli of different sizes depending upon the

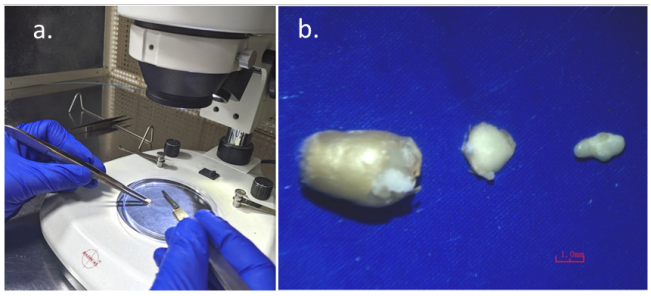

Fig. 1. Showing endosperm, scutellum and embryonic axis excised with scalpel blade under stereo zoom microscope. (a) Embryonic axis removal under stereo zoom microscope, (b) Endosperm, scutellum, embryonic axis

Table 1. Various types of CIM with supplemented MS and different concentrations of exogenous plant growth regulators (PGRs)

Media name	Name and conc. of PGRs	Media name	Name and conc. of PGRs
CIM 1	None	CIM 6	Picloram (1.5 mg/l)
CIM 2	Dicamba (1 mg/l)	CIM 7	Picloram (2 mg/l)
CIM 3	Dicamba (1.5 mg/l)	CIM 8	2,4-D (1 mg/l)
CIM 4	Dicamba (2 mg/l)	CIM 9	2,4-D (1.5 mg/l)
CIM 5	Picloram (1 mg/l)	CIM 10	2,4-D (2 mg/l)

wheat genotype and type of CIM used. After 21 days of incubation in CIM, the calli were ready to transfer on SIM media. Callus size was measured using a radical stereo zoom microscope (Model no. RSM-9), India with Radical procam software. Callus weight was recorded using an electronic microbalance Mettler Toledo (model no. MS 105DU), USA. The CIE is calculated by using the following formula.

$$CIE = \frac{\text{Number of calli}}{\text{Total number of explant}} \times 100$$

Shoot Induction

Three week old calli from CIM 4 were sub-cultured to the different combinations of SIM. Supplemented MS media for SIM contains MS media (Himedia (PT021-1L)) 4.4g/litre with 1 g/l casein hydrolysate, 750 mg/l glutamine and 3% maltose as a carbon source. pH is set to 5.7–5.8 with 0.5N NaOH. To provide support to the calli, 0.4% phytagel is used as a solidifying agent and the media was autoclaved. Various combinations of different growth regulators and inducers were added to autoclaved media after cooling to around 50°C. A total of 29 types of shoot induction media SIM 1–SIM 29 indicated in Table 2 were prepared by using various combinations of cytokinin (BAP, Zeatin), auxins (NAA,2,4-D), and inducers $CuSO_4$. $CuSO_4$ (20 mg/l) was used as an inducer of multiple shoots of SIM in SIM24–SIM2. Eighteen calli were placed in each plate with SIM. The Petri plates were placed in 16/8 hours of photoperiod at 22 ± 2°C for 21 days (Zale et al. 2004). SIE was calculated using the following formula.

$$CIE = \frac{\text{Number of calli with shoots}}{\text{Total number of calli placed}} \times 100$$

Root induction media

MS media accompanied with 3% sucrose was dissolved in distilled water and pH was set to 5.7 to 5.8 using 0.5 N NaOH. Phytagel (0.4%) is used as a solidifying agent to provide support to regenerating calli. Different concentrations of auxins Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA) and their combination were used to determine root induction response in regenerated shoots. Table 3 shows three types of root induction media (RIM) with different concentrations of auxins in MS media. RIM is poured into

Table 2. Different types of SIM with supplemented MS and different amounts of PGRs

Media name	Name and conc. of PGRs	Media name	Name and conc. of PGRs
SIM 1	Without any PGRs	SIM 16	Zeatin (5 mg/l), 2,4-D (0.25 mg/l)
SIM 2	BAP (2.5 mg/l), NAA (0.25 mg/l)	SIM 17	Zeatin (5 mg/l), 2,4-D (0.5 mg/l)
SIM 3	BAP (2.5 mg/l), NAA 0.5 mg/l)	SIM 18	BAP (2.5 mg/l), Zeatin (2.5 mg/l), NAA (0.25 mg/l)
SIM 4	BAP (2.5 mg/l), 2,4-D (0.25 mg/l)	SIM 19	BAP (2.5 mg/l), Zeatin (2.5 mg/l), 2,4-D (0.25 mg/l)
SIM 5	BAP (2.5 mg/l), 2,4-D (0.5 mg/l)	SIM 20	BAP (2.5 mg/l), Zeatin (2.5 mg/l), NAA (0.25 mg/l), 2,4-D (0.25 mg/l)
SIM 6	BAP (5 mg/l), NAA (0.25 mg/l)	SIM 21	BAP (5 mg/l), Zeatin (5 mg/l), NAA (0.25 mg/l)
SIM 7	BAP (5 mg/l), NAA (0.5 mg/l)	SIM 22	BAP (5 mg/l), Zeatin (5 mg/l), 2,4-D (0.25 mg/l)
SIM 8	BAP (5 mg/l), 2,4-D (0.25 mg/l)	SIM 23	BAP (5 mg/l), Zeatin (5 mg/l), NAA (0.25 mg/l), 2,4-D (0.25 mg/l)
SIM 9	BAP (5 mg/l), 2,4-D (0.5 mg/l)	SIM 24	BAP (2.5 mg/l), Zeatin (2.5 mg/l), NAA (0.25 mg/l), $CuSO_4$ (20 mg/l)
SIM 10	Zeatin (2.5 mg/l), NAA (0.25 mg/l)	SIM 25	BAP (2.5 mg/l), Zeatin (2.5 mg/l), 2,4-D (0.25 mg/l), $CuSO_4$ (20 mg/l)
SIM 11	Zeatin (2.5 mg/l), NAA (0.5 mg/l)	SIM26	BAP (2.5 mg/l), Zeatin (2.5 mg/l), NAA (0.25 mg/l), 2,4-D (0.25 mg/l), $CuSO_4$ (20 mg/l)
SIM 12	Zeatin (2.5 mg/l), 2,4-D (0.25 mg/l)	SIM 27	BAP (5 mg/l), Zeatin (5 mg/l), NAA (0.25 mg/l), $CuSO_4$ (20 mg/l)
SIM 13	Zeatin (2.5 mg/l), 2,4-D (0.5 mg/l)	SIM 28	BAP (5 mg/l), Zeatin (5 mg/l), 2,4-D (0.25 mg/l), $CuSO_4$ (20 mg/l)
SIM 14	Zeatin (5 mg/l), NAA (0.25 mg/l)	SIM 29	BAP (5 mg/l), Zeatin (5 mg/l), NAA (0.25 mg/l), 2,4-D (0.25 mg/l), $CuSO_4$ (20 mg/l)
SIM 15	Zeatin (5 mg/l), NAA (0.5 mg/l)		

'Jam' bottles to provide more space for rooting as well as shooting. Multiple shoots were separated gently and subcultured on RIM. The jam bottles with shoots were kept at 22 ± 2 °C for 16/8 light/dark hours. Within 5–8 days rooting was started and after 1 more week, regenerated plantlets were ready for hardening.

Hardening of plantlets

The plantlets with fully formed roots and shoots were removed gently from jam bottles to avoid injury to the roots and root caps. Plantlets were washed with distilled water to eliminate the remaining semi-solid media from roots and to avoid further bacterial and fungal contamination due to media. Further, these plantlets were transferred to 6×6 inches pots with autoclaved soil and soil rite in 1:3 proportions. Liquid basal Hoagland media (TS 1094) was used to provide water and nutrients to establishing plantlets. Immediately, transparent polybags were used to cover the pots to maintain light and humidity. The pots were placed at 22 ± 2°C for a 16/8 light/dark. During hardening, after 3 days of transfer to the pots, small pores were created into polybags to fasten acclimatization. More pores in polybags were created on alternate days till the 10th day. The polybags were removed completely on the 10th day. Now the hardened and acclimatized plants were shifted to green houses for further growth and development till maturity. This whole process from scutellum to transfer in the greenhouse took 8-9 weeks as shown in Fig. 2.

Table 3. Different RIM containing full MS with different concentrations of Auxins

Media name	Name and conc. of PGRs
RIM 1	IAA (0.2 mg/l)
RIM 2	IBA (0.2 mg/l)
RIM 3	IAA (0.2 mg/l) + IBA (0.2 mg/l)

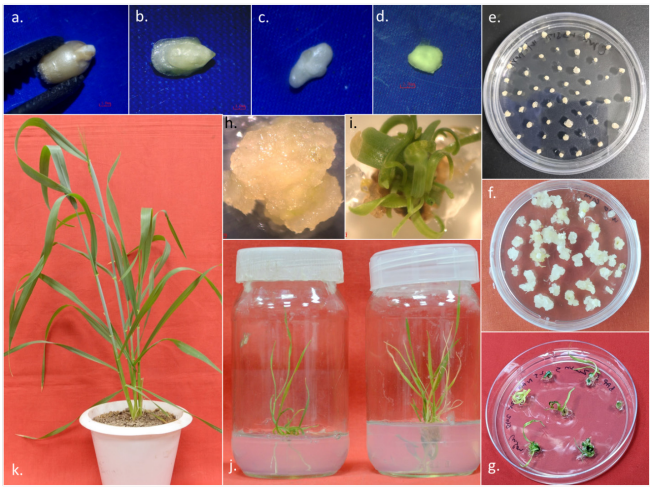

Fig. 2. Different stages of wheat regeneration. (a) Wheat mature seed, (b) Mature embryo with embryonic axis, (c) Excised embryonic axis, (d) Mature embryo without embryonic axis, (e) 5 days old calli at CIM 4, (f) 3 weeks old calli at CIM 4, (g) Regenerated calli after 3 weeks in RM 29, (h) Embryogenic calli, (i) Regenerating calli, (j) Shoots on root induction media RIM3 and (k) Plant tissue culture raised wheat plant at booting stage.

Flow chart of *in-vitro* regeneration protocol

Flow chart depicting all the steps involved in the *in-vitro* regeneration of wheat is shown in Fig. 3.

Statistical analysis

All trials employed a completely randomized design with three replications for each treatment of each genotype. All parameters were analyzed statistically using two-way ANOVA. Significant differences were analyzed by Duncan's multiple range tests, at a significance level of $p \leq 0.05$ using statistical software R version 4.2.2.

Results

Explant sterilization and incubation

Seed sterilization carried out by using a combination of different sterilizing agents (Tween20 for 30 minutes, 70% ethanol for 90 seconds, and 2% w/v sodium hypochlorite for 10 minutes) showed 100% contamination-free callus when the excised scutella from the mature and immature embryos were used as explants.

Callus induction

The callus induction response of ten combinations of media was evaluated and represented in Table 4. Four parameters viz., callus induction efficiency (CIE), callus size, callus weight and precocious germination, were recorded for both mature as well as immature scutellum as explants. CIM1 without any growth regulator didn't show any callus induction till 2 months of observation but around 60% of mature and 50% of immature scutellum showed precocious germination in CIM1. However, precocious germination was not observed in any other CIM except CIM1. All other CIM showed variable CIE. An increase in PGRs concentration resulted in increased CIE. CIM4 with dicamba @ 2 mg/l concentration showed the highest CIE, size and weight. Improved response of immature over mature embryos was observed for all parameters tested in the study. Scutella in media containing dicamba and picloram showed more CIE than in media containing 2,4-D.

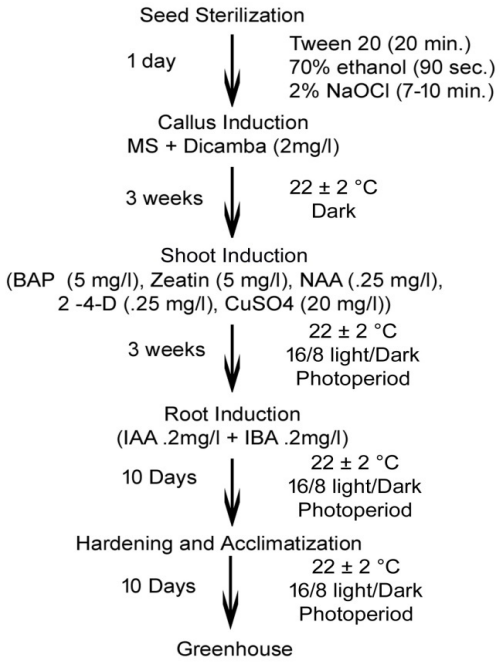

Fig. 3. Flow chart of *in-vitro* regeneration protocol

While comparing CIE of all three genotypes, HD 2967 and HD 3171 were found more responsive than Bobwhite in all combinations as shown in Table 5.

Shoot Induction

The induced calli were to be regenerated into a whole plant to examine the response of different SIM. Three weeks old embryogenic calli induced on CIM 4 were further sub-cultured to different SIM. All shoot induction media from SIM1 to SIM29 were able to induce shoots but showed variable shoot induction efficiency (SIE) indicated in Table 6. SIM29 containing (BAP (5 mg/l) + zeatin (5 mg/l) + NAA (0.25 mg/l) + 2,4-D (0.25 mg/l) + $CuSO_4$ (20 mg/l)) showed maximum average SIE of 95.68 and 98.15% from mature and immature scutellum-derived calli, respectively. The shoot induction response of two cytokinins BAP and zeatin was tested @ 2.5 and 5 mg/l concentrations and found both cytokinins were more effective for shoot induction @ 5 mg/l concentration. $CuSO_4$@ 20 mg/l strength was used as an inducer for multiple shootings and upto 9 shoots were observed in SIM 27 to SIM 29. Among genotypes, all three cultivars showed almost equal SIE. All the above regeneration parameters were recorded after 3 weeks of incubation of embryogenic calli on different SIM. Within 1 week of incubation, almost all embryogenic calli started showing green spots. Within 2 more weeks of incubation, the calli started shooting. The multiple shoots from the regenerated calli were separated and transferred to the RIM. Some of the green calli with poorly developed shoots were also transferred to RIM.

SIE in all genotypes was found to be similar with a non-significant difference, but the average number of shoots that emerged in Bobwhite was more than HD 2967 and HD 3171 as shown in Table 7.

Root induction

The healthy shoots of a minimum of 2 cm from SIM were separated and transferred to different RIM in jam bottles. Roots started emerging within 1 week of sub-culturing in RIM. Within 2 weeks of incubation on RIM all the shoots had developed healthy roots of around 2-4 cm. Irrespective of the genotypes used in present study, all three RIM showed 100% root induction in the regenerated shoots. It was also observed that when the un-regenerated green calli from SIM were transferred directly to RIM, 65% of these green calli had developed shoots (upto 4-5 shoots per callus) along with roots within 2 weeks.

Table 4. CIE in different culture media after 3 weeks of incubation. MSt-Mature Scutellum, IMSt-Immature Scutellum. According to Duncan's multiple range test (p = 0.05), means within columns followed by the same letter are not significantly different

Media name	Name and conc. of PGRs	CIE with MSt	CIE with IMSt	Callus size with MSt	callus size with IMSt	Callus weight with MSt	callus weight with IMSt
CIM 1	None	0.00^{f}	0.00^{g}	0.00^{h}	0.00^{g}	0.00^{i}	0.00^{i}
CIM 2	Dicamba (1 mg/l)	92.44^{bcd}	93.33^{de}	0.78^{d}	0.85^{d}	54.91^{ef}	60.19^{e}
CIM 3	Dicamba (1.5 mg/l	94.22^{bc}	95.56^{bc}	0.90^{b}	0.98^{b}	70.58^{b}	75.51^{b}
CIM 4	Dicamba (2 mg/l)	97.33^{a}	98.22^{a}	1.02^{a}	1.08^{a}	82.84^{a}	85.83^{a}
CIM 5	Picloram (1 mg/l)	91.11^{d}	91.56^{de}	0.67^{f}	0.74^{e}	47.88^{g}	52.58^{f}
CIM 6	Picloram (1.5 mg/l)	92.89^{bcd}	93.78^{cd}	0.80^{d}	0.86^{d}	57.38^{de}	63.30^{d}
CIM 7	Picloram (2 mg/l)	95.11^{ab}	96.89^{ab}	0.85^{c}	0.90^{c}	61.85^{c}	68.61^{c}
CIM 8	2,4-D (1 mg/l)	88.44^{e}	89.33^{f}	0.58^{g}	0.66^{f}	39.63^{h}	49.45^{g}
CIM 9	2,4-D (1.5 mg/l)	90.22^{de}	91.11^{ef}	0.72^{e}	0.76^{e}	53.56^{f}	58.66^{e}
CIM 10	2,4-D (2 mg/l)	92.00^{cd}	93.33^{de}	0.80^{d}	0.85^{d}	59.05^{cd}	63.18^{d}

Table 5. Comparison of CIE among three bread wheat genotypes. MSt- Mature Scutellum, IMSt- Immature Scutellum. According to Duncan's multiple range test (p = 0.05), means within columns followed by the same letter are not significantly different

Genotype	CIE with MSt	CIE with IMSt	Callus size with MSt	Callus size with IMSt	Callus weight with MSt	Callus weight with IMSt
Bobwhite	81.73^{b}	82.40^{b}	0.66^{c}	0.72^{c}	48.05^{c}	52.78^{c}
HD 2967	84.13^{a}	85.47^{a}	0.73^{b}	0.78^{b}	53.49^{b}	58.38^{b}
HD 3171	84.27^{a}	85.07^{a}	0.74^{a}	0.80^{a}	56.73^{a}	62.03^{a}

Table 6. Results of shoot induction response of different genotypes in 29 types of culture media.MSt- Mature Scutellum, IMSt-Immature Scutellum. According to Duncan's multiple range test (p = 0.05), means within columns followed by the same letter are not significantly different

Media name	Name and conc. of PGRs	SIEfrom MSt	SIE fromIMSt	Avg. no. of shoots from MSt	Avg. no. of shoots from IMSt
SIM 1	Without any PGRs	46.30^{i}	53.089^{l}	1.44^{n}	1.89^{m}
SIM 2	BAP (2.5 mg/l), NAA (0.25 mg/l)	61.11^{gh}	68.52^{ijk}	2.33^{klm}	2.89^{kl}
SIM 3	BAP (2.5 mg/l), NAA (0.5 mg/l)	61.73^{gh}	68.52^{ijk}	2.44^{klm}	3.44^{ghijkl}
SIM 4	BAP (2.5 mg/l), 2,4-D (0.25 mg/l)	61.11^{gh}	67.90^{jk}	2.44^{klm}	2.89^{kl}
SIM 5	BAP (2.5 mg/l), 2,4-D (0.5 mg/l)	58.02^{gh}	66.67^{k}	3.11^{hijk}	3.67^{fghijk}
SIM 6	BAP (5 mg/l), NAA (0.25 mg/l)	72.22^{def}	83.33^{g}	2.00^{lmn}	2.67^{l}
SIM 7	BAP (5 mg/l), NAA (0.5 mg/l)	70.37^{ef}	83.33^{g}	3.56^{ghi}	3.89^{efghi}
SIM 8	BAP (5 mg/l), 2,4-D (0.25 mg/l)	69.75^{f}	78.40^{h}	3.44^{ghij}	3.89^{efghi}
SIM 9	BAP (5 mg/l), 2,4-D (0.5 mg/l)	67.90^{f}	77.78^{h}	2.44^{klm}	2.89^{kl}
SIM 10	Zeatin (2.5 mg/l), NAA (0.25 mg/l)	62.96^{g}	72.22^{i}	1.67^{mn}	3.00^{jkl}
SIM 11	Zeatin (2.5 mg/l), NAA (0.5 mg/l)	61.11^{gh}	71.60^{ij}	2.67^{jkl}	3.22^{hijkl}
SIM 12	Zeatin (2.5 mg/l), 2,4-D (0.25 mg/l)	62.35^{gh}	72.22^{i}	2.67^{jkl}	3.78^{efghij}
SIM 13	Zeatin (2.5 mg/l), 2,4-D (0.5 mg/l)	62.41^{gh}	70.37^{ijk}	2.44^{klm}	3.00^{jkl}
SIM 14	Zeatin (5 mg/l), NAA (0.25 mg/l)	77.16^{c}	84.57^{g}	3.67^{fghi}	4.11^{efg}
SIM 15	Zeatin (5 mg/l), NAA (0.5 mg/l)	75.93^{cd}	85.80^{g}	2.56^{kl}	3.33^{ghijkl}
SIM 16	Zeatin (5 mg/l), 2,4-D (0.25 mg/l)	74.70^{cde}	83.33^{g}	3.67^{fghi}	4.00^{efgh}
SIM 17	Zeatin (5 mg/l), 2,4-D (0.5 mg/l)	72.22^{def}	87.04^{fg}	2.67^{jkl}	3.11^{ijkl}
SIM 18	BAP (2.5 mg/l), Zeatin (0.25 mg/l), NAA (0.25 mg/l)	79.63^{c}	85.19^{g}	3.67^{fghi}	4.00e^{fgh}
SIM 19	BAP (2.5 mg/l), Zeatin (2.5 mg/l), 2,4-D (0.25 mg/l)	77.78^{c}	85.18^{g}	3.89^{efgh}	4.33^{ef}

SIM 20	BAP (2.5 mg/l), Zeatin (2.5 mg/l), NAA (0.25 mg/l), 2,4-D (0.25 mg/l)	77.16^{c}	90.12^{ef}	3.00^{ijk}	3.67^{fghijk}
SIM 21	BAP (5 mg/l), Zeatin (5 mg/l), NAA (0.25 mg/l)	88.27^{b}	93.21^{bcde}	4.55^{de}	5.33^{d}
SIM 22	BAP (5 mg/l), Zeatin (5 mg/l), 2,4-D (0.25 mg/l)	89.51^{b}	94.44^{abcd}	4.00e^{fg}	4.56^{e}
SIM 23	BAP (5 mg/l), Zeatin (5 mg/l), NAA (0.25 mg/l), 2,4-D (0.25 mg/l)	92.59^{ab}	96.91^{ab}	4.44^{ef}	5.44^{d}
SIM 24	BAP (2.5 mg/l), Zeatin (0.25 mg/l), NAA (0.25 mg/l), $CuSO_4$ (20 mg/l)	89.51^{b}	91.36^{de}	6.00^{bc}	6.78^{c}
SIM 25	BAP (2.5 mg/l), Zeatin (2.5 mg/l), 2,4-D (0.25 mg/l), $CuSO_4$ (20 mg/l)	89.51^{b}	92.59^{cde}	5.22^{cd}	5.67^{d}
SIM 26	BAP (2.5 mg/l), Zeatin (2.5 mg/l), NAA (0.25 mg/l), 2,4-D (0.25 mg/l), $CuSO_4$ (20 mg/l)	90.12^{b}	91.36^{de}	6.00^{bc}	6.44^{c}
SIM 27	BAP (5 mg/l), Zeatin (5 mg/l), NAA (0.25 mg/l), $CuSO_4$ (20 mg/l)	92.59^{ab}	95.68^{abc}	6.67^{b}	7.67^{ab}
SIM 28	BAP (5 mg/l), Zeatin (5 mg/l), 2,4-D (0.25 mg/l), $CuSO_4$ (20 mg/l)	93.21^{ab}	95.06^{abcd}	5.78^{c}	7.11^{bc}
SIM 29	BAP (5 mg/l),Zeatin (5 mg/l), NAA (0.25 mg/l), 2,4-D (0.25 mg/l), $CuSO_4$(20 mg/l)	95.68^{a}	98.15^{a}	7.44^{a}	8.22^{a}

Table 7. Genotype-wise shoot induction efficiency. MSt-Mature Scutellum, IMSt-Immature scutellum.According to Duncan's multiple range test (p = 0.05), means within columns followed by the same letter are not significantly different

Genotype	SIE from MSt	SIE from IMSt	No. of shoots from MSt	No. of shoots from IMSt
Bobwhite	74.14^{a}	81.61^{a}	3.92^{a}	4.51^{a}
HD 2967	75.54^{a}	82.50^{a}	3.54^{b}	4.25^{b}
HD 3171	74.58^{a}	82.50^{a}	3.49^{b}	4.16^{b}

Hardening

Fully grown plantlets with well-developed shoots (10-12 cm height) and roots were transferred for hardening and acclimatization. All the plantlets were survived during hardening and acclimatization without any loss. Regeneration percentage from the mature embryo-derived explants to plantlets after hardening was 93.13% whereas in the case of immature embryo-derived explants, it was 96.40%.

Discussion

Wheat tissue culture and *in-vitro* regeneration is a very important components in crop improvement programs that could be utilized for embryo rescue in wide specific hybridization and genetic transformation (Ltifi et al. 2019; Santra et al. 2017). Sterilization of explants is the first and foremost step for any plant tissue culture-related experiment. If an explant is left infected with bacteria/ fungus or other microorganisms following sterilization, these contaminants can proliferate and spread utilizing nutrients from culture media, resulting in explant destruction and experiment failure. But the modified method developed in this study is highly efficient for explant sterilization with no contamination. The combination and concentration of various disinfectants and their treatment time are crucial for explant sterilization. Earlier reports mentioned that a duration of 25 minutes with 25% (Bie et al. 2020), 15 minutes with 25% (Liu et al. 2022) and 7 minutes with 10% (Hayta et al. 2021) of sodium hypochlorite treatment was required at the final stage of sterilization, but 10 minutes of treatment with 2% sodium hypochlorite was found sufficient enough for seed sterilization. The explant used for callus formation in this study was excised scutellum, which was found better than the earlier reports where the whole embryo was used (Tamimi and Othman 2021; Xhulaj and Doriana 2019). Excised scutellum as explant was reported earlier, and their result showed 67.6 and 90% regeneration efficiencies from mature and immature embryo-derived scutellum, respectively (Yadav et al. 2020) but regeneration efficiencies 93.13 and 96.40% were obtained in present study using mature and immature embryo-derived scutellum respectively, which are significantly higher. This efficient protocol will be very useful for genetic transformation, as excised scutellum as explant was reported to be successful for genetic transformation (Gil-Humanes et al. 2017; Liang et al. 2022). Three auxins, namely, dicamba, 2,4-D and picloram were assessed for their CIE at different concentrations. Callus induction was observed in nine different media (CIM 2 to CIM 10) after 3rd day of incubation. However, CIM 4 containing 2 mg/l dicamba outperformed other treatments with an average CIE of 97.33 and 98.22%. CIM 4 with 2 mg/l dicamba showed a larger size of callus around 6 mm within 1 week in both mature and immature scutellum. After 3 weeks of incubation, the average size of the callus had reached 1.02 cm and 1.08 cm and the average weight was up to 82.84 and 85.83 mg for the mature and immature scutellum

explants, respectively, and these results are consistent with the previous reports (Eshagi et al. 2021). A high CIE, callus size and callus weight was observed using dicamba than 2,4-D and picloram, which could be due to the rapid metabolism of dicamba (Filippov et al. 2006). Calli grown on media containing 2,4-D and dicamba were colorless, while in picloram were of light yellow color. Many of the earlier studies showed precocious germination during callus formation (Krishnaveni et al. 2019; Miroshnichenko et al. 2017) but in present study, the precocious germination was fully controlled by combined effect of excision of embryonic axis and PGRs in CIM. Removing the embryonic axis results in reduced precocious germination and eliminates the need to excision plumule and radical-derived shoots and roots and further sub-culturing to another CIM.

After 3 weeks of incubation in CIM 4, the calli were placed onto the SIM for shoot development. All 29 SIMs produce shoots in calli, but with different SIE. Furthermore, SIM 29 containing BAP (5 mg/l), Zeatin (5 mg/l), NAA (0.25 mg/l), 2,4-D (0.25 mg/l), $CuSO_4$ (20 mg/l) showed a maximum SIE of 95.68% and 98.15% in mature and immature embryo (scutellum) explant derived calli. The average number of shoots in SIM 29 is 8.22 that could be the combined effect of PGRs and $CuSO_4$. It was reported that Cu^{++} ions serve as trace nutrients required for many enzymes and are actively involved in redox reactions and nucleic acid metabolism (Printz et al. 2016). $CuSO_4$ also acts as a fungicide, and probably the low doses of it is helping in preventing fungal contamination during the shoot induction (Dos Santos et al. 2019). The dead callus tissue (black color) certainly hindered root induction during rooting. The dead callus tissue was cut and removed while separating multiple shoots from the regenerated calli. However, no reports on removal of dead callus tissue were found so far.

Two auxins, IAA and IBA, separately and in combination @ 0.2 mg/l concentration, could produce 100% roots within 10 days of sub-culture. This suggests that rooting is not a problem and could be achieved with any of these media. During hardening 100% survival was observed when shoots with well-developed roots were transferred to pots with potting mixture. In the present study, regeneration efficiency from the number of explants to regenerated plantlets after hardening depended on CIE and SIE, where rooting and survival after hardening was found to be 100% during the regeneration process.

It was reported that several genetic factors were found upregulated during regeneration. These genetic factors are named as 'regeneration related genes'. In wheat, upregulated *TaTCP-1, TaWOX5* and *GRF4–GIF1* regeneration related genes were found to promote regeneration and regulation of somatic embryogenesis of wheat (Debernardi et al. 2020; Li et al. 2020; Wang et al. 2022). But the present study was more focused about the use of exogenous plant growth regulators to improve wheat regeneration. Most available *in-vitro* regeneration protocols emphasize that their efficiency was genotype-dependent (Mendoza and Kaeppler 2002; Ozgen et al. 1998). In the present study, three different genotypes of hexaploid wheat were used, of which HD2967 and HD3171 were high-yielding popular varieties, with the purpose to develop a robust protocol across different genotypes. The present study showed that the protocol is extremely successful with very high regeneration efficiency for the varied genotypes like Bobwhite as well as HD 2967 and HD3171, which could be used for genetic transformation. Previously reported regeneration efficiency was found upto 85% using mature and immature embryos as explants (Aadel et al. 2016; Chopra et al. 2022). But 93.13 and 96.40% regeneration efficiency using scutellum derived from mature and immature embryos, respectively, has been obtained, which is sufficiently high for any transformation experiment. Many reports mention that immature embryos are better for regeneration than mature embryos (Aadel et al. 2016; Ahmadpour et al. 2018). The important aspect of this protocol is that both mature and immature embryo-derived explants could give rise to very successful regeneration for three diverse genotypes. Therefore, the protocol could be used to regenerate wheat plants throughout the year using mature embryos when the availability of immature embryos is limited. In conclusion, a simple and robust protocol for *in-vitro* wheat regeneration of two elite wheat varieties HD2967 and HD3171 is developed in this study using scutellum derived from both immature/mature embryos, which will be very useful for various wheat improvement-related experiments in these varieties.

Author's contribution

Conceptualization of research (PKM, P, JM); Designing of the experiments (PKM, P, JM); Collection of experimental materials (SP, RB); Execution of lab experiments and data collection (SP, AP), Analysis of data and interpretation (SP, JM); Preparation of the manuscript (SP, PKM, JM, AP, RB, GK, JCP, PKS, VT).

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