

RESEARCH ARTICLE

Responses of explant type of wheat (*Triticum aestivum* L.) genotypes to different tissue culture media

Imran Mahmood* and Abdul Razzaq

Department of Agronomy, Faculty of Crop and Food Sciences, Pir Mehr Ali Shah, Arid Agriculture University, Rawalpindi-46300, Pakistan.

Revised: 17 November 2016; Accepted: 16 February 2017

Abstract: Wheat recalcitrance to *in vitro* culture is one of the most important and crucial steps for its genetic transformation, and is directly associated with the ability of the explant to regenerate, composition of the media and genotype. Callus induction and regeneration potential of various explants, viz, mature embryo, immature embryo, endosperm supported (ES) mature embryo and shoot apical meristem, taken from the same plants of two wheat genotypes (AS-2002 and GA-2002) were investigated using Murashige and Skoog (MS) basal callus induction medium supplemented with 2, 4, 6 or 8 mg/L of 2,4-dichlorophenoxyacetic acid (2,4-D) and regeneration media comprising various combinations of auxins and cytokinins. *In vitro* culture response of the explants taken from the same plant varied from each other and was influenced by the genotype and media composition. Immature embryo culture of genotype AS-2002 exhibited the highest callusing ability (84.75 %) followed by the mature embryo culture of genotype GA-2002 (78.50 %). Similarly, immature embryos cultured on MS basal medium supplemented with 4 mg/L 2,4-D and mature embryos cultured on MS basal medium supplemented with 6 mg/L 2,4-D exhibited the highest callusing ability of 90.83 and 88.33 %, respectively. Immature embryos of genotype GA-2002 cultured on MS based regeneration medium supplemented with 0.2 mg/L 2,4-D, 0.5 mg/L zeatin and 0.5 mg/L benzylaminopurine (BAP) showed a significantly higher regenerating potential. ES mature embryos of genotypes GA-2002 and AS-2002 cultured on regeneration medium comprising 0.4 mg/L 2,4-D, 1.0 mg/L zeatin and 1.5 mg/L BAP showed maximum regeneration potentials of 59.67 and 59.0 %, respectively. Appraisal of the responses of the four explants used showed that endosperm-supported mature embryo is the most suitable explant for plant regeneration in wheat.

Keywords: Auxins, cytokinins, *in vitro*, regeneration, somatic embryogenesis, totipotency.

INTRODUCTION

The world population is projected to grow from 6 to 8 billion by 2020 (Wagiran *et al.*, 2010). Efforts are being made to address the increasing food demand of the world population by breeding genotypes with increased yield potential and resistance/tolerance to a wide range of biotic and abiotic stresses through genetic transformation, coupled with conventional breeding approaches. Up till now, genetic modification of wheat was carried out through gene gun and agrobacterium-mediated transformation involving callus phase (Razzaq *et al.*, 2011), where the major bottleneck is not the delivery of the genes but the establishment of long-term cultures and finally the regeneration of transformed plants.

Wheat (*Triticum aestivum* L.) is amongst the most recalcitrant cereals to *in vitro* culture (Razzaq *et al.*, 2011), since its embryogenic callus formation and regeneration of transformed plants, besides genotypic and media composition (Chen *et al.*, 2006; Mahmood *et al.*, 2012c; Gill *et al.*, 2014), are explant dependent (Sarker & Biswas, 2002; Chen *et al.*, 2006; Gill *et al.*, 2014). In cereal tissue culture, not all cells express totipotency (Shah *et al.*, 2003) and the choice of a favourable explant and genotype is required for the success in tissue culture approaches. Therefore, the success of any callus-based transformation strategy depends largely upon the regeneration capability of the target explant and the genotype (Chugh & Khurana, 2003), and efforts are being made to increase their callusing and regeneration efficiencies by using various combinations of phytohormones (Mahmood *et al.*, 2012c).

* Corresponding author (imran_agri@yahoo.com)

Generally, immature embryo is preferred as an explant source for tissue culture and genetic transformation studies of wheat due to its superb callusing and regeneration potential (Sarker & Biswas, 2002; Vendruscolo *et al.*, 2008; Aguado-Santacruz *et al.*, 2011; Mahmood *et al.*, 2012a, 2012b, 2012c; Gill *et al.*, 2014; Yang *et al.*, 2015). However, the production of immature embryos throughout the year is a space, time and labour intensive task and appears to be not feasible. In addition, the lack of competence of immature embryos in certain elite lines is still a barrier to routine production of transgenic commercial genotypes. Therefore, alternative explants and regeneration systems are needed for efficient transformation of cereal crops to avoid or reduce the above limitations.

The selection of a promising explant, genotype and the development of a reliable regeneration system with an intervening callus phase would certainly augment the genetic transformation of wheat (Mahmood *et al.*, 2012c). Explants such as the shoot apex (Aguado-Santacruz *et al.*, 2011), mature seed (Shah *et al.*, 2003), isolated mature embryos (Vendruscolo *et al.*, 2008; Gill *et al.*, 2014), endosperm supported (ES) mature embryo (Birsin & Ozgen, 2004; Aydin *et al.*, 2011) and coleoptiles (Benkirane *et al.*, 2000) are deployed to study tissue culture responses of wheat, and are found more convenient and easily available throughout the year. However, comparative studies regarding callusing and regeneration potential of these explants taken from the same plants of various genotypes in response to various combinations of growth regulators are limited.

The present study is an attempt to test the hypothesis that tissue culture responses of wheat explants taken from the same plant differ from each other and is dependent on the genotype and media composition. In addition, callusing and regeneration potential of various explants (mature embryo, immature embryo, ES mature embryo and shoot epical meristem) of wheat genotypes was explored, and a reliable callus induction and regeneration protocol for each explant was established with the future aim of exploitation of somaclonal variation, and biolistic and agrobacterium-mediated genetic transformation for breeding biotic and abiotic stress tolerant plants of wheat.

METHODOLOGY

Plant material

Preliminary studies conducted have shown that wheat genotypes GA-2002 and AS-2002 have the highest

tissue culture responses among seven genotypes tested (Mahmood *et al.*, 2012c). Therefore, genotypes GA-2002 and AS-2002 were selected for the present study. Tissue culture responses of various explants, *viz.*, immature embryo, mature embryo, ES mature embryo and shoot apical meristem, taken from the same plants of wheat genotypes AS-2002 and GA-2002 were tested, and the callus induction and regeneration media were standardised for each explant.

Sterilisation

The explants were surface sterilised aseptically with 90 % ethanol for 5 min followed by rinsing with 3 changes of sterile distilled water. The explants were again sterilised with a 6.5 % solution of sodium hypochlorite containing 0.1 % Tween-20 for 30 min. Finally, the explants were thoroughly washed with 4 changes of sterile distilled water.

Explants preparation for callus induction

The seeds of both genotypes were sown in earthenware pots and explants were collected as explained below.

Immature embryo

The seeds of genotypes were raised in pots and immature caryopses at the milk ripe to soft dough stages (about 15 – 18 ds post-anthesis) were collected from the middle portion of the spikes. The spikes were not detached from the plants. Immature embryos were aseptically excised from caryopses and placed on induction medium with the scutellum facing up. The remaining spikes/caryopses of the plants from which immature embryos were collected, were raised to maturity and the seeds were collected for mature embryo, ES mature embryo and shoot epical meristem explants.

Mature embryo

The mature caryopses were surface sterilised and immersed overnight in sterile distilled water. The embryos were excised and placed on induction medium facing the scutellum side up.

Endosperm supported mature embryo

After surface sterilisation, the mature seeds were immersed in sterile distilled water for 4 h at 30 °C. Mature embryos of the immersed seeds were aseptically removed slightly (not set free from endosperm) with a scalpel. The seeds with removed embryos were placed furrow downwards in sterile Petri dishes containing induction medium.

Shoot apical meristem

The sterilised seeds were soaked for germination. Before emergence of coleoptiles apical meristems (3 mm sections of shoot apices) were dissected. Vertical cross slits were made at the base of each dissected apex to enhance callus induction. The explants were placed horizontally on induction medium and incubated.

Callus induction

MS basal media (Murashige & Skoog, 1962) supplemented with 30 g/L sucrose, 6 g/L agar and various concentrations of 2,4-D (2, 4, 6 or 8 mg/L) were used to induce callus from various explants of wheat. Prior to autoclaving at 121 °C for 20 min, pH of the medium was adjusted to 5.8. The explants were incubated in total darkness at 25 ± 1 °C for 21 ds, replacing the media after each week. The callus induction potential of explants of both genotypes were calculated on percentage basis.

Regeneration

The regeneration potential of the explants (immature embryo, mature embryo, ES mature embryo and shoot apical meristem) with an intervening callus phase was evaluated in this step. Fresh calli were induced from each explant using their respective best suited callus induction medium (MS basal medium + 30 g/L sucrose + 6 g/L agar and best suited concentration of 2,4-D for callus induction for the respective explants of each genotype), as determined in the previous step of this study. The calli were cultured on fresh maintenance medium (half of the best suited concentration of 2, 4-D for callus induction of the respective genotype) for a further period of 3 wks, replacing the callus maintenance medium after every 14 – 21 days. Morphogenic calli (nodular and solid calli with white to pale yellow surface) showing high potential were then shifted on to regeneration media (MS basal medium + 30 g/L sucrose + 6 g/L agar + various combinations of phytohormones). The calli were incubated at 26 °C with 16 h light and 8 h dark photoperiod. After 25 – 30 ds, calli with clearly differentiated shoots were scored as regenerating calli. Each piece of regenerating callus was counted as one regardless of the number of shoots and roots. The various regeneration protocols tested are given in Table 1.

Experimental design

The experiment was laid out following a completely randomised design (CRD) with factorial arrangements, and replicated thrice. The data collected were analysed

using analysis of variance (ANOVA) and least significant difference (LSD) ($\alpha = 0.05$) using Statistics 8.1.1.0 software.

Table 1: Treatments/regeneration protocols tested in the study (combinations of various concentrations of phytohormones)

Media	2,4-D (mg/L)	Zeatin (mg/L)	BAP (mg/L)
M ₁	0.0	1.0	0.0
M ₂	0.2	0.5	0.5
M ₃	0.4	1.0	1.0
M ₄	0.6	1.5	1.5

RESULTS AND DISCUSSION

The callusing and regeneration potential of various explants (mature embryo, immature embryo, ES mature embryo and shoot apical meristem) of the two wheat genotypes, viz. GA-2002 and AS-2002 were investigated by using various callus induction and regeneration protocols. The callogenesis response of various explants of cv. GA-2002 and AS-2002 varied significantly between 24.92 to 84.75 % (Table 2). The callogenesis response of both immature and mature embryos were significantly the highest compared to the rest of the explants with mean callus induction percentages of 80.21 and 79.86 %, respectively (Table 2). However, *in vitro* culture response of the explants was influenced by the genotypes and media composition. Immature embryo culture of genotype AS-2002 exhibited the highest callusing ability (84.75 %) followed by the mature embryo culture of genotype GA-2002 (78.50 %). Similarly, the immature embryo cultured on MS basal medium supplemented with 4 mg/L 2,4-D and the mature embryo cultured on MS basal medium supplemented with

Table 2: Callogenesis response of various explants of genotype GA-2002 and AS-2002

Explants	Callogenesis percentage (%)		
	GA-2002	AS-2002	Mean
Immature embryo	75.67 ^{CD}	84.75 ^A	80.21 ^A
Mature embryo	78.50 ^{BC}	81.25 ^B	79.86 ^A
ES mature embryo	69.17 ^E	73.33 ^D	71.25 ^B
Shoot apical meristem	24.92 ^G	30.17 ^F	27.54 ^C
Mean	62.06 ^B	67.38 ^A	

LSD: genotype = 1.621; explants = 2.292; genotype × explants = 3.242
Entries sharing common letters do not differ significantly ($\alpha = 0.05$)

6 mg/L 2,4-D exhibited the highest callusing abilities of 90.83 and 88.33 %, respectively. On the contrary, the least callus frequency (10.83 %) was observed for shoot epical meristems with 2 mg/L of 2,4-D (Table 3).

In general, genotype AS-2002 exhibited a significantly higher callus induction percentage than cv. GA-2002 with respective callus induction percentages of 67.38 and 62.06 % (Table 2).

Table 3: Callogenesis response of explants of wheat genotypes to various concentrations of auxins (2,4-D)

Explants	Callogenesis percentage (%)			
	2 mg/L	4 mg/L	6 mg/L	8 mg/L
Immature embryo	78.33 ^{DE}	90.83 ^A	84.33 ^{BC}	67.33 ^G
Mature embryo	73.33 ^F	83.83 ^{BC}	88.33 ^{AB}	74.00 ^{EF}
ES mature embryo	55.83 ^H	72.83 ^F	80.33 ^{CD}	76.00 ^{DEF}
Shoot epical meristem	10.83 ^K	27.83 ^J	35.50 ^I	36.00 ^I
Mean	54.58 ^D	68.83 ^B	72.12 ^A	63.33 ^C

LSD: explants × 2,4-D = 4.585

Entries sharing common letters do not differ significantly ($\alpha = 0.05$)

Non-significant differences were recorded in the regeneration potential of immature embryo and ES mature embryo culture with respective mean regeneration potentials of 45.54 and 46.33 % (Table 4). However, the regeneration potential of the explants was genotype and media specific (Figure 1). Immature embryo of the genotype GA-2002 cultured on MS based regeneration medium supplemented with 0.2 mg/L 2,4-D, 0.5 mg/L zeatin and 0.5 mg/L benzylaminopurine (BAP) showed significantly the highest regenerating potential (60.0 %). ES mature embryos of genotypes GA-2002 and AS-2002 cultured on regeneration medium comprising 0.4 mg/L 2,4-D, 1.0 mg/L zeatin and 1.0 mg/L BAP showed the maximum regeneration potentials of 59.67 and 59.0 %,

Table 4: Regeneration response of various explants of genotype GA-2002 and AS-2002

Explant	Regeneration response (%)		
	GA-2002	AS-2002	Mean
Immature embryo	48.33 ^A	42.75 ^C	45.54 ^A
Mature embryo	42.17 ^{CD}	40.25 ^D	41.21 ^B
ES mature embryo	45.42 ^B	47.25 ^{AB}	46.33 ^A
Shoot epical meristem	27.75 ^E	28.42 ^E	28.08 ^C
Mean	40.92 ^A	39.67 ^B	

LSD: genotype × explants = 2.140

Entries sharing common letters do not differ significantly ($\alpha = 0.05$)

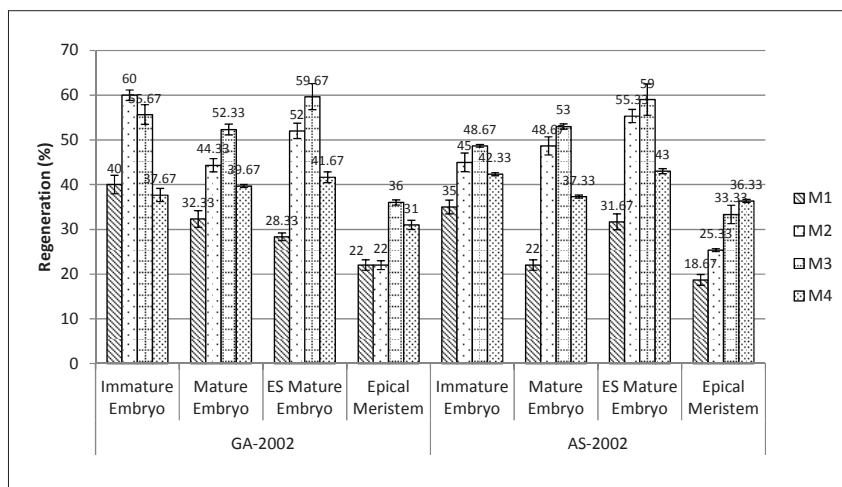


Figure 1: Regeneration response of various explants of genotype GA-2002 and AS-2006 to various regeneration media

respectively. The least regeneration potential (18.67 %) was observed for shoot epical meristems of genotype AS-2002 (Figure 1).

Genotype GA-2002 exhibited a significantly higher regeneration potential (mean 40.92 %) than genotype AS-2002 (mean 39.67 %; Table 4). In general, regeneration medium M₃ (0.4 mg/L of 2,4-D, 1.0 mg/L zeatin and 1.0 mg/L BAP) was found to be superior for regeneration of both wheat genotypes followed by M₂ (0.2 mg/L of 2,4-D, 0.5 mg/L zeatin and 0.5 mg/L BAP), with mean regeneration responses of 49.71 and 44.08 %, respectively (Table 5).

Table 5: Regeneration response of wheat genotypes to various regeneration media

Regeneration media	Regeneration response (%)		
	GA-2002	AS-2002	Mean
M ₁	30.67 ^F	26.83 ^G	28.75 ^D
M ₂	44.58 ^C	43.58 ^C	44.08 ^B
M ₃	48.50 ^B	50.92 ^A	49.71 ^A
M ₄	37.50 ^E	39.75 ^D	38.62 ^C

LSD values: genotype × regeneration media = 2.140

Entries sharing common letters do not differ significantly ($\alpha = 0.05$)

In contrast to other monocotyledons, not all wheat genotypes are responsive to tissue culture procedures due to lack of secondary growth through cambium tissues (Shah *et al.*, 2009). The regeneration ability of explants is under the influence of at least two complementary dominant genes; or different genes or combinations of genes may govern the different stages from callus induction to plantlet regeneration (Jia *et al.*, 2009). Therefore, genotypes and explants vary in their ability to regenerate whole plants. Also, varying tissue culture responses of the explants and genotypes are associated with the cytoplasmic composition of the cells (Ma *et al.*, 1987). In addition, variable tissue culture responses of wheat genotypes are mainly due to the genotypic and media interaction. Conditions conducive for tissue culture responses of one genotype are usually not conducive for another genotype of the same species (Mahmood *et al.*, 2012c). Due to this reason genotype AS-2002 and GA-2002 varied for tissue culture responses. Some reports have suggested that there are no notable variations in the callus induction and regeneration potential of various genotypes of wheat (Sarker & Biswas, 2002).

Auxins play a vital role in gene activation involved in cell division and dedifferentiation (Dudits *et al.*, 1991). Also, cell division, callus proliferation, regeneration and recovery of transformed plants are mainly determined by the type and concentration of auxins (Barro *et al.*, 1999). Various phytohormones including indole-3-acetic acid (IAA), BAP and kinetin (Kn) are being used alone or in combinations for the regeneration of various explants with an intervening callus phase (Noor *et al.*, 2009; Mahmood *et al.*, 2012c). However, the interactions of phytohormones like auxins and cytokinins are complex and more than one combination of two substances or changes in their relative proportion is likely to produce optimum results (Mahmood *et al.*, 2012c), and they influence rate and growth patterns of the explants. Similarly, auxins are critical for callus induction and have a negative role in explant regeneration, and are therefore reduced or excluded from regeneration medium. Hence, the developed callus can be enforced to form shoots by decreasing the auxin or increasing the cytokinin concentrations in culture media (Mahmood *et al.*, 2012c).

Unlike callogenesis from immature embryos at lower concentrations of auxins, a relatively higher concentration of auxins (2,4-D) was required to induce calli from mature embryos, ES mature embryos and epical meristem (Table 3), and this fact is supported by Fazeli-Nasab *et al.* (2012) and Gill *et al.* (2014). Mature embryos contain more old cells and more differentiated tissues than immature embryos, which necessitate the use of a high concentration of auxins for dedifferentiation. In the case of ES mature embryo culture, the endosperm can absorb some proportion of the added plant growth regulators, which requires a higher concentration of auxins (Aydin *et al.*, 2011). Profuse callus induction from immature embryo and ES mature embryo is reported by the use of 2 mg/L and 8 mg/L of 2,4-D, respectively (Özgen *et al.*, 1998). Generally, 5.5 – 6.0 mg/L of 2,4-D is considered optimum for callogenesis of various explants including immature embryos, mature embryos, seeds, endosperm, root tips and shoot bases (Sarker & Biswas, 2002).

An optimum concentration of auxins and cytokinins results in maximum regeneration of explants (Mahmood *et al.*, 2012c). For example, regeneration medium supplemented with 0.2 mg/L IAA, 0.5 mg/L Kn and 0.5 mg/L of BAP was proved excellent for obtaining maximum regeneration of callus induced from immature embryos (Mahmood *et al.*, 2012c). Similarly, for shoot regeneration from immature embryonic calli of wheat

0.5 mg/L BAP, 0.5 mg/L Kn and 25 mg/L tyrosine have shown the best response. On the contrary, 0.5 mg/L BAP, 0.5 mg/L IAA and 40 mg/L tyrosine have been reported best for shoot regeneration of mature embryonic calli of wheat (Sarker & Biswas, 2002).

In cereal tissue culture, embryogenic callus formation and regeneration potential besides genotype and media composition (Mahmood *et al.*, 2012c), are also dependent on the explant source because not all cells are totipotent (Shah *et al.*, 2003). Various explants taken even from the same plant of a genotype differ in their regeneration potential (Sarker & Biswas, 2002). Therefore, the choice of a favourable explant is inevitable for the success of tissue culture based transformation of cereals. Most of the studies and reviews have shown that immature embryos are the best explants in terms of regeneration (Yang *et al.*, 2015). The results reported in the present study also indicated that explants of both genotypes have different callusing abilities (Table 2), and that the callogenesis response of explants is genotype and media dependent (Table 3). Yang *et al.* (2015) have also reported significant interaction effects between genotypes and explant types, and revealed that different explants of various wheat lines have different callusing potentials. However, calli derived from immature embryos had higher differentiation and regeneration potentials than those from mature embryos (Yang *et al.*, 2015). Similarly in wheat, the callus induction percentage of mature embryos is significantly higher than ES mature embryos cultured in the absence of endosperm (non-endosperm supported culture), regardless of the 2,4-D concentration (2 or 8 mg/L 2,4-D) used in induction medium (Chen *et al.*, 2006). However, endosperm supported calli had a higher regeneration potential than the non-endosperm supported ones mainly because of higher oxalate oxidase activity (Chen *et al.*, 2006).

The results presented here depicted that immature embryo and ES mature embryo had no significant differences in regeneration potential under the tested conditions (Figure 1), and ES mature embryo can be a valuable explant for genetic transformation of wheat.

CONCLUSION

In vitro culture response of explants taken from the same plant of wheat genotypes differs from each other, and is influenced by the genotype and media composition. Immature and mature embryos had the highest callusing ability (80.21 and 79.86 %, respectively) than the rest of the explants. However, ES mature embryo explants and immature embryo explants had non significant differences in regeneration potential, with regeneration

potentials of 46.33 and 45.54 %, respectively, showing that ES mature embryo explants are an alternative to immature embryo explants of wheat, which is available throughout the year unlike immature embryos. The wheat genotypes vary in their tissue culture responses with the maximum regeneration potential in GA-2002 (40.92 %).

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