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Callus induction and plant regeneration from mature embryos of some Iranian wheat (*Triticum aestivum* L.) genotypes

Shahnam Azizi Dargahlou*, Ebrahim Dorani Uliaie, Ali Bandehagh

Department of Plant Breeding and Biotechnology, Faculty of Agriculture, University of Tabriz, Tabriz, Iran

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Abstract

Wheat is one of the most widely planted crop across the world. A simple, efficient and credible *in-vitro* culture is necessary for genetic improvement of wheat. In this study we evaluated the effect of genotype and different combination and concentration of PGRs and sugar content as carbon source on callus induction and plant regeneration from mature embryos. To do so, the embryos were transferred to MS medium supplemented with 2 mg/L 2,4-D for callus induction; the calli were then transferred to basal MS medium without PGRs for plant regeneration. Analysis of variance showed significant difference ($P<0.01$) among the genotype son callus induction, callus weight, and shoot regeneration. Callus induction were observed in all genotypes and percentage of callus induction ranged from 60 to 100%. The highest percentage of shoot regeneration obtained in the Aflak (%26) in PGRs-free basal MS medium. Medium containing BAP in combination with a week auxin including IAA and IBA, showed high regeneration capacity compared to media supplemented with BAP (0.5 and 2mg/L). Medium supplemented with 2mg/L BAP along with 0.5mg/L IAA showed the highest percentage of shoot regeneration (37.5%). Using 15 and 30g/L sugars in PGRs-free 1/2 MS basal medium did not show significant influence on shoot regeneration whereas these media displayed better shoot regeneration in compare to medium containing 0.5mg/L BAP. Aseptic culture is an indispensable basis for genetic engineering of plants therefore results of this study can be used in genetic transformation of wheat to improve agronomic traits.

***Corresponding Author:** Shahnam Azizi Dargahlou ✉ shahnambio@gmail.com

Introduction

Wheat is one of the most critical crops in the world. More than 120 countries in the world produce wheat, which to meet one-fifth the need for the world's calorie (Donald *et al.,* 2005). In recent years, production of wheat has declined because of climate changes, intensifying various biotic and abiotic stresses (Joshi *et al.*, 2007).

Genetic engineering leading to genetic transformation of crops is considered as an effective method to improve plants to be tolerant or resistant against different challenges arising from various biotic and abiotic stresses. Genetic engineering of wheat is dependent on efficient and reliable systems of callus induction and plant regeneration. Callus induction and regeneration capacity of wheat are influenced by genotype (Vendruscolo *et al.,* 2008), explant source (Redway *et al.*, 1990), geographical origin (Hess and Carman, 1998), physiological status of the donor plants, the culture medium and the interactions between them (Ren *et al.*, 2010). Various explants including leaf segments (Yu *et al.*, 2012), meristematic shoot segments (Sharma *et al.*, 2004), coleoptiles (Benkirane *et al.*, 2000), endosperm supported embryos (Özgen *et al.*, 1998), mature embryos (Yu and Wei, 2008), thin mature embryo fragments (Delporte *et al.*, 2001) and immature embryos (Jia *et al.,* 2009) have been used in wheat tissue culture. The highest rates of callus induction and plant regeneration of wheat have been obtained using immature embryo as an explant (Sarker and Biswas, 2002). Strong potential of immature embryos in response to *in-vitro* culture has made it a widely used exultantly researchers. But some limiting factors are impeding use of immature embryos such the need to grow donor plants for year-round to supply continuously samples, requiring greenhouse space as well as demanding extra labor works and cost. Finding the most suitable developmental stage of immature embryos for successful culture is another limiting factor (Jun ying *et al.*, 2006). Mature embryos are potentially useful alternative to immature embryos because they can be stored in the form of dried seeds, isolated easily, available throughout the year (Ding *et al.*, 2009) and come with minimal variability in physiological conditions (Yu *et al.*, 2008). When mature embryos are used as explant source, frequency of plant regeneration is low as compared to immature embryo culture (Jun-ying *et al.*, 2006).

Success in plant tissue culture of wheat depends largely upon the average effect of several important factors including explant tissue, culture medium, plant genotypes, and their interaction (Khan *et al.*, 2015). Accordingly, the first objective of this study was to screen Iranian wheat cultivars for callus induction and plant regeneration by using mature embryos as explant source to introduce some promising genotypes for subsequent works. The second goal was to improve shoot capacity in once of promising genotypes (Kaskogen) by using different PGRs and different amount of carbon source (sugar).

Materials and methods

Plant materials

Seeds of 15 cultivars of wheat (*Triticum aestivum* L.) (Table 1), were provided from the Seed and Plant Improvement Institute, Karaj, Iran. Mature embryos of these cultivars used as explant source.

First experiment

In order to screen 15 genotypes of wheat in response to callus induction and plant regeneration, mature seeds were first surface-sterilized in 5% commercial bleach, then imbibed in sterile distilled water for overnight at room temperature. Before isolation of mature embryos, imbibed seeds were sterilized with 70% (vol/vol) ethanol for 90S and 5% commercial bleach for 5min. In each sterilization step, we used hand-shaking and three time washing with sterile distilled water. For callus induction, mature embryos were aseptically slightly removed with a scalpel. The embryos with scutellum upwards were placed onto sterile 10cm petri dishes containing MS (Murashige and Skoog, 1962) medium supplemented with 2mg/L 2,4-D. The pH of the media was adjusted to 5.7 and autoclaved for 15min at 121°C under 1.1kg/cm² pressure.

The cultures were incubated in total darkness at 25±1°C for six weeks. For plant regeneration, calli were transferred to PGRs-free MS medium containing MS mineral salts, sugar (30g/L) and agar (7g/L) in plates. The plates were incubated at 25±1°C with 16-8h photoperiod (1500lux). The number of calli with shoot regeneration were counted after five weeks. Each petridish with 8explants was considered as a repeat.

Second experiment

At the end of the first experiment, once of promising genotype (kaskogen) was selected for subsequent work. In this stage, we tested effects of two sugar amounts (15 and 30g/L) as carbon source, and different types and concentrations of PGRs (Fig. 4) to improve regeneration capacity; one 12 cm petridish with six callus was considered as a repeat. After five weeks the number of shoot regenerating calli were counted for all treatments.

Both experiments were done in a completely randomized design with three replicates per treatments. Mean of treatments were compared using Duncan's Multiple Range Test.

Table 1. Mean comparison of 15 Iranian wheat cultivars for callus induction from mature embryos, on MS medium supplemented with 2.0mg/L 2,4-D and their response to shoot regeneration on PGRs-free basal MS medium.

Origin cultivars	Callus weight (mg)	Callus induction rate	Shoot regeneration rate
Aflak	42.46 g	66 de	26 + a*
Kaskogen	92 b	93.33 ab	21.66 ab
Sardari	58.1 cdef	95.66 ab	17.33 abc
Sivand	55.83 cdefg	68.66 de	12 abc
Shahpasand	141.46 a*	100 a	11.33 abc
Gonbad	54.1 defg	100 a	10.66 abc
Parsi	43.06 g	76.66 cd	10 abc
Gaspar	43.03 g	100 a	5.33 bc
Pishgam	45 fg	100 a	5.33 bc
Sepahan	47.83 efg	85.66 bc	5.33 bc
Chamran	67.26 c	60 e	4.66 bc
Pishtaz	45.13 fg	100 a	4/66 bc
Almot	45.5 fg	89.33 ab	0 c
Sayson	61.8 cd	95.66 ab	0 c
Karaj3	59.83 cde	91.66 ab	0 c

*Means followed by the same letter are not statistically significant (p<0.01).

Results

Callus induction

Response of genotypes to callus induction medium were greatly difference; there was a significant difference between genotypes for callus fresh weight and callus induction at 1% probability (Table 1). Among the genotypes, Shahpasand and Aflak showed the highest (141.46mg) and lowest (42.46mg) callus fresh weight, respectively. Speed of callus proliferation was very different among the cultivars according to their different weights (Fig. 1.), whereas initiation of callus induction among the genotypes proved the same and observed in all genotypes after 2-4 days. In terms of callus morphology, in the initiation stage of the callus induction, many cultivars showed soft, juicy and scaly callus that later became amorphous.

Among the genotypes, Shahpasand, Pishtaz, Gonbad, Pishgam and Gaspar showed 100% callus induction rate and Chamran with 60% callus induction was the lowest compared to others. Among the genotypes only Shahpasand exhibited germination in callus induction medium (Fig.1.) which indicating it's resistant to low concentration of 2, 4-D.

Shoot regeneration

Later, calli were transferred to regeneration medium (MS without PGRs) (Aydin *et al.*, 2011).

Response of cultivars to regeneration were so different (Table 1., Fig. 2.). Calli of most cultivars demonstrated greening along with more proliferation in regeneration medium.

Some cultivars produced adventitious roots and a few cultivars showed shoot regeneration (Fig. 2.). Greening of callus was an adverse indicator of shoot regeneration; so Aflak with lowest callus greening showed maximum shoot regeneration.

Percentage of shoot regeneration were between 0 to 26%. Aflak showed highest percentage of shoot regeneration (26%) and Almot, Karaj3 and Sayson didn't show regenerated callus.

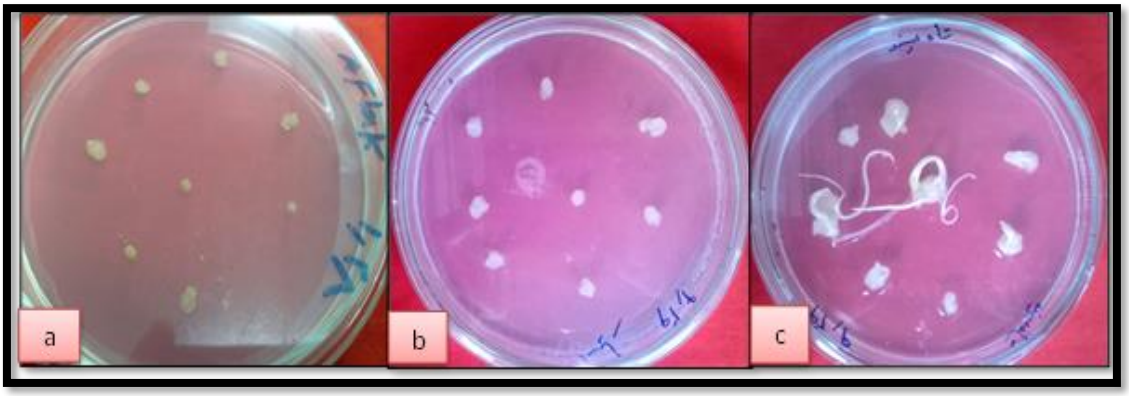

Fig. 1. Different responses of wheat genotypes in terms of callus induction rate and size of callus after six weeks on callus induction medium a: Aflak b: Gaspar c: Shahpasand.

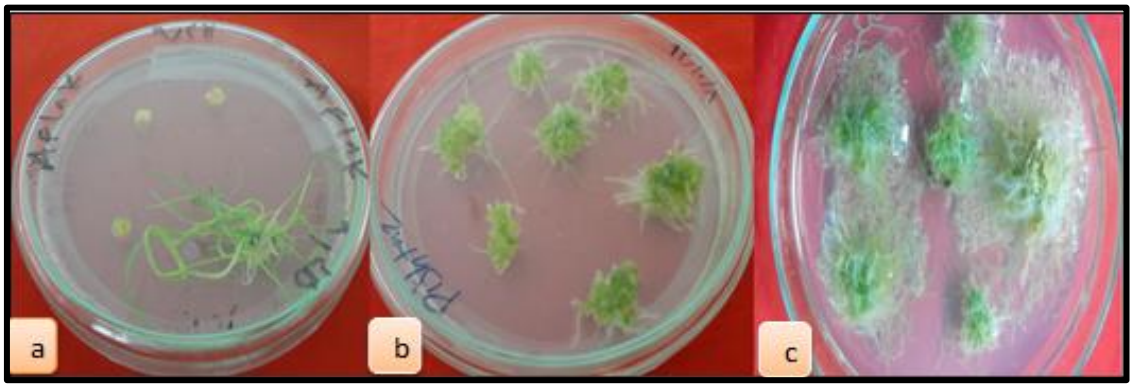

Fig. 2. Different response of wheat genotypes in regeneration medium a: lees greening with highest shoot regeneration b: greening along with proliferation c: produces more adventitious roots.

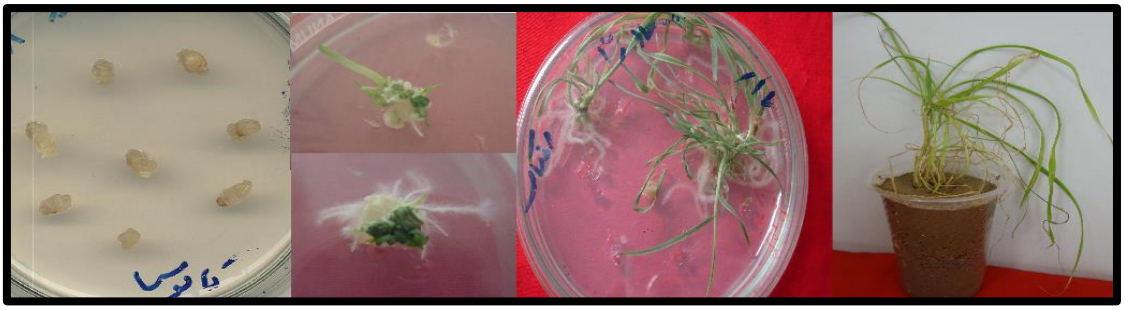

Fig. 3. Different stages in indirect regeneration of whole plant from wheat mature embryos in aseptic culture.

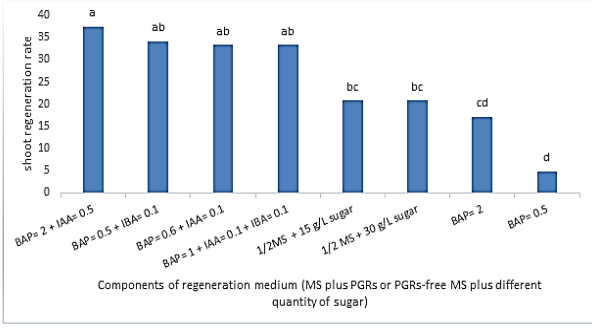

Fig. 4. Effects of different amounts of sugar and PGRs on efficiency of plant shoot regeneration in Kaskogen.

*Means followed by the same letter are not significantly different at the 0.01 probabil-ity level.

Effect of PGRs and sugar for improving shoot regeneration capacity in Kskogen

We found that significant difference between PGRs concentration regarding to shoot regeneration (Table 2). Combination of a weak auxin such as IAA and IBA with BAP significantly increased shoot regeneration in Kaskogen. Medium supplemented with 2mg/L BAP in combination with 0.5mg/L IAA and medium containing 0.5mg/L BAP showed the highest (37.5%) and lowest (4.76%) shoot regeneration, respectively. The effect of sugar in media lacking PGRs for shoot regeneration was evaluated. Adding 30g/L and 15g/L sugars as carbon source to 1/2 MS medium showed no significant effect on shoot regeneration.

Discussion

First experiment: Effect of genotype on callus induction and plant regeneration

We observed a significant effect in callus fresh weight and callus induction rate at the 1% probability existed among the wheat genotypes. 2,4-D is the most common used of auxin in plant tissue culture (Slater *et al*, 2008) and the most widely utilized auxin in wheat cultures (Sharma *et al*, 1995). In the recent study we used 2mg/L 2,4-D in callus induction medium and, in this medium, mature embryos of all genotypes initiated callus induction after 2-4 day. This observation was in consistent with previous results (Mendoza and Keappler, 2002; Yu *et al.*, 2008) where initiation of callus occurred after 3 days.

In recent study, we discovered that a crucial difference existed between the 15 genotypes of bread wheat in callus induction rate. This suggests that the induction of callus from mature embryo of wheat is under the genetically control. Callus induction rate rangedfrom60 to 100%, 88.17% on average. This results approximately equal with Chauhan and colleagues study (2007), those who claimed achievement of 82-85% and 77-79% percentage of callus induction in *T. aestivum* and *T. durum,* respectively, using 2mg/L 2,4-D. Gul-Nasicilar *et al.* (2006) applied 2mg/L 2,4-D for callus induction from mature embryo of *Triticum aestivum and* reported various Callus induction frequencies from 25.67% to 53% after 2 weeks. The callus induction frequency was recorded between 11.6 to 89.6% in a medium containing 8mg/L 2,4-D in various genotypes of wheat (Chen *et al*, 2006); however, these results were not robust compared to the results of current study.

If one researcher determined to used 2,4-D for callus induction we suggested 2mg/L 2,4-D for these reasons, here, using 2mg/L 2,4-D showed 88.17% callus induction on average which is efficient result, high levels of growth regulators such as 2,4-D has been implicated in somaclonal variation in different species of plants including cereal crops (Murata, 1989; Ziauddin and Kasha, 1990) as well as high concentration of this hormone would be decline the regeneration capacity. Also we recommend pretreatment of mature seeds in imbibed duration time with auxins such as 2, 4-D would be increase the response to *in vitro* culture by prevent the initiate of regeneration phase. In terms of callus weight,15 Genotypes of wheat showed significant difference and Shahpasand with 141.46 mg ranked best genotype for this trait. Producing high amount of callus is desirable in experiment considering cell suspension and investigation of soma clonal variation.

For shoot regeneration from 15 genotypes of wheat, we used PGRs-free MS medium. In this medium the value of shoot regeneration rate was observed between 0 to 26%. Delporte *et al.* (2001) reported an average of 11% of regeneration capacity in their study.

Different response to shoot regeneration in PGRs-free MS medium is the indication of different concentration and combination of endogenous PGRs present in different genotypes of wheat. Mathias and Simpson (1986) reported that genotype is important factor in wheat regeneration in comparison to culture medium. Mitic and co-workers (2006) analysis the effect of genotype and environmental condition on 96 genotypes of wheat in response to *in vitro* culture and announced genotype is the most important for determining regeneration potential in wheat.

Different response of 15 types of wheat genotypes to shoot regeneration, listed in first table and superior natural genotypes for this trait can be used in genetic engineering of wheat. In wheat, similar to other plants, it is assumed that different responses to tissue culture to be controlled by interaction of large number of genes (polygenes) (Varshney and Altpeter, 2001).

With this information on hand, screening wheat genotypes in response to tissue culture is one of the methods employed to find out top genotypes of wheat for regeneration capacity.

Second experiment: Improvement of shoot regeneration capacity in Kaskogen

We applied different concentration of BAB, IAA, IBA and two amounts of sugar (15 and 30g/L) for improvement of shoot regeneration capacity in Kaskogen. We found out that applyingweak auxin in combination with cytokines increases shoot regeneration capacity in Kaskogen. highest shoot regeneration rate (37.5%) was achieved in medium containing 2mg/L BAP in combination with 0.5mg/L IAA; In agreement with research of Satyvathi *et al.* (2004) which showed, combination of BAP and IAA worked the best as a PGRs for plant regeneration. In the regeneration stage using BA along with low concentrations of IAA (0.5mg/L) significantly increased shoot regeneration rate (Kumar *et al.*, 2015). A range of auxins in combination with cytokines have an essential role in multiple shoot regeneration in many tree species (Anis *et al.*, 2012).

Moreover, here, media without PGRs showed efficient shoot regeneration in compared to medium containing 0.5 BAP (Table 2). Aydin *et al.* (2011) and Mendoza and Kaeppler (2002) used MS medium containing 0.5mg/L BAP for plant regeneration. Almost all aspects of plant development are regulated by plant hormones which may act individually or in a related manner. Shoot regeneration is crucially important in realization of potential cell and tissue culture techniques for plant improvement. Auxins which are essential for callus induction, play a negative role in plant regeneration and are generally reduced or excluded from culture media used for shoot regeneration (Purnhauser *et al,* 1987).

With using two different quantities (15 and 30g/L) of sugar as carbon source in regeneration medium we not found significant effect on regeneration capacity of Kaskogen. Li *et al* (2006) reported that, sucrose concentration of medium ranging from 3% to 9% had no significant effects on callus induction of mature embryos of wheat. Variety of carbon sources (reducing or non-reducing) are used in culture media depending on genotypes and specific stages of growth. Addition of sugar to the culture media helps in the maintenance of osmotic potential of cells and conservation of water (Hazarika, 2003).

Therefore compatible quantity and type of carbon source would be improved *in vitro* response. Here we detected regeneration capacity of wheat is not so sensitive to amount of sugar and we can use 15g/L sugar instead of 30g/L in regeneration medium.

Conclusion

İn this study we indicated that callus induction in wheat is not a so hard process compared to regeneration phase as the highest of callus induction and shoot regeneration rate among the 15 genotypes of wheat were 100 and 26.0%, respectively, which showed a significant gap between these two stages. We did not distinguish any relationship between callus induction and plant regeneration stage as Aflak with lowest callus induction rate showed highest shoot regeneration rate. It is implicated that callus induction and plant regeneration may be controlled independently from each other (Sears and Deckard, 1982).

Finally, genotype has a crucial effect on callus induction and plant regeneration and priority of genotypes attributed to these characters illustrated in first table. In testing of best PGRs for plant regeneration, mediums which containing BAP in combination with weak auxin showed high capacity for plant regeneration in comparison to using BAP alone. These achievements can be used in genetic improvement of wheat by non-classical methods (Gene gun, Agro bacterium mediated transformation and etc.).

Abbreviations

PGRs (plant growth regulators),
2,4-D (2,4-Dichlorophenoxyacetic acid),
IAA (Indole-3-acetic acid),
IBA (Indole-3-butyric acid),
BA (6-Benzyladenine).

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