International Journal of Advanced Biotechnology and Research (IJBR)
ISSN 0976-2612, Online ISSN 2278–599X,
Vol-7, Issue-3, 2016, pp1134-1141
http://www.bipublication.com

Research Article

Comparison Study On *In Vitro* morphogenesis of Mature and Immature Wheat (*Triticum aestivum* L.) Embryos

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[Received-05/09/2016, Accepted-12/09/2016, Published-27/09/2016]

ABSTRACT

The present study comparedthe *in vitro* performance of two stages of wheat embryos;mature and immature embryos explantswere cultured in MS medium supplemented with different concentrations of 2,4-Dfor callus induction,mature embryosgenerally showed ostensibly preference to the immature embryo and achieved the highest fresh weight value (62.0±0.01a) at 2.0 mg/l 2,4-D. Calluses derived were transferred to regeneration mediasupplemented with different levels of activated charcoal, sucrose and silver nitrate. Mature embryo derived calluses achieved the highestpercentages (50-100%) of nodular callus at all types of regeneration mediatested compared to immature embryo derived callusespercentages (8-66%).Shootregenerationfrequencies obtained were better in the mature embryo derived callus cultures, where it achieved 25% regeneration percentage at 3.0mg/l activated charcoal;16% regeneration percentage at 60.0 g/l sucrose and 25% regeneration percentage at 10.0 ml/l silver nitrate, while immature embryo derived callus cultures achieved 8% regeneration percentage at all activated charcoal concentrations, failed to regenerate shoots in sucrose concentrations and the highest regeneration percentage obtained was 8% at silver nitrate treatments.

The present study showed the mature embryo culturespreference to immature embryo culturesfor callus induction, embryogenesis and plantlet regeneration in Elnileen wheat cultivar.

Keywords: Wheat, embryo culture, embryogenesis, regeneration, ethylene inhibitor, osmolarity

INTRODUCTION

Regeneration is one of the critical steps of plant transformation from *in vitro* culture and is a key point in biotechnological improvement of wheat (Keresa*et al*., 2001). Comparatively; monocotyledons are regarded as difficult *in vitro* material particularly in Gramineae family (Sears and Deckard, 1982), as a member of the family, wheat is also a recalcitrant crop that limits the utilization of tissue culture technique for crop improvement (Vasil and Vasil, 1986).

Relatively few tissue culture reports are available on mature or immature zygotic embryos of triticale when compared to other cereals, it has therefore been necessary to optimize conditions for regeneration prior to attempting genetic transformation and selection of favorable somaclonal variant strains (Atak*et al*, 2008).A variety of explants have been used for wheat callus culture, such as immature embryos (Machii*et al*., 1998), immature leaves (Wang and

Wei, 2004), anthers (Lazar *et al*., 1990), immature inflorescence (Sharma *et al*., 1995) and mature embryos (Delporte*et al*., 2001; Yu *et al*., 2008).

Optimize conditions for the development of high frequency plant regeneration systems is an important step towards application of tissue culture to plant breeding and somaclonal variations can be exploited in certain breeding programmes (Malik *et al*., 2004).

In vitro regeneration of wheat is possible from different explants such as mature and immature embryos, seeds, endosperm, leaves, shoot bases and root tips (Sarker and Biswas, 2002). Most previous investigations have used immature embryos and microspores as well as immature inflorescences as explants with high totipotency in all major cereals including wheat (Sahrawat*et al*., 2003). A number of workers have reported the regeneration of wheat plants from callus culture derived from various plant parts but the frequency of green plant regeneration was very low (Ahmed *et al*., 2002; Ayse and Kenanturgut, 2006).

The importance of plant growth regulators has been demonstrated for a large number of cereals,especially the levels of synthetic auxins for callus induction and plant regeneration. One of the basic components of a medium influencing somatic embryogenesis of cereals is the type of auxin (Przetakiewicz*et al*., 2003). Commonly 2,4-D considers to be the main phytohormone used for induction of somatic embryogenesis in wheat (Dmitry *et al*., 2009).

The objective of the present study is to comparethe *in vitro* morphogenesis of two stages of wheat embryos using some treatments are known to promote somatic embryo maturation and regeneration such as activated charcoal (Johansson *et al*.,1982), osmotic treatment (Zhou *et al*., 1991), ethylene inhibitor AgNO3 (Purnhauser*et al*., 1987).

METHODOLOGY

The plant material was seeds of Elnileen wheat cultivar whichbroughtfrom the Wheat Research Center, Agricultural Research Corporation, Wad- Madani, Sudan.

Mature embryos sterilization and culture for callus induction

Mature seeds were washed under tap water for ten minutes, and then immersed in distilled water for five hours at room temperature for imbibition, under aseptic condition; seeds were surface sterilized with ethanol 70% for 3 minutes then disinfested with sodium hypochlorite(Clorox) 100% for 20 minutes, rinsed several times in sterile distilled water. The mature embryos were then aseptically isolated from the embedded seeds and placed in MS medium supplemented with different concentrations of 2,4-D (0.0, 1.5, 2.0, 3.0 and 4.0 mg/l), 25 ml medium was dispensed/bottle; then the media were autoclaved at 121º C and 15 psi for 15 minutes.

The embryos cultures were incubated in completely dark condition at 25±2 ºC for a month. Data were recorded for callus fresh weight and analyzed in excel program using ANOVA table.

Immature embryos sterilization and culture for callus induction

Wheat seeds were grown in pots in the plastic house in the winter season; approximately12 days after green spikes appearance, the heads were collected and washed under running tap water for 30 minutes, under aseptic condition heads were rinsed with ethanol (70%) for 3 minutes, then washed in sterile distilled water once,then immersed in sodium hypochlorite(Clorox) 100% for 20 minutes with continuous shaking, and then rinsed several times in sterile distilled water.

The green immature seeds were removed from the spikes and placed in sterile Petri dishes; the immature embryos were aseptically isolated using sterile forceps and scalpels and placed inMS medium supplemented with different concentrations of 2,4-D (0.0, 1.5, 2.0, 3.0 and 4.0 mg/l), 25 ml medium was dispensed/bottle; then autoclaved at 121º C and 15 psi for 15 minutes.

The immature embryos cultures were incubated in completely dark condition at 25±2 ºC for a month. Data were recorded for: number of regenerated plantlets, regeneration percentage and nodular callus percentage.

Embryogenesis and plant regeneration

Calluses derived from mature and immature embryo cultures were transferred to regeneration media for somatic embryos maturationand subsequent plantlet regeneration,MS medium were supplemented with: a)different concentrations (0.0, 0.25, 0.5, 1.0 and 3.0g/l) of activated charcoal, b) different levels (0.0, 15, 30, 60 and 120 g/l) of sucrose, c) different concentrations (0.0, 5.0, 10.0, 15.0 and 20.0 ml/l) of silver nitrate.Calluses cultures were incubated under 8/16 photoperiod at 25±2 ºC for six weeks.

RESULTS

Callus induction from mature and immature embryos

To compare callogenesis between mature and immature embryos they were cultured in MS medium supplemented with different concentrations (0.0, 1.5, 2.0, 3.0 and 4.0 mg/l) of 2,4-D. Analysis of variance showed no significant differences between the two explants in callus induction frequency; however mature embryo showed ostensibly preference to the immature embryo (Figure 1.); where it achieved the highest fresh weight value (62.0±0.01a) at 2.0 mg/l 2,4-D (Table 1.).

Embryogenesis and plant regeneration in mature and immature embryos

In order to evaluate somatic embryogenesis and subsequent plantlet regeneration frequency for both explants some treatments are known to promote somatic embryo maturation and plant regeneration were used.

Calluses derived from mature and immature cultures were transferred to MS media supplemented with different levels of activated charcoal ranged from 0.25 to 3.0 g/l, different concentrations of sucrose ranged from 15.0 to 120.0 g/l and graded concentrations of silver nitrate ranged from 5.0 to 20.0 ml/l. Mature embryo derived calluses achieved the highest percentages (50-100%) of nodular callus at all types of regeneration media tested compared to immature embryo derived calluses percentages (8-66%).

Shoots regeneration frequencies were also better in the mature embryo derived callus cultures, where it achieved 25% regeneration percentage at 3.0 mg/l activated charcoal (Table 2, Figure 1.); 16% regeneration percentage at 60.0 g/l sucrose (Table 3, Figure 1.)and 25% regeneration percentage at 10.0 ml/l silver nitrate (Table 4, Figure 1.), while immature embryo cultures achieved 8% regeneration percentage at activated charcoal concentrations (Table 2.), failed to regenerate shoots at sucrose concentrations (Table 3.) and the highest regeneration percentage obtained was 8% at silver nitrate treatments (Table 4.).

DISCUSSION

In agreement with our results; Ozagen*et al*., 1998 reported that both mature and immature embryos have been used extensively in tissue culture protocols, but mature embryos were found to be a better choice in comparison to immature embryos; in addition mature wheat embryos have a high frequency of callus induction and regeneration capacity.

In contrast, immature embryos were reported as the best for callus induction and shoot regeneration (Sarker and Bisswas, 2002); in agreement with his findings Redway*et al*., 1990 reported that immature embryos are the most frequently used explants source for plant regeneration from callus culture of wheat and have the highest rates of callus induction and plant regeneration.

However, the use of immature embryos is limited by wheat growing season or requires expensive growth chambers, as it can't be supplied throughout the year.Use of mature embryos saves time and space, and reduces greenhouse costs (Zale *et al*., 2004).

Both somatic embryogenesis and regeneration are complex morphogenic processes and have been shown to be genotype-dependent in cereals (Maes*et al.*, 1996). In agreement with our study (Birsin and Ozgen, 2004; Parmaksiz and Khawar, 2006) reported that the use of MS medium without plant growth regulators was very fruitful in maturing somatic embryos and obtaining plantlets in triticale. Delporte*et al.,* 2001 reported that somatic embryos are formed on nutrient medium with a reduced 2,4-D concentration.

In agreement with our findings; the beneficial effects of activated charcoal may be attributed to removal of inhibitory substances present in the medium or originating from the cultured tissue (Johansson *et al*.,1982), adsorption of undesirable/inhibitory substances and adsorption of growth regulators and other organic compounds (Ebert *et al.*,1993).

Our results, however, contrasted with those of Lashermes, 1992which showed negative effects of activated charcoal on anther culture in wheat attributing that to the binding ability of charcoal to inhibitory as well as enhancing substances in the medium.

Type and concentration of carbon source also determined the efficiency of embryogenic callus formation, the carbohydrates serve not only as an energy supply but also influence osmolarity of the culture medium, the importance of an increased osmotic value for improvement of embryogenic callus formation and for maintenance of long term embryogenic capacity was demonstrated for wheat (Ryschka*et al*., 1991;).

Several authors have reported the inhibition of embryogenesis by ethylene (Auboiron*et al*., 1990). Silver nitrate has been shown to be effective in improving somatic embryogenesis and plant regeneration in cereal crops, such as rice (Adkins *et al*., 1993), maize (Huang and Wei,2004), pearl millet (Jessy*et al*., 1993) and barley (Castillo *et al*., 1998). Furthermore, Fernandez *et al.*, 1999 observed that the number of somatic embryos increased with increasing $AgNO_3$ concentrations up to 22 fold, they also found that using 1.0 mg/l $AgNO_3$ enhanced the induction of direct somatic embryogenesis on a large scale in durum wheat immature embryo culture.

CONCLUSION

Callogenesis, embryogenesis and plant regeneration was found more efficient inmature embryo derived cultures of Elnileen wheat cultivar; which is required thing as the research can be carried out on wheat improvement throughout the year, whereas the mature embryo is available throughout the year andcan be stored in the form of dried seedsso it overcome the limitations of using immature embryo, while the immature embryo needs specific needs and the suitable stage for culture strictly limited.

ACKNOWLEDGEMENT

The author is grateful to the Wheat Research Center, Agricultural Research Corporation for providing the wheat seeds.

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Table 1. Effect of wheat embryo type on callus induction after 6 weeks of culture.

Embryo type	2,4-D concentration (mg/l)	Mean±SE Callus fresh weight (mg)
Mature embryo	1.5	51.0±0.01 a
	2	62.0±0.01 a
	3	47.0±0.01 a
	4	35.0±0.01 a
Immature embryo	1.5	46.0±0.01 a
	2	46.0±0.01 a
	3	44.0±0.01 a
	4	56.0±0.01 a

Means with the same letter are not significantly different

Table 2. Effect of activated charcoal on embryogenesis and plant regeneration from mature and immature embryo of Elnileen wheat cultivar.

Activated charcoal con. (g/l)	Mature embryo			Immature embryo		
	No. regenerated plantlet	Plantlet regeneration %	Nodular callus%	No. regenerated plantlet	Plantlet regeneration %	Nodular callus%
0.0	0.0	0.0	100	0.0	0.0	0.0
0.25	1	8	100	2	8	41
0.5	1	8	100	1	8	41
1.0	1	8	100	7	8	41
3.0	5	25	100	1	8	41

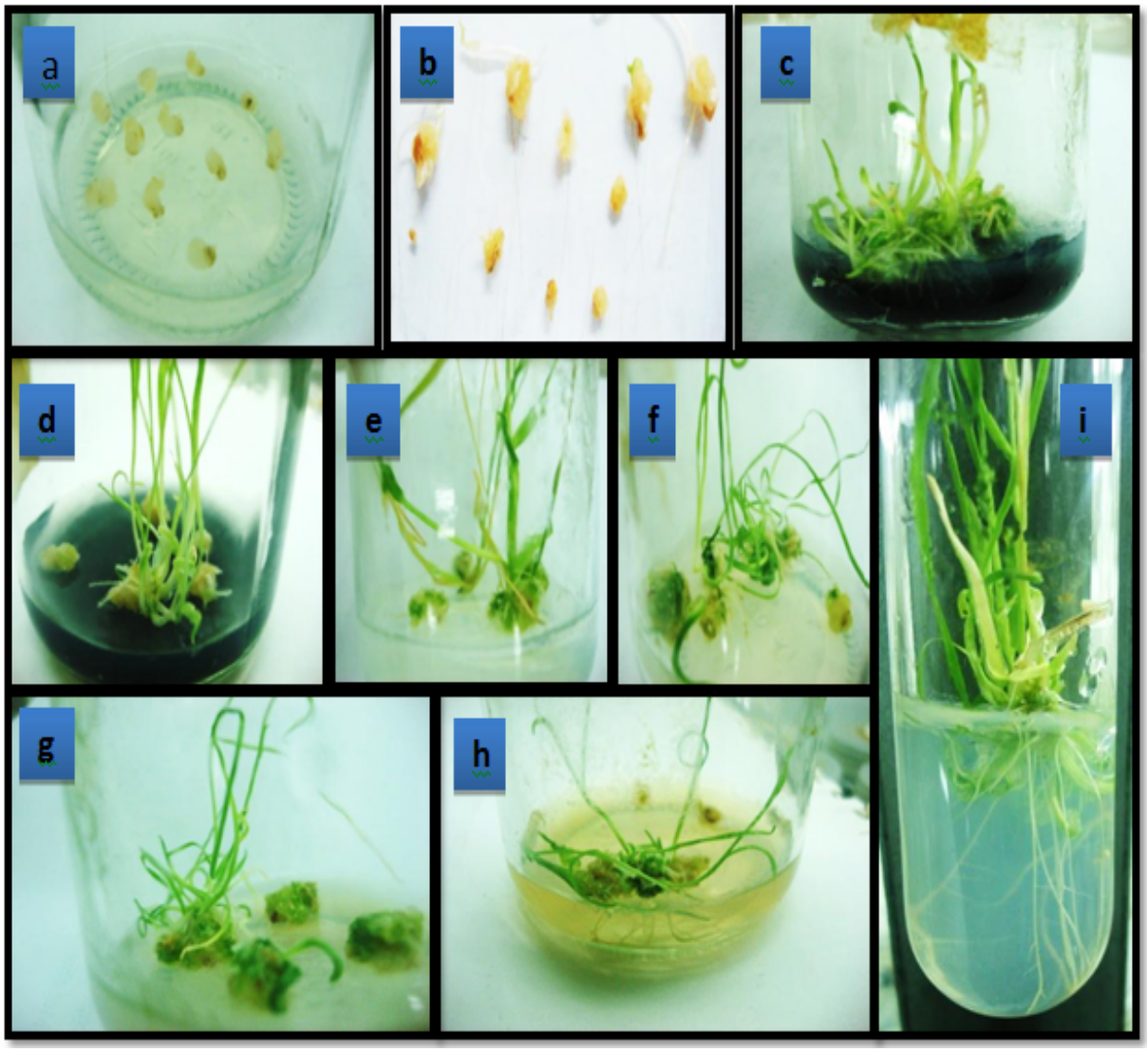
Table 3. Effect of sucrose on embryogenesis and plant regeneration from mature and immature embryo of Elnileen wheat cultivar.

Sucrose con. (g/l)	Mature embryo			Immature embryo		
	No. regenerated plantlet	Plantlet regeneration %	Nodulated callus%	No. regenerated plantlet	Plantlet regeneration %	Nodulated callus%
0.0	0.0	0.0	91	0.0	0.0	0.0
15	0.0	0.0	100	0.0	0.0	16
30	0.0	0.0	100	0.0	0.0	8
60	5	16	91	0.0	0.0	66
120	0.0	0.0	50	0.0	0.0	0.0

Table 4. Effect of Silver nitrate on embryogenesis and plant regeneration from mature and immature embryo of Elnileen wheat cultivar.

Silver nitrate con. (ml/l)	Mature embryo			Immature embryo		
	No. regenerated plantlet	Plantlet regeneration %	Nodulated callus%	No. regenerated plantlet	Plantlet regeneration %	Nodulated callus%
0.0	0.0	0.0	91	0.0	0.0	0.0
5	0.0	0.0	100	0.0	0.0	33
10	3	25	100	5	8	33
15	0.0	0.0	100	2	8	33
20	1	8	91	2	8	41

Fig 1. Callus induction and plant regeneration inwheat mature and immature embryos.



(a) Mature embryos cultured in MS medium supplemented with 2.0 mg/l 2,4-D (b) Immature embryos cultured in MS medium supplemented with 2.0 mg/l 2,4-D (c) Plantlet regeneration from mature embryo cultured in MS medium supplemented with 3.0 g/l activated charcoal (d) Plantlet regeneration from immature embryo cultured in MS medium supplemented with 1.0 g/l activated charcoal (e) Plantlet regeneration from mature embryo cultured in MS medium supplemented with 60.0 g/l sucrose (f) Plantlet regeneration from mature embryo cultured in MS medium supplemented with 10.0 mg/l silver nitrate (g) Plantlet regeneration from mature embryo cultured in MS medium supplemented with 20.0 mg/l silver nitrate(h) Plantlet regeneration from immature embryo cultured in MS medium supplemented with 10.0 mg/l silver nitrate(i) Regenerated shoots successfully rooted in MS medium.