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RESEARCH ARTICLE

Development of an efficient and reproducible regeneration system in wheat (*Triticum aestivum* L.)

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Abstract The availability of reproducible regeneration system through tissue culture is a major bottleneck in wheat improvement program. The present study has considered to develop an efficient callus induction and regeneration system using mature and immature embryos as explants in recently released agronomically superior spring wheat varieties. An efficient sterilization process was standardized using 0.1% $HgCl_2$ and 70% ethanol for both seeds and embryos. The maximum possible combinations of plant growth regulators (PGRs) were evaluated for their effect on different wheat regeneration processes through tissue culture starting from callus to root induction. Picloram is found as an effective auxin with 87.63–98.67% callus induction efficiency in both explants. Supplementation of $CuSO_4$ along with 2,4-D, zeatin in regeneration medium significantly enhanced the multiple shoot induction. The shoot development was achieved using full strength Murashige and Skoog's (MS) medium and root induction using half MS medium without PGRs. The optimized medium and method has resulted up to 100% regeneration irrespective of the genotype used with high reproducibility. Thus, the standardized regeneration system can be used in the regeneration of healthy plants from embryos rescued from interspecies crosses, transgenic production, induced mutation breeding and recently developed genome editing techniques for the procreation of wheat plants having novel traits.

Keywords *Triticum aestivum* · Mature/immature embryos · 2,4-D · Picloram · Dicamba · Zeatin · Regeneration

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
BAP	6-Benzylaminopurine
Dicamba	3,6-Dichloro-2-methoxybenzoic acid
IAA	Indole-3-acetic acid
NAA	1-Naphthaleneacetic acid
PGRs	Plant growth regulators
Picloram	4-Amino-3,5,6-trichloro-2-pyridinecarboxylic acid
RH	Relative humidity
TDZ	Thidiazuron
Zeatin	6-(4-Hydroxy-3-methylbut-2-enylamino)purine

Introduction

Wheat globally contributes about 30% of total grain cereals production (FAO 2016). The current world population of 7.3 billion is expected to be about 9 billion by 2050 (UN 2015). Hence, there is an urgent need for a second green revolution in cereal crops worldwide to fulfill food requirement for this tremendously growing global population. It is essential to adapt both conventional and genetic engineering based breeding strategies in crop improvement programs to achieve this food requirement in the shortest

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possible time. Tissue culture is an integral part of biotechnology breeding and provides an added advantage to crop improvement programmes. The hindrance of different cereal crops to regeneration through callus is a major bottleneck in any crop improvement program including wheat (Parmar et al. 2012).

A good and efficient callus induction system in wheat highly depends on sterilization process, type of explants, genotypes, media composition and its pH, growth hormones, inducers and incubation conditions (Parmar et al. 2012; Mamrutha et al. 2014). Different explants have been tested as starting material for wheat callus cultures, such as immature embryos (Hakam et al. 2015), leaf segment (Yu et al. 2012), anthers (Redha and Suleman 2011), immature inflorescence (Kavas et al. 2008), microspores (Shariatpanahi et al. 2006) and mature embryos (Parmar et al. 2012) which showed the variable response for callus induction and regeneration. Immature embryos have been found most suitable explant source due to its high callus induction and regeneration capabilities (Redway et al. 1990). However, the immature embryos have limited seasonal availability and difficult to obtain during off-season. Furthermore, the most suitable stage for their efficient culture is also strictly restricted to 12–20 days post-anthesis (Zale et al. 2004), limiting their application for in vitro culture and genetic transformation. A regeneration system based on mature embryos may overcome these limitations (Zale et al. 2004).

The presence of plant growth regulators (PGRs) and their concentration in the culture media highly affect the callus induction and regeneration. In general, the PGRs concentration varies for optimum callus growth, development, and regeneration (Kothari et al. 2004) for particular plant species and specific explant. The first successful regeneration in wheat was reported by Zhou and Lee (1984). They investigated the effect of different auxins on mature embryo culture. Most frequently used auxin to induce callus in wheat is 2,4-dichlorophenoxyacetic acid (2,4-D), a synthetic auxin and potent herbicide, at a concentration of 1–2 mg/l. It is followed by 3,6-dichloro-2-methoxybenzoic acid (dicamba), another PGR and herbicide that shares similarities in structure and activity to 2,4-D (Bahieldin et al. 2000; Ren et al. 2010) and 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid (picloram) (Mendoza and Kaeppler 2002; Satyavathi et al. 2004). Till date, many regeneration media were optimized by adding cytokinins [kinetin, 6-benzylaminopurine (BAP), thidiazuron (TDZ), zeatin (6-(4-hydroxy-3-methylbut-2-enylamino)purine)] along with auxins indole-3-acetic acid (IAA), 1-naphthaleneacetic acid (NAA), 2,4-D, picloram (Fennell et al. 1996; Fahmy et al. 2006; Benderradji et al. 2012). Additionally, some inducers, such as $CuSO_4$, $AgNO_3$, were found to be useful to enhance the regeneration rate (Yu et al. 2008). Przetakiewicz et al. (2003) reported good regeneration from callus culture on hormone-free full strength MS medium. Further, some studies were carried out to improve the existing regeneration protocol in wheat (Parmar et al. 2012; Hakam et al. 2015). The wheat *cv*. Bobwhite and Chinese spring are world recognized wheat genotypes and are extensively used in tissue culture and genetic engineering (Zale et al. 2004; Agarwal et al. 2009). However, these genotypes are agronomically inferior and developed protocols are highly genotype dependent (Zale et al. 2004). Limited information exists on established regeneration protocol in agronomically superior wheat genotypes.

Hence, the present study was undertaken in agronomically superior Indian wheat genotypes to develop a robust, reproducible and genotype independent regeneration system. The sterilization conditions for both seeds and embryos were standardized and the effect of different PGRs in various concentrations and combinations were optimized for regeneration.

Materials and methods

Plant material

The seeds of six recently released high yielding Indian wheat (*T. aestivum* L.) genotypes (DBW 88, DBW 90, DBW 93, DPW 621-50, HD 3086, and WH 1105) were procured from the germplasm unit of the Indian Council of Agricultural Research-Indian Institute of Wheat and Barley Research (ICAR-IIWBR), Karnal (India). The genotypes were grown in the experimental field of ICAR-IIWBR. Spikes of all the genotypes were collected after 18–20 days of post-anthesis (Zadok's scale 75–85) for immature embryos.

Explants sterilization

Mature and immature embryos of wheat were used as explants in the present study. Mature/immature seeds of wheat were washed with sterile distilled water (SDW) twice and sterilized with 0.1% $HgCl_2$ followed by two SDW washing, then with 70% ethanol and rinsed with SDW three times. The different treatment durations 30, 60, 120, 180, 240 and 300 s for each disinfectant viz. 0.1% $HgCl_2$ and 70% ethanol were performed. After sterilization, mature seeds were soaked in SDW in a sterilized petri plate, sealed with parafilm and incubated overnight at 10 °C (not required for immature seeds). Embryos were excised from mature/immature seeds using a sterile needle under laminar air flow. Excised embryos were sterilized in similar manner as for the seeds, but the durations for

treatment were 10, 15, 20, 30, 60, 120 and 180 s for both disinfecting agents. Finally, these embryos were washed thrice with SDW to remove the excess disinfectants and retained in SDW until they were placed in the culture medium.

Callus induction

Murashige and Skoog (MS) medium (supplementary Table S1) (34.41 g/l) was used for various media preparations supplemented with different PGRs of variable concentrations (Table 1) and was solidified using 8 g/l agar (HI-MEDIA®, India, catalogue no. PCT0901). About 40–60 embryos with scutellum side-up were placed per petri plate for individual callus induction medium having different concentrations of auxins (2,4-D, picloram, dicamba, NAA) (Table 1) ranging from 1.5 to 4.0 mg/l. The petri plates were sealed with parafilm and incubated in the dark at 24 ± 1 °C for 20 days.

Regeneration

Twenty days calli were obtained from mature and immature embryos from all six genotypes using callus induction

Table 1 Composition of different culture media used for callus induction, regeneration and root induction

Medium name	PGRs (mg/l)	Medium name	PGRs (mg/l)
Callus Induction medium (CI) (full MS medium[a] 34.14 g/l, agar 8 g/l, pH 5.8)			
CI1	Full MS without any PGRs	CI7	Picloram (2.5)
CI2	2,4-D (1.5)	CI8	Dicamba (1.5)
CI3	2,4-D (2.0)	CI9	Dicamba (2.0)
CI4	2,4-D (2.5)	CI10	Dicamba (2.5)
CI5	Picloram (1.5)	CI11	2,4-D (4.0) + NAA (2.0)
CI6	Picloram (2.0)	CI12	2,4-D (2.0) + Picloram (2.0)
Regeneration medium (RM) (full MS medium 34.14 g/l, agar 8 g/l, pH 5.8)			
RM1	Full MS without any PGRs	RM21	BAP (1.5) + Kinetin (1.5) + IAA (0.5)
RM2	BAP (1.5)	RM22	BAP (1.0) + Kinetin (2.0) + IAA (0.5)
RM3	BAP (2.0)	RM23	BAP (0.5) + Kinetin (2.5) + IAA (0.5)
RM4	BAP (2.5)	RM24	2,4-D (0.1)
RM5	Kinetin (1.5)	RM25	2,4-D (0.1) + Zeatin (1.0)
RM6	Kinetin (2.0)	RM26	2,4-D (0.1) + Zeatin (2.0)
RM7	Kinetin (2.5)	RM27	2,4-D (0.1) + Zeatin (3.0)
RM8	BAP (2.0) + IAA(0.1)	RM28	2,4-D (0.1) + Zeatin (4.0)
RM9	BAP (2.0) + IAA(0.2)	RM29	2,4-D (0.1) + Zeatin (5.0)
RM10	BAP (2.0) + IAA(0.3)	RM30	2,4-D (0.1) + $CuSO_4$ (12)
RM11	BAP (2.0) + IAA(0.4)	RM31	2,4-D (0.1) + $CuSO_4$ (15)
RM12	BAP (2.0) + IAA(0.5)	RM32	2,4-D (0.1) + $CuSO_4$ (18)
RM13	BAP (2.0) + 2,4-D(0.2)	RM33	2,4-D (0.1) + $CuSO_4$ (21)
RM14	BAP (2.5) + Kinetin (0.5) + IAA (0.1)	RM34	2,4-D (0.1) + $CuSO_4$ (25)
RM15	BAP (2.0) + Kinetin (1.0) + IAA (0.1)	RM35	2,4-D (0.1) + Zeatin (5.0) + $CuSO_4$ (12)
RM16	BAP (1.5) + Kinetin (1.5) + IAA (0.1)	RM36	2,4-D (0.1) + Zeatin (5.0) + $CuSO_4$ (15)
RM17	BAP (1.0) + Kinetin (2.0) + IAA (0.1)	RM37	2,4-D (0.1) + Zeatin (5.0) + $CuSO_4$ (18)
RM18	BAP (0.5) + Kinetin (2.5) + IAA (0.1)	RM38	2,4-D (0.1) + Zeatin (5.0) + $CuSO_4$ (21)
RM19	BAP (2.5) + Kinetin (0.5) + IAA (0.5)	RM39	2,4-D (0.1) + Zeatin (5.0) + $CuSO_4$ (25)
RM20	BAP (2.0) + Kinetin (1.0) + IAA (0.5)		
Rooting medium (RTM) (half MS medium 17.21 g/l, agar 8 g/l, pH 5.8)			
RTM1	Half MS without any PGRs	RTM5	NAA (0.2)
RTM2	IAA (0.1)	RTM6	IAA (0.1) + NAA (0.1)
RTM3	IAA (0.2)	RTM7	IAA (0.2) + NAA (0.2)
RTM4	NAA (0.1)		

[a] MS medium composition and preparation (see supplementary Table S1)

(CI6) medium containing picloram 2.0 mg/l and transferred to regeneration media (RM) having different PGRs in various combinations. A total thirty-nine different regeneration media containing several combinations of PGRs, cytokinins (BAP, kinetin, zeatin) ranging from 0.5 to 5 mg/l and auxins (IAA and 2,4-D) ranging from 0.1 to 0.5 mg/l were tested. These media were supplemented with or without different concentration of $CuSO_4$ (12–25 mg/l) as listed in Table 1. The incubation conditions for regeneration were relative humidity (RH) with 55–65%, temperature 24 ± 1 °C and 16/8 h photoperiod with a cool-fluorescent light 10 µmol m^{-2} s^{-1} for the first week. In the second week, each individual shoots were separated out from regenerated calli and placed in the tube (25 × 150 mm) having full MS medium (RM1) without any growth hormone for shoot development. The incubation conditions for the second week remained same except the light intensity was increased to 52 µmol m^{-2} s^{-1}. The number of multiple shoots developed per callus was recorded after 15 days of culture.

Root induction

The developed shoots were placed in 300 ml culture bottles containing 50 ml half MS supplemented with different combinations of IAA and NAA. A total of seven root induction media (RTM) were used (Table 1). The incubation conditions for root induction were RH 55–65%, temperature 24 ± 1 °C and light 52 µmol m^{-2} s^{-1} for 1-week duration.

Hardening and acclimatization

The plantlets having well-established shoot and root network were transferred to 4″ plastic pots having an equal proportion of soil-peat mix and watered with 50 ml Hoagland solution (Supplementary Table S2). The plantlets were covered with transparent polythene bags for 10 days under the same conditions as of root induction to harden the plantlets. Subsequently, the polythene bags were punctured and removed after 2 days. Later, the hardened plants were transferred to 8″ sized pots, and grown up to maturity in controlled lab conditions.

Data analysis

A completely randomized design with three replications per treatment of each genotype was used in all experiments. The actual callus induction efficiency was calculated as calli without precocious germination divided by total explants and multiplied by 100. The morphological characters like callus growth rate, colour, size, weight, were also recorded. Callus size was measured using vernier calliper micrometer and callus weight was recorded using electronic microbalance (Mettler Toledo, India). The data was analysed using the SAS statistical software program, PROC GLM, SAS version 9.3 (SAS Institute Inc., Cary, NC, USA) at different statistical significance level using ANOVA and Tukey–Kramer's test.

Results

Explants sterilization

The explant sterilization is a crucial step in tissue culture, which affects both explants viability and regeneration efficiency. Different durations of sterilization were tested for both seeds and embryos using 0.1% $HgCl_2$ and 70% ethanol to find out optimum sterilization period. The viability of mature seed was reduced after exceeding the sterilization time more than 120 s for both sterilizing agents (0.1% $HgCl_2$ and 70% ethanol). Bacterial contamination was observed in 60 s and less than 60 s sterilization of seeds. For mature and immature embryos, complete sterilization was achieved at 20 s sterilization with 0.1% $HgCl_2$ followed by 20 s of 70% ethanol treatment without affecting explants viability. Hence, the optimum duration for seeds and embryos sterilization was 120 and 20 s, respectively for both disinfectants.

Callus induction

The callus induction was tested on twelve combinations of media and all tested callus induction media showed variable embryogenic callus induction efficiency in all six wheat genotypes (DBW 88, DBW 90, DBW 93, DPW 621-50, HD 3086, and WH 1105) along with different percentage of precocious germination (Table 2). The results showed optimum embryogenic callus induction obtained from callus induction 6 (CI6) medium (picloram 2.0 mg/l) ranging from 87.63 to 97.73% and 95.4 to 98.67% for mature and immature embryos respectively (Table 3). Though, other CI media showed up to 100% callus induction, but was with slow growth rate or with high percentage of precocious germination. CI1 without PGRs showed only precocious germination in both explants (Fig. 2b). There was no callus induction using CI1 (Table 2). In the comparative analysis of three auxins viz. 2,4-D, picloram and dicamba in callus induction, dicamba showed very fast callus growth rate in both explants with increase in concentration along with high precocious germination. 2,4-D showed minimum precocious germination but with slow rate of callus induction. Picloram showed optimum callus induction with less precocious germination than dicamba. The higher concentration, i.e. 2.5 mg/l of

Table 2 The response of wheat genotypes for embryogenic callus induction

Medium	Mature embryos				Immature embryos			
	Callus induction (%)	Precocious germination (%)	Callus size (cm)	Callus weight (mg)	Callus induction (%)	Precocious germination (%)	Callus size (cm)	Callus weight (mg)
CI1	0	100^{a}	0	0	0	100^{a}	0	0
CI2	97.96^{a}	2.04^{g}	0.41^{j}	38^{i}	100^{a}	0	0.50^{h}	42^{j}
CI3	96.79^{ab}	3.21^{fg}	0.50^{h}	43^{h}	99.67^{a}	0.33^{e}	0.50^{h}	43^{j}
CI4	95.00^{b}	5.00^{f}	0.52^{gh}	47^{g}	99.37^{a}	0.63^{e}	0.50^{h}	46^{i}
CI5	92.51^{c}	7.49^{e}	0.58^{f}	74^{e}	96.37^{b}	3.63^{d}	0.58^{f}	75^{f}
CI6	92.52^{c}	7.48^{e}	0.68^{e}	85^{d}	96.53^{b}	3.47^{d}	0.77^{e}	86^{e}
CI7	90.98^{cd}	9.03^{de}	0.73^{d}	87^{d}	95.64^{bc}	4.36^{cd}	0.80^{d}	87^{d}
CI8	87.71^{e}	12.29^{c}	0.82^{c}	108^{c}	92.26^{d}	7.75^{b}	0.90^{c}	114^{c}
CI9	85.76^{ef}	14.24^{bc}	0.97^{b}	120^{b}	91.54^{d}	8.46^{b}	1.00^{b}	124^{b}
CI10	85.26^{f}	14.74^{b}	1.07^{a}	127^{a}	92.67^{d}	7.33^{b}	1.10^{a}	136^{a}
CI11	92.21^{c}	7.79^{e}	0.45^{i}	50^{g}	96.27^{bc}	3.73^{cd}	0.47^{i}	50^{h}
CI12	89.96^{d}	10.04^{d}	0.53^{g}	54^{f}	94.53^{c}	5.47^{c}	0.57^{g}	53^{g}

Data of each trait represents the average values of six wheat genotypes (DBW 88, DBW 90, DBW 93, DPW 621-50, HD 3086 and WH 1105)

The figure followed by same superscript letters are not significantly different according to Tukey's multiple comparison test at $P < 0.01$. CI6 gave the optimal callus induction

Table 3 Callus induction response of six Indian wheat genotypes in CI6

Genotype	Mature embryos				Immature embryos			
	Callus induction (%)	Precocious germination (%)	Callus size (cm)	Callus weight (mg)	Callus induction (%)	Precocious germination (%)	Callus size (cm)	Callus weight (mg)
DBW 88	97.73^{a}	2.27^{c}	0.70^{a}	86^{a}	96.77^{a}	3.23^{a}	0.77^{a}	86^{a}
DBW 90	94.93^{ab}	5.07^{bc}	0.67^{a}	86^{a}	96.17^{a}	3.83^{a}	0.73^{a}	85^{a}
DBW 93	89.13^{c}	10.87^{a}	0.63^{a}	86^{a}	96.63^{a}	3.37^{a}	0.80^{a}	86^{a}
DPW 621-50	90.30^{bc}	9.70^{ab}	0.70^{a}	85^{a}	95.53^{a}	4.47^{a}	0.73^{a}	85^{a}
HD 3086	95.37^{a}	4.63^{c}	0.70^{a}	85^{a}	95.40^{a}	4.60^{a}	0.77^{a}	86^{a}
WH 1105	87.63^{c}	12.37^{a}	0.70^{a}	85^{a}	98.67^{a}	1.33^{a}	0.80^{a}	86^{a}

The figure followed by same superscript letters are not significantly different according to Tukey's multiple comparison test at $P < 0.01$

each growth hormone showed maximum callus induction rate as well as high precocious germination than their respective lower concentrations (1.5 and 2.0 mg/l). Embryogenic calli developed on callus induction media containing 2,4-D and dicamba were of white colour while media having picloram were of light yellow. The picloram at 2.0 mg/l was found to be optimum to achieve the required callus growth rate along with good quality callus in both types of explants (Table 2). The other tested CI media were CI11 [2,4-D (4.0 mg/l) + NAA (2.0 mg/l)] and CI12 [2,4-D (2.0 mg/l) + Picloram (2.0 mg/l)]. The callus induction was not satisfactory in these media also as compared to picloram 2.0 mg/l. The results obtained for all tested twelve callus induction media were collinear for both the explants. However, the embryogenic callus induction efficiency was found higher and precocious germination was low in case of immature embryos compared to mature embryos.

Regeneration

A total 39 different hormonal combination media tested for regeneration showed good regeneration, but with a variable growth rate and efficiency (Table 4). The effect of BAP alone and with kinetin and IAA in different concentrations was tested. RM14 containing BAP at 2.5 mg/l, kinetin 0.5 mg/l and IAA 0.1 mg/l showed regeneration efficiency ranging from 80 to 84.5% and 83.4 to 87.9% using mature and immature embryos, respectively. RM24 containing only 2,4-D (0.1 mg/l) also showed good regenerations of about 80.73% (mature embryos) and 89.60% (immature embryos) on an average in all genotypes with less number

Table 4 The response of wheat genotypes for regeneration in different RM media

Medium	Mature embryos		Immature embryos		Medium	Mature embryos		Immature embryos	
	Reg (%)	SH	Reg (%)	SH		Reg (%)	SH	Reg (%)	SH
RM1	51.51^{m}	1	61.61^{l}	1	RM21	71.68^{fghij}	1	69.98^{k}	1
RM2	21.28^{q}	1	20.88^{o}	1	RM22	71.28^{ghij}	1	73.47^{ijk}	1
RM3	38.93^{n}	1	40.71^{mn}	1	RM23	72.45^{fghi}	1	60.53^{l}	1
RM4	57.10^{lm}	1	47.83^{m}	1	RM24	80.73^{ed}	1	89.60^{cde}	1
RM5	31.91^{op}	1	37.18^{n}	1	RM25	81.82^{ed}	1	83.23^{defgh}	1
RM6	34.47^{no}	1	41.35^{mn}	1	RM26	83.03^{cbde}	1	86.47^{defg}	1
RM7	27.81^{p}	1	37.15^{n}	1	RM27	85.09^{cbd}	1	87.77^{def}	1
RM8	66.34^{jk}	1	73.74^{ijk}	2	RM28	87.83^{cb}	2	90.63^{bcd}	2
RM9	64.95^{k}	1	73.99^{ijk}	1	RM29	88.41^{b}	2	96.80^{abc}	3
RM10	66.68^{ijk}	1	73.67^{ijk}	1	RM30	96.72^{a}	3	97.41^{ab}	2
RM11	65.91^{jk}	1	72.77^{jk}	1	RM31	98.23^{a}	3	99.17^{a}	3
RM12	67.68^{hijk}	1	77.14^{hijk}	1	RM32	97.89^{a}	3	100^{a}	3
RM13	61.91^{kl}	1	73.02^{ijk}	1	RM33	96.52^{a}	4	100^{a}	4
RM14	82.30^{bed}	1	85.60^{defg}	2	RM34	97.59^{a}	5	100^{a}	4
RM15	71.68^{fghij}	2	79.37^{ghij}	2	RM35	100^{a}	8	100^{a}	8
RM16	74.57^{fg}	1	80.41^{fghi}	2	RM36	100^{a}	9	100^{a}	11
RM17	72.29^{fghi}	1	76.91^{hijk}	1	RM37	99.48^{a}	11	100^{a}	13
RM18	66.91^{ijk}	2	79.97^{ghij}	1	RM38	100^{a}	12	100^{a}	15
RM19	77.28^{ef}	2	74.49^{ijk}	2	RM39	100^{a}	14	100^{a}	17
RM20	73.23^{fgh}	2	82.32^{efgh}	2					

Data of each trait represents the average values of six wheat genotypes (DBW 88, DBW 90, DBW 93, DPW 621-50, HD 3086 and WH 1105)

Reg regeneration, *SH* average number of shoots per embryo

The figure followed by same superscript letters are not significantly different according to Tukey's multiple comparison test at $P < 0.01$. RM36 gave the optimal regeneration

of shoots per callus. The concentrations of zeatin (1, 2, 3, 4 and 5 mg/l) and $CuSO_4$ (12, 15, 18, 21, 25 mg/l) were tested individually and together with 2,4-D (0.1 mg/l) in regeneration medium. Zeatin showed its inductive effect on shoot regeneration, but found suitable at 5 mg/l in regeneration medium. Less than 5 mg/l zeatin showed less shoot induction in both mature and immature embryo explants in all tested genotypes. A linear increasing trend of multiple shoot induction was observed with increase in $CuSO_4$ concentration in regeneration medium. The maximum regeneration frequency was observed in regeneration medium RM35 [2,4-D (0.1 mg/l) + zeatin (5 mg/l) + $CuSO_4$ (12 mg/l)], and onwards media having 2,4-D, zeatin, and $CuSO_4$ in different concentration in case of mature embryos. From RM32 medium onwards, the 100% regeneration was observed using immature embryos and RM32 showed the less number of multiple shoots in calli compared to others (Fig. 1). The regeneration medium RM36 [(2,4-D (0.1 mg/l) + zeatin (5 mg/l) + $CuSO_4$ (15 mg/l)] was found as an optimum regeneration medium as it showed more individual healthy plantlets compared to RM39. The RM39 showed more rosette leaves with high $CuSO_4$ concentration (Fig. 1). Mature and immature embryos derived calli from six genotypes gave 100% regeneration with variable number of shoots per explant in RM36 medium (Supplementary Table S3). The plantlet recovered from different regeneration media showed good shoot development in RM1 (full strength MS) having no plant growth hormones. The immature embryos gave more shootlets as compared to mature embryos from all six genotypes.

Root induction of regenerated plantlets

All tested root induction media showed root initiation. However, the optimum root network was observed within a week in rooting medium 1 (RTM1) i.e. medium without any PGRs, for all tested genotypes in both immature and mature embryos. The root initiation rate was higher in RTM1 followed by RTM7 having IAA (0.2 mg/l) and NAA (0.2 mg/l).

Hardening and acclimatization

All plants recovered from RTM1 survived under hardening process and acclimatized in controlled conditions.

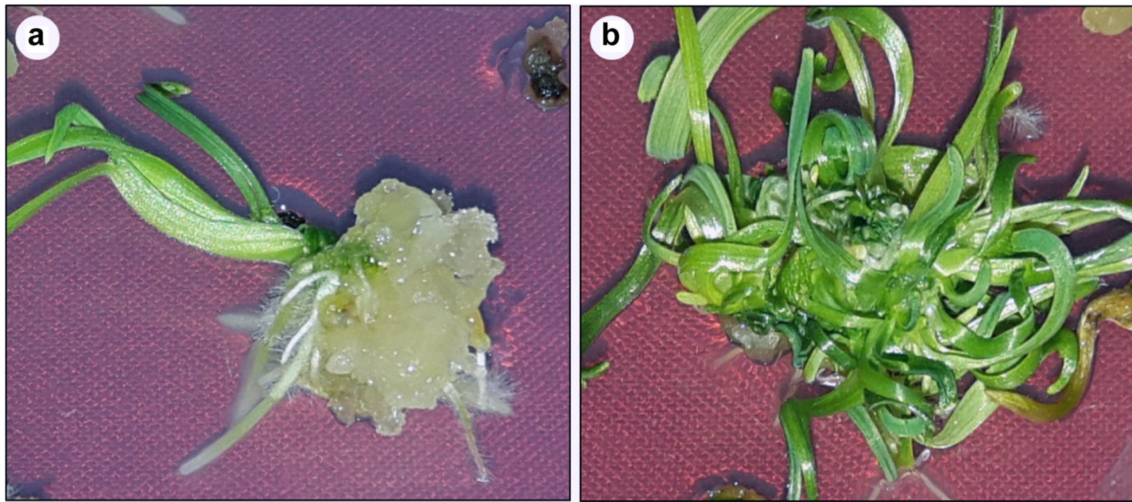

Fig. 1 Effect of different concentrations of $CuSO_4$ in regeneration media along with 2,4-D 0.1 mg/l and zeatin 5 mg/l, **a** RM29 (without $CuSO_4$), and, **b** RM39 ($CuSO_4$ 25 mg/l)

Morphologically healthy wheat plants were regenerated from this protocol. The complete regeneration of wheat plant from the explants to hardening took about 52–60 days as shown in Fig. 2.

Discussion

A robust genotype independent regeneration system for wheat is the need of the hour to transfer desired genes using *Agrobacterium*-mediated method or by biolistic method or with other tissue culture based methods. This study emphasized on evaluation of the PGRs influence on callus induction and regeneration and to find out their optimal concentrations using both immature and mature embryos in agronomically superior Indian wheat genotypes. The explants sterilization is the most important step in tissue culture. Earlier reported sterilizing agents such as sodium hypochlorite, calcium hypochlorite, mercuric chloride, ethanol and their long term treatment with explants caused explant death and consequently reduced the regeneration efficiency (Fillipov et al. 2006; Chauhan et al. 2007). In the present study, the higher regeneration efficiency was maintained by sterilization using 0.1% $HgCl_2$ and 70% ethanol for 120 s for seed and 20 s (each disinfectant) for embryos followed by SDW washing after each step. Only the seeds sterilization was reported in previous studies for wheat (Parmar et al. 2012; Hakam et al. 2015). To our best knowledge, this is the first report on embryos sterilization before their use in callus induction. Hence, the optimized sterilization process for both seeds and embryos will reduce both duration and contamination risk in wheat tissue culture.

Some studies have already reported the effect of growth regulators on callus induction and regeneration in wheat (Fillipov et al. 2006; Chauhan et al. 2007). But these studies were carried out in limited number of wheat genotypes and with less number of PGR combinations in the media. Earlier reports, claims 2,4-D has optimum callus induction capacity in wheat (Yu et al. 2008). However, our results indicated that 2,4-D has the slowest rate of callus induction as compared to dicamba followed by picloram. Dicamba showed high callus induction rate among the tested auxins but it also has highest precocious germination. Some reports (Hunsinger and Schauz 1987; Papenfus and Carman 1987; Redway et al. 1990) also claims that the dicamba shows rapid callus induction than 2,4-D. These reports suggest that dicamba is consumed very rapidly by cellular metabolism in wheat tissue. In contrast, 2,4-D is a highly stable auxin and shows strong resistance to enzymatic degradation and conjugation in the plant cell (Moore 1989).The rapid rate of dicamba consumption from callus induction medium than 2,4-D induces the precocious germination of somatic embryos (Mendoza and Kaeppler 2002). In our study, by considering maximum combinations and different concentration of auxins, picloram was identified as suitable auxin at optimum concentration of 2.0 mg/l for good quality embryogenic callus induction within 20 days using mature and immature embryos irrespective of the genotypes used.

Shoot regeneration is another crucial step after callus induction in tissue culture. BAP and kinetin were tested at different concentration and combinations. The absence of 2,4-D in regeneration medium showed the shoot induction as well as the root induction. The root induction suppressed to a large extent by supplying 2,4-D alone in regeneration medium or with other growth regulators, only shoot induction occurred. It was evident that the inclusion of 2,4-D in combination with cytokinins is valuable for regeneration

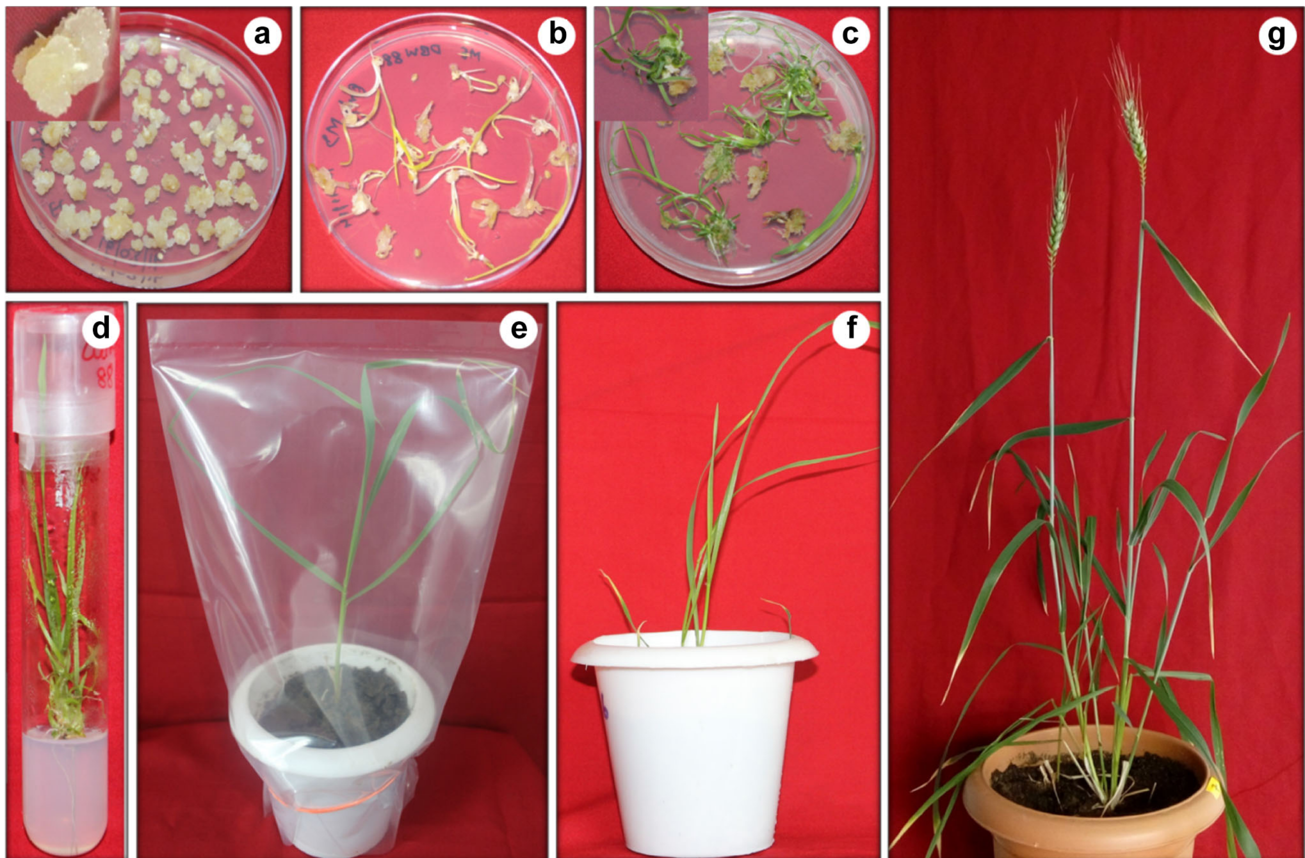

Fig. 2 Schematic representation of standardized protocol, **a** embryogenic calli in CI6, **b** precocious germination in CI1 (only full MS) having no PGRs, **c** regeneration in RM36, **d** shoot development of plantlet in RM1, **e** hardening of recovered plantlet, **f** plant acclimatization in soil and peat mix containing pot, **g** plant generated through optimized protocol grown up to maturity

(Chauhan et al. 2007). From the current study, RM36 [2,4-D (0.1 mg/l) + zeatin (5 mg/l) + $CuSO_4$ (15 mg/l)] showed optimal regeneration within 1 week among thirty-nine tested regeneration media. Chauhan et al. (2007) used zeatin and TDZ individually and in different combination and found zeatin as superior to TDZ. $CuSO_4$ acts as stress inducing agent in regeneration medium and promotes shoots. The Cu^{2+} is known to be a cofactor of many important enzymes implicated in biological processes, suggesting these Cu enzymes could play an important role in plant tissue culture (Sparks et al. 2014). As $CuSO_4$ concentration increased, the amount of green tissue per callus was increased. The excessive concentration of $CuSO_4$ showed more wrinkled plantlets with rosette leaves. Hence, the optimum concentration was 15 mg/l which gave individual plantlets having good and healthy leaves. The immature embryos showed fast callus induction rate and higher regeneration than mature embryos, which conferred immature embryos as a good source of explants for wheat tissue culture. Nevertheless, the mature embryos also showed the good callus induction and regeneration in the standardized media. The accessibility of immature embryos throughout the year is a very major hurdle due to limited seasonal availability. Results also indicate that the selected genotypes have not much significant influence on callus induction and regeneration. However, most of the earlier published protocols were genotype dependent (Zale et al. 2004; Yu et al. 2008). Overall, the standardized regeneration method showed 100% regeneration efficiency irrespective of the genotype used in both mature and immature embryos as explant. Hence, a robust and reproducible regeneration protocol to our best knowledge has been reported in agronomically superior wheat genotypes for the first time. And these genotypes immediately after gene transfer can be easily used in the breeding program with the added advantage of a gene.

Conclusion

Establishment of a reproducible regeneration system is the essential prerequisite for effective transgenics development in wheat improvement. In the current research, the sterilization process was effectively standardized using 0.1%

$HgCl_2$ and 70% ethanol for both seed and embryos. The effect of different PGRs on callus induction, regeneration and root induction was also analyzed. Among the tested auxins, picloram (2.0 mg/l) was found to be most suitable for embryogenic callus induction. A combination of 2,4-D + zeatin + $CuSO_4$ showed good regeneration. The MS medium without any PGRs gave good rooting. The optimized media showed good callus induction and regeneration in both mature and immature embryos as explant irrespective of the genotype used. All these standardized media and protocol can be effectively used in different wheat genotypes, for faster introgression of useful transgenes through *Agrobacterium* or by any other tissue culture based method for rapid wheat improvement.

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Authors' contribution MHM conceived the project and designed the experiments with AG. RK conducted experiments. AK assisted RK during Research. RK, MHM, and KV wrote the manuscript. RajK and VT guided and extended facilities for research. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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