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Effect of embryoids age, size and shape for improvement of regeneration efficiency from microspore-derived embryos in wheat (*Triticum aestivum* L.)

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Abstract

For rapid production of doubled haploid plants through isolated microspore culture is very promising target and useful tool in crop plants. But there are some problems for low rates of embryogenesis, regeneration, albinism and all genotype do not show in androgenetic response. Therefore, the aim of this investigation were to screen some androgenetic responsive cultivars, efficient and reproducible plant regeneration system through isolated microspore cultura of wheat. For improvement of regeneration efficiency experiments were conducted on embryoids age, size and shape on regeneration. Different sizes of embryos were classified into three categories, such as large (> 2.0 - 3.0 mm), medium (1.0 - 1.9 mm) and small (< 1.0 mm). Results indicated that size of the embryo is an important factor for efficient regeneration. It was observed that large embryos produced higher percentage of green plantlets and small embryos showed low regeneration. Embryoids age and shape are also very important factor for regeneration. The present investigation demonstrated that transfer of embryos to semi-solid regeneration medium at an early age, e.g. three - five weeks, showed significant and effective results for the production of regenerated green plants in comparison to prolonged age (six - eight weeks). Transfer of embryos to the regeneration medium after six weeks produced four - five times higher albinos than the earlier age. This results indicate that embryo shape (torpedo, heart and globular) plays an important role for regeneration. Large and heart shaped embryoids produced higher percentage of green plantlets and lower albinos in all cases. This investigation has increased the knowledge for efficient plant regeneration system through proper microspore-derived embryoids using in age, size and shaped in wheat microspore culture.

Key words: *Triticum aestivum*, microspore-derived embryoids, Regeneration, Albinism

Introduction

Since the discovery by Guha and Maheshwari (1994) the immature pollen could be induced to bypass normal development within the anther and the production of haploid plants, first realized in *Datura innoxia* Mill. Afterwards many reports have been done for production of doubled haploids in cereals and other crops. The technique of regenerating fertile plants from isolated microspores represents a potential tool for different biotechnological approaches. Till now progress through anther and isolated microspore culture are reported in cereals and other crops e.g. wheat (Datta and Schmid, 1996; Cistué et al., 2009; Slama-Ayed et al., 2010), rice (Raina and Irfan, 1998; Bikash and Mandal, 2001; Suriyan et al., 2009); barley (Jahne and Lörz, 1999; Jacquard et al., 2006; Shim et al., 2009), maize (Nägeli et al., 1999; Obert and Barnabás, 2004), *Brassica* (Weber et al., 2005; Möllers et al., 1994), *Capsicum* (Barany et al., 2005), carrot (Górecka et al., 2010), *Datura* (Iqbal and Wijesekara, 2007), *Nicotiana* (Touraev et al., 1996a), etc. The success achieved in anther culture of cereals encouraged several workers to establish regeneration systems for isolated microspores in wheat. But success in microspore culture is predominantly dependent on the genotype of the anther donor material. Genetic factors are also important in determining the age of anther and microspore culture response. Several workers observed that the growing conditions of the donor plants might have a profound influence on the induction of androgenic embryo and its development (Ali and Jones, 2000; Islam et al., 2001). In cereal crops, still the major problem is albinisms for androgenetic study. Many factors have been found to affect the degree of albinisms, such as the genotype and physiological state of the donor plants (Torp and Andersen, 2009; Jacquard et al., 2006, Wojnarowiez et al., 2004). The problem is partly solved by using fully developed embryos in respect of their shape and size is very important for better regeneration and avoiding the albinisms. In microspore culture of wheat highest percentage of green plants was obtained when large (>4 mm) embryos were transferred after 25 days in regeneration medium (Kunz et al., 2000). Cistué et al. (1995) also reported that induction time in culture was directly correlated with the production of albino regenerants. Hoffmann et al. (1990) examined histological conditions of several androgenic callus types in wheat and reported that soft and aqueous callus tissue, which consisted of long tubular cells, was not regenerable. De Buyser and Henry (1979) transferred prolonged aged embryos in regeneration medium i.e. after 45 days of culture at 26 - 27°C and observed its retarded regeneration. Till now there are very few reports on the shape and age of embryos for regeneration efficiency. Therefore, the purpose of the present study was to develop an efficient and reproducible plant regeneration system through isolated microspore culture of wheat.

Materials and methods

For this study the spring wheat (*Triticum aestivum* L.) genotypes, namely Barkat, Kanchan and Pavon 76 were taken as plant materials for its good androgenetic response (Islam et al., 2001). Donor plants were grown in the greenhouse (1999) with approximately 25/18ºC day/night temperature and 16/8 h light/dark in ETH-Zurich, Switzerland. Spikes were

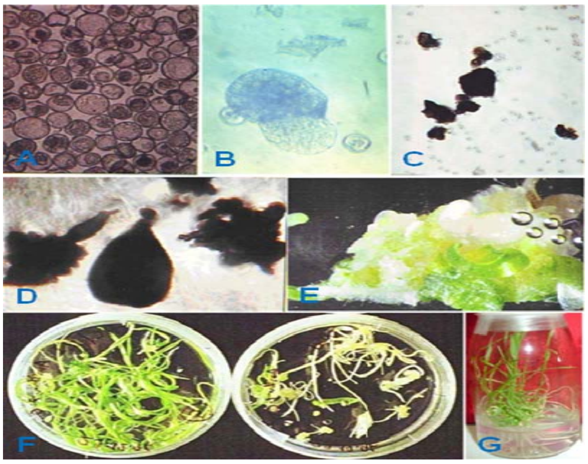

Fig 1. Stages of microspore development and regeneration from microspore-derived embryoids in wheat. A: Microspore development after 5 days of culture. B: Dividing microspore after 10 days of culture. C: Different shape (heart) and sizes of embryos after 3 weeks of culture. D: Torpedo and globular shape of embryos after 4 weeks of culture. E: Callus with regenerable structures. F. Green and albino regenerated plantlets. G. Microspore-derived plants.

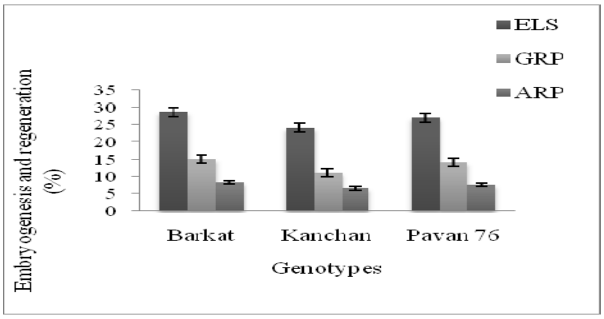

Fig 2. Embryogenesis and plant regeneration efficiency for three wheat genotypes through isolated microspore culture. Vertical bars indicate standard error.

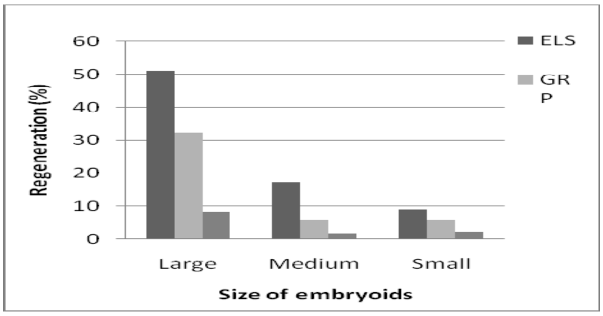

Fig 3. Effect of embryoids size on plant regeneration

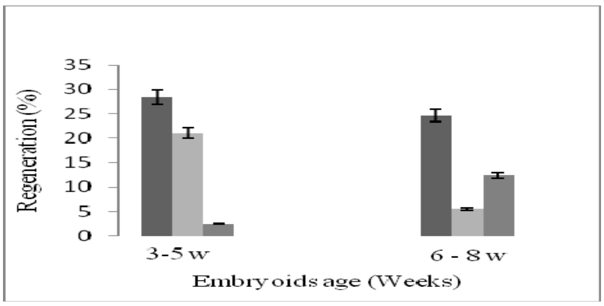

Fig 4. Effect of embryoids age on plant regeneration. Vertical bars indicate standard error.

Table 1. Regeneration efficiency of different shapes of microspore-derived embryoids in wheat

Shape of embryos	Total embryos	Green plants (%)	Albinos (%)
Torpedo	304	63.16	19.08
Heart	252	90.48	9.52
Globular	272	76.47	17.64

Green and albino plants are calculated per 100 embryos.

harvested shortly after just emerging of flag leaf and when the microspores were at mid to late uninucleate stage as determined by 1% aceto-carmine staining test. Harvested spikes were then subjected to cold pretreatment in dark for 3 - 15 days at 4°C. Cold pre-treated spikes were surface sterilized with 70% ethanol and anthers were removed from the spikes using a fine tweezers. Then microspores were released into the medium by squeezing the anthers with a sterile glass rod or by homogenizer. The suspension was diluted with optimum range of liquid AMC (induction) medium (Kunz et al., 2000) and filtered through a sieve with a 100 µm stainless steel mesh and centrifuged at 1000 rpm for 3 - 4 minutes. The pellet was then carefully re-suspended in induction medium and transferred to sterile Petri dishes following the protocol of Puolimatka et al. (1996) and incubated at 28ºC for embryo induction. Microspore developmental stages were observed after five days of culture initiation (Fig A, B).

After three - four weeks in culture the embryos and calli, 1-3 mm in diameter, were removed weekly and transferred to regeneration medium (Fig C, D) and cultures were placed in a chamber at 16/8 hr light/dark regime and 27°C and relative humidity as 80% for regeneration. Regenerated plantlets were transferred to plant growth medium (PM), (Schmid, 1990) for good root and shoot formation. The effect of embryo age (transferring time) on regeneration from microspore-derived embryoids of Barkat was considered for this study. The cultures were divided into two age groups, e.g. (a) three - five and (b) six - eight weeks. Firstly, selected embryoids were transferred in regeneration medium within three - five weeks and secondly; embryoids were transferred within six - eight weeks. For both the age groups, approximately 12×10^5 microspores (120 anthers) of each genotype were cultured into 20 Petri dishes (Corning, surface treated, 35 × 10 mm). To observe regeneration efficiency embryo sizes were determined by superimposing the Petri dish with a transparent grid (1 mm). Embryoids were classified into three categories (sizes): large (>2.0 - 3.0 mm), medium (1.0 - 1.9 mm) and small (<1.0 mm). In another findings different shapes of embryos e.g. torpedo, heart and globular were taken from Barkat and cultured to observe their regeneration ability. Embryo's shape was identified with a stereoscopic microscope after three weeks of culture and transferred to regeneration medium within 21 - 35 days of culture (Fig E). Data were recorded on the basis of number of embryo like structures (ELS) per 10^5 microspore and total regenerated plantlets (TRP), green regenerated plants (GRP) and albino regenerated plants (ARP) per 100 embryos.

For statistical analysis, data were transformed by the ArcSin√P function for converting their multiplicative inter-effects of the traits into additive ones and subjected to ANOVA. Significance level of 0.05 and 0.01 were compared the independent effect of each factor to evaluate the effect of embryo size and age on regeneration. The analysis was computed following the working schedule of Gomez and Gomez (1976).

Table 2. Analysis of variance for the effect of genotype, size and age of embryos on plant regeneration.

Sources of variation	df	ELS		TRP		GRP		ARP	
		MS	F. value	MS	F. value	MS	F. value	MS	F. value
Variety	2	22.16	44.32**	19.34	15.85**	11.82	4.38	2.52	5.04
Size	2	1489.79	2979.58**	1443.57	1183.25**	1122.9	415.89**	193.58	387.16**
Variety × Size	4	11.68	23.36**	18.08	14.82*	10.45	3.87	1.51	3.02
Age	1	48.91	97.82**	78.75	64.55**	688.58	255.02**	462.59	925.18**
Variety × Age	2	0.39	0.78	0.95	0.78	2.11	0.78	2.32	4.64
Size × Age	2	23.17	46.34**	19.50	15.98**	169.80	62.89**	152.67	305.34**
P. error	4	0.50	-	1.22	-	2.70	-	0.50	-
Total	17	186.37	-	183.69	-	197.30	-	69.62	-

ELS = per 10^5 microspores. TRP, GRP and ARP recorded per 100 embryos.

*, ** indicating significant at 0.05 and 0.01 level of significance, respectively.

Results and discussion

Three wheat cultivars from Bangladesh were tested for isolated microspore culture and found that 28.63%, 24.05% and 26.94% embryoids in Barkat, Kanchan and Pavon 76, respectively per 10^5 microspores (Fig 2). Hoffmann et al. (1991) and Ouyang (1986) reported that microspore-derived green plantlet yield of the field-grown spring wheat were several times higher than the greenhouse grown plants.

For microspore culture the physiological or developmental conditions of the donor plants are very important for induction and regeneration. It was found that the large (L) embryos showed significantly highest percentage of green plantlets (32.30), whereas medium (M) and small (S) embryos showed greatly decreased regeneration (Fig 3). Moreover, when the embryos were transferred to regeneration medium within three - five weeks then it showed significantly higher percentage of green plantlets (21.07 %) and least number of albinos. But when the embryos were transferred after six weeks then decreased green plantlets and increased significantly higher number of albino plants (Fig 4). It was observed that many factors have been found to affect the degree of albinism. However, when embryo size was larger than > 3.00 mm then it produced a highest number of regenerated green plantlets per 100 embryos along with least number of albino plants. If embryo size was smaller than 1.00 mm it showed the lowest regeneration potential and the highest albinism. To overcome the albinism and improved green plant regeneration shape of embryos was considered as an important factor in the present study. It was observed that, heart shaped embryos showed highest percentage of regeneration than other two i.e. torpedo and globular shape. A very high percentage of regenerated green plants (90.48%) and less number of albino plants (9.52%) were found when different sizes of heart shaped embryos were transferred to the regeneration medium within three - five weeks of culture initiation (Table 1).

The effect of genotype, embryoids size and age and their interactions on induction and regeneration traits were tested at the 0.05 and 0.01 level of significance by F-test (Table 2). Embryo's age, size and age - size interaction on the induction and regeneration were found to be highly significant, whereas, the genotype and genotype - embryo's size also showed highly significant effect on the formation of embryo like structures and showed significant effect on plant regeneration. The present investigation about the effect of embryo age, size and shape on plant regeneration potential were evaluated. It clearly demonstrated that an early transfer of embryos or embryo like structures (ELS) into the regeneration medium within three - five weeks is more efficient for regeneration of green plantlets in comparison to prolonged culture (six - eight weeks) in AMC (Fig F, G). It might be due to the accumulation of gametoclonal variations induced by the relatively high 2, 4-D content of AMC. The quality of embryos (size and shape) also showed significant effect on the regeneration efficiency. Large embryos yielded more green plantlets than other embryos of the same age. Furthermore, the percentage of albino plants was negetively correlated with the embryo size. Hu and Kasha (1997) determined two sizes of embryos e.g. large (>2.0 mm) and small (0.5 - 2.0 mm) and only the large sized embryos were transferred into differentiation medium for regeneration. They reported that the frequency of number of green and albino plant regeneration is dependent on embryoids size. Touraev et al. (1996b) reportad that albino plant production is not only dependent on genotype, it is also correlated with the embryo size. Bruins and Snijders (1995) also examined the effect of size of embryo on regeneration and they classified the embryos into three groups (0.5 - 0.75, 1.0 - 1.25 and 1.50 - 2.0 mm), and demonstrated that the green plantlet production from 0.5 - 0.75 mm sized embryos was more efficient than the larger (>1.0 mm) embryos. Hu and Kasha (1997) also transferred large sized embryos (>2.0 mm) to regeneration medium after 30 days, and obtained very higher percentage of green plants (18 - 43 %) and lower number of albinos (5 - 8 %). Kunz et al. (2000) reported that the prolongation of embryo culture in induction medium up to 45 days caused a dramatic drop in the regenertaion efficiency. It was observed that a very high percentage of green plantlets and low number of albinos were recorded from large (2.0 - 3.0 mm) embryos, but <1.0 mm embryos failed to show good regeneration. However, it differs from the observation of Bruins and Snijders (1995), they obatined significantly better results on regeneration from >1.0 mm embryos. But they did not mention any effect of age of embryo on regeneration and albino production. The present results on the influence of embryo size and age on regeneration agreed well with the findings of Kunz et al. (2000), Hu and Kasha (1997), De Buyser and Henry (1979). Cistué et al. (1995) mentioned that the duration of culturing time was highly correlated with the production of albino plants. In this study, only 2.39% albino plants were observed when the embryos were transferred within three - five weeks and 12.40% albinos were counted when embryos transferred after six - eight weeks. Similar type of work has been reported by De Buyser and Henry (1979).

In conclusion, different shape of embryos showed significant effect on the variability of regeneration. Torpedo and globular embryos also showed good results but production of albino was two - three times higher than the heart shaped embryoids. Finally, it has been proved that embryoid's age, size and shape are important factors for the production of increased green plantlets and reduced number of albino. Large and hearts shaped embryos were appeared to be the best performer for green plant regeneration.

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References

Ali MA, Jones JK (2000) Microspore culture in *Corchorus olitorius*: effect of growth regulators, temperature and sucrose on callus formation. Indian J Exp Biol. 38(6): 593-597.

Barany I, Gonzalez MP, Fadon B, Mityko J, Risueno MC, Testillano PS (2005) Microspore-derived embryogenesis in pepper (*Capsicum annuum* L.): subcelluar rearrangements through development. Biol Cell. 97(9): 709-22.

Bikash C, Mandal AB (2001) Microspore embryogenesis and fertile plantlet regeneartion is a salt susceptible × salt tolerant rice hybrid. Plant Cell Tiss Org Cult. 65: 141-147.

Bruins MBM, Snijders CHA (1995) Inheritance of anther culture derived green plantlet regeneration in wheat (*Triticum aestivum* L.). Plant Cell Tiss Org Cult. 43: 13-19.

Cistué L, Ziauddin A, Simion E, Kasha KJ (1995) Effects of culture conditions on isolated microspore response of barley cultivar Igri. Plant Cell Tiss Org Cult. 42: 163-169.

Cistué L, Romgosa I, Batlle F, Echávarri B (2009) Improvement in the production of doubled haploids in durum wheat (*Triticum turgidum* L.) through isolated microspore culture. Plant Cell Rep. 28(5): 727-735.

Datta SK, Schmid JE (1996) Prospects of artificial seeds from microspore-derived embryos of cereals. In: Jain SM, Sopory SK, Veilleux RE (eds) *In Vitro* Haploid Production in Higher Plants, Kluwer Academic Publishers, Vol. 2: 353-365.

De Buyser J, Henry Y (1979) Androgenèse sur des tendres en cours de selection I. L'obtention des plants *in vitro*. Z. Pflanzenzücht 83: 49-56.

Górecka K, Kowalska U, Krzyżanowska D, Kiszezak W (2010) Obtaining carrot (*Daucus carota* L.) plants in isolated microspore cultures. J Appl Genet 51:141-147.

Gomez KA and Gomez AA (1976) Statistical Procedures for Agricultural Research. IRRI (Ed.), Philippines, pp. 10-119.

Guha S, Maheshwari SC (1964) *In vitro* production of embryos from anthers of ***Datura.*** Nature 204: p 497.

Hoffmann B, Schumann G, Krüger HU (1990) Histological observations on morphogenesis from androgenetic tissue of *Triticum aestivum* L. I. Callus Tissue. Arch Züchtungsforsch 20(3): 179-186.

Hoffmann B, Krüger HU, Schumann G (1991) *In vitro* androgenesis in wheat (*Triticum aestivum* L.) II. The influence of donor plant growth environment. Arch Züchtungsforsch 21(3): 237-244.

Hu T, Kasha KJ (1997) Improvement of isolated microspore culture of wheat (*Triticum aestivum* L.) through ovary co-culture. Plant Cell Rep. 16(8): 520-525.

Iqbal MCM, Wijesekara KB (2007) A brief temperature pulse enhances the competency of microspores for androgenesis in *Datura metel.* Plant Cell Tiss Org Cult. 89: 41-149.

Islam SMS, Bari MA, Amin MN, Schmid JE (2001) *In vitro* plant regeneration through anther culture of some Bangladeshi wheat varieties. Plant Tissue Cult. 11(1): 31-39.

Jacquard C, Asakaviciute R, Hamalian AM, Sangwan RS, Devaux P, Clement C (2006) Barley anther culture: effects of annual cycle and spike position on microspore embryogenesis and albinism. Plant Cell Rep. 25(5): 375-81.

Jähne GA, Lörz H (1999) Protocols for anther and microspore culture of barley. Methods Mol Biol. 111: 269-79.

Kunz C, Islam SMS, Berberat J, Peter SO, Büter B, Stamp P, Schmid JE (2000) Assessment and improvement of wheat microspore derived embryo induction and regeneration. J Plant Physiol. 156: 190-196.

Nägeli M, Schmid JE, Stamp P, Büter B (1999) Improved formation of regenerable callus in isolated microspore culture of maize: impact of carbohydrates, plating density and time of transfer. Plant Cell Rep. 19: 177-184.

Obert B, Barnabás B (2004) Colchicine induced embryogenesis in maize. Plant Cell Tiss Org Cult. 77:283-285.

Ouyang J (1986) Induction of pollen plants in *Triticum aestivum*. In: Hu H, Yang H (Eds.) Haploids of higher plants *in vitro*. Springer-Verlag, Berlin, pp. 26-41.

Puolimatka M, Laine S, Pauk J (1996) Effect of ovary co-cultivation and culture medium on embryogenesis of directly isolated microspores of wheat. Cereal Res Comm. 24(4): 393-400.

Raina SK, Irfan ST (1998) High-frequency embryogenesis and plantlet regeneration from isolated microspores of indica rice. Plant Cell Rep. 17: 957-962.

Shim YS, Pauls KP, Kasha K (2009) Transformation of isolated barley (*Hordeum vulgare* L.) microspores: I. The influence of pretreatments and osmotic treatment on the time of DNA synthesis. Genome 52:166-174.

Slama-Ayed O, De Buyser J, Picard E, Trifa Y, Amara HS (2010) Effect of pre-treatment on isolated microspores culture ability in durum wheat (*Triticum turgidum* subsp. *Durum* Desf.). J. Plant Breed and Crop Sci. 2:30-38.

Schmid JE (1990) *In vitro* production of haploids in *Triticum **spelta.*** In: Bajaj YPS (Eds.) Biotechnology in Agriculture and Forestry, Vol. 13, pp 363-381.

Suriyan, C, Bootsaya S, Aussanee P, Chalermpol K (2009) An efficient procedure for embryogenic callus induction and doubled haploid plant regeneration through anther culture of Thai aromatic rice (*Oryza sativa* L. subsp. *indica*). In vitro Cell Dev Biol - Plant 45: 171-179.

Torp AM, Andersen SB (2009) Albinism in microspore culture. In: Touraev A, Forster BP, Jain SM (eds) Advances in haploid production in higher plants. Springer, The Nederlands, pp 155-160.

Touraev A, Ilham A, Vicente O, Heberle-Bors E (1996a) Stress induced microspore embryogenesis in tobacco: an optimized system for molecular studies. Plant Cell Rep. 15: 561-565.

Touraev A, Indrianto A, Wratschko I, Vicente O, Heberle-Bors E (1996b) Efficient microspore embryogenesis in wheat (*Triticum aestivum* L.) induced by starvation at high temperature. Sex Plant Repr. 9(4): 209-215.

Weber S, Ünker F, Friedt (2005) Improved doubled haploid production protocol for *Brassica napus* using microspore colchicine treatment *in vitro* and ploidy determination by flow cytometry. Plant Breed. 124:511-513.

Wojnarowiez G, Caredda S, Devaux P, Sangwan R, Clement C (2004) Barley anther culture: assessment of carbohydrate effects on embryo yield, green plant production and differential plastid development in relation with albinism. J Plant Physiol. 161(6): 747-755.