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Efficient Microspore Isolation Methods for High Yield Embryoids and Regeneration in Rice (*Oryza sativa* L.)

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Abstract**—**Through anther and microspore culture methods, complete homozygous plants can be produced within a year as compared to the long inbreeding method. Isolated microspore culture is one of the most important techniques for rapid development of haploid plants. The efficiency of this method is influenced by several factors such as cultural conditions, growth regulators, plant media, pretreatments, physical and growth conditions of the donor plants, pollen isolation procedure, etc. The main purpose of this study was to improve the isolated microspore culture protocol in order to increase the efficiency of embryoids, its regeneration and reducing albinisms. Under this study we have tested mainly three different microspore isolation procedures by glass rod, homozeniger and by blending and found the efficiency on gametic embryogenesis. There are three types of media *viz.* washing, pre-culture and induction was used. The induction medium as AMC (modified MS) supplemented by 2, 4-D (2.5 mg/l), kinetin (0.5 mg/l) and higher amount of D-Manitol (90 g/l) instead of sucrose and two types of amino acids (L-glutamine and L-serine) were used. Out of three main microspore isolation procedure by homogenizer isolation (P_4) showed best performance on ELS induction (177%) and green plantlets (104%) compared with other techniques. For all cases albinisims occurred but microspore isolation from excised anthers by glass rod and homogenizer showed lesser numbers of albino plants that was also one of the important findings in this study.

Keywords**—**Androgenesis, pretreatment, microspore culture, regeneration, albino plants, *Oryza sativa*.

I. Introduction

RICE (*Oryza sativa* L.) is one of the most important cereals crop in Bangladesh and also in the world. The population of rice eaters are increasing day by day and the number of rice consumers will probably two fold by the year of 2020 [1]. Agricultural genetics is one of the easier parts of the solution. Use of unconventional techniques such as doubled haploids breeding through anther and microspore culture is becoming more useful in the speeding up of the application of conventional plant breeding methods [2]. The main advantage of using doubled haploid techniques is the rapid homozygosity of the descendants, results a time saving procedure for the development of new varieties [3], [4]. But for advance biotechnological work to cereal crops have recalcitrant to recombinant techniques mainly because of problems in establishing regenerable cell and tissue cultures systems [5], [6]. Isoalted microspore culture is an important and useful tool in plant breeding for production of homozygous line and combined study with genetic transformation fertile homozygous plants can be produced rapidly [7], [8]. To overcome the high reactivity of somatic tissues such as anther wall, multicellular hair-type structures, anther connective and parenchymatous vascular bundle microspore culture is an important technique [9], [10]. Microspore embryogenesis is an elegant system also for genetic transformation and could provide a practical alternative for the production of doubled haploid plant species in which regeneration from somatic cells is difficult, especially in the recalcitrant cereals [11]. It has been reported that most of the genotypes, the production of embryos per anther obtained in isolated microspore culture is generally higher than in anther culture [12]. In certain plant species such as *Hordium vulgare* the isolated microspore culture procedure has produced five fold green plants compared to anther culture [13], [14]. Till there are some reports on isolated microspore culture in cereal and other crops with success, e.g. barley [15]-[17]; durum wheat [18], rice [19], [20]; rye [21], maize [22], [23], wheat [24]-[28], *Brassica napus* [29], [30], carrot [31], chickpea [32], *Corchorus* [33]; horse chestnut [34], *Medicago* [35], pepper [36]-[38], For androgenetic research still a great lacking of embryo induction and high percentage of albinos among regenerated plants [39]. Albino plants are very often produced during the regeneration of microspore-derived plants in cereals [40]. The frequency of albinos may vary from 5 -100% in rice anther culture [41]. Till many researches have been done on several experiments in different aspects for influencing of androgenesis. But there is not enough report on rice and some other crops about the suitable microspore isolation procedure. Under this circumstance this research project has been undertaken to develop an efficient embryoids induction and regeneration methods using different isolation techniques of microspore using Bangladeshi rice cultivars.

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II. Materials and Methods

A. Microspore Donor Plants and Different Steps for Production of Doubled Haploids in Rice

To optimize different microspore isolated procedure in rice, IR 34 variety were used as plant material that showed good androgenetic performance previously reported by [42] Seeds were grown in the experimental field at the Institute of Biological Sciences during the rice cultivation period (2012-2013). Spikes were harvested and subjected to cold pre-treatment at 4ºC~5ºC for 3-15 days in dark. Anthers containing microspores at the late uninucleated to early binucleate stage were excised from the central part of the spikes. Microspore conditions were observed by 1% aceto-carmine staining under microscope prior to culture. Different steps of microspore isolation and subsequent procedure of doubled haploids (DH) production are shown in Fig. 1.

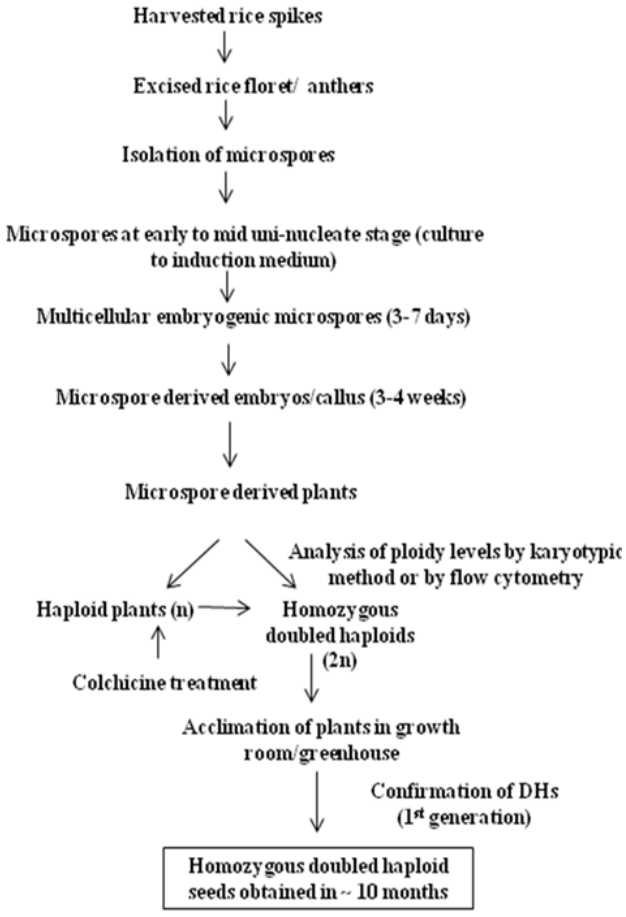

Fig. 1 A flow chart showing on different steps of rice microspore culture for doubled haploid production

B. Culture Media

Three types of media *viz.* PC (pre-culture), WM (washing) and induction (AMC) were used for this study [24] that is shown in Table I. The WM and AMC were modified by AM [43]. The pH of media was adjusted at 5.8 before autoclaving.

C. Methods for Different Microspore Isolation Procedure

Squeezed of Rice Floret by Glass Rod (P_1)

Cold pretreated spikes were surface sterilized with 70% ethanol and anthers were removed with a fine tweezers (forceps) and kept in sterile petri dishes with 4-ml washing medium. In this case microspores were released from rice floret (along with anthers lemma and palea were closed) by squeezing with a sterile glass rod. The suspension was diluted with 15ml WM and filtered through a sieve with a 100µm mesh and centrifuged for 4 minutes with 750rpm. Then the sediment was carefully resuspended in 3ml induction medium and transferred to a sterile petri dish (35 × 10mm). Petri dishes were sealed with parafilm and incubated at 28±2ºC in dark for embryo induction. After 3-4 weeks the targeted embryos and calli, those were around 1-3mm in diameter, were removed weekly and transferred to the regeneration medium. Then regenerated plantlets were transferred to plant medium [43] for root and shoot development.

Squeezed of Excised Anther by Glass Rod (P_2)

In this case microspores were released in the medium from excised anther by squeezing with a sterile glass rod. The subsequent procedure was followed as described previously in P_1.

Squeezed of Rice Floret by Homogenizer (P_3)

Microspore isolation by homogenizer, sterilized rice floret was placed in a 50ml tube with 20-25ml washing medium (WM). Rice floret was squeezed by polytron (PT-1200C, Kinematica AG, Switzerland) at medium speed for 45-60 seconds. The anthers and debris were removed by passing a 100µm stainless steel mesh sieve and the microspores were collected and the solution was washed by WM and further centrifuged with 750 rpm for 3 minutes. The debris was removed through a 100µm stainless steel mesh sieve and the microspores were collected. The subsequent procedure was followed as described previously in P_1.

Microspore Isolation from Anthers by Homogenizer (P_4)

In this case microspores were released in the medium from excised anther by squeezing with homogenizer (polytron). The subsequent procedure was followed as described previously in P_1 and P_3.

Squeezed of Rice Floret by Blender (P_5)

Rice floret placed in a sterilized microblender chamber containing 20-30ml liquid WM. The microblender chamber covered with aluminum foil during blending and anthers were blended with low speed for few seconds. Then the debris was removed through a 100µm stainless steel mesh sieve and microspores were collected following the subsequent procedure as described previously P_1.

Microspore Isolation from Anthers by Blender (P_6)

In this case microspores were released in the medium from excised anther by squeezing with a blender. The subsequent procedure was followed as described previously in P_1 and P_3.

TABLE I
CHEMICAL COMPOSITION OF PRECULTURE (PC), WASHING (WM) AND INDUCTION (AMC) MEDIA FOR RICE MICROSPORE CULTURE

Media composition	PC- Preculture medium	WM- Washing medium (mg/l)	AMC- Induction medium (mg/l)
KNO_3	-	500	1000
$(NH_4)_2SO_4$	-	50	100
$Ca(NO_3)_2.4H_2O$	-	50	100
KH_2PO_4	-	100	200
$MgSO_4.7H_2O$	-	62.5	125
KCl	-	17.5	35
$MnSO_4.4H_2O$	-	4.4	4.4
$ZnSO_4.7H_2O$	-	1.5	1.5
H_3BO_3	-	1.6	1.6
KI	-	0.8	0.8
$FeCl_3$	-	27.0	27.0
Na_2EDTA	-	37.3	37.3
Thiamin HCl	-	1.0	1.0
2, 4-D	-	2.5	2.5
Kinetin	-	0.5	0.5
Sucrose	-	60 g/l	-
D-Manitol	80.0 g/l	-	90 g/l
L-glutamine	-	1000	1000
L-serine	-	100	100
L-ascorbic acid	50 mg/l	-	-
L-proline	150 mg/l	-	-

pH of all media was: 5.8.

D. Data Recording

Data were recorded on the basis of embryo yield and its regeneration potentials from three major microspore isolation methods. For Control, excised anthers were directly cultured in induction medium (AMC). For microspore isolation procedure data were recorded on the basis embryogenesis (number of regenerated embryoids, embryoids per 10^5 microspores from 100 anthers) and for regeneration e.g. total regenerated plantlets (TRP), green (GRP) and albino plants (ARP) per 100 embryos. Five replications were considered and 5×10^5 microspores were used from 500 anthers for all cases. In case of control 500 anthers were inoculated in induction medium.

III. RESULTS AND DISCUSSION

Through isolated microspore culture methods it may possible to overcome the production of embryoids from somatic tissues such as anther wall, multicellular hair-type structures, anther connective and parenchymatous vascular bundle. To found the embryo yield and its regeneration potentials from six microspore isolation methods e.g. P_1 = squeezed of rice floret by glass rod, P_2 = squeezed of excised anthers by glass rod, P_3 = squeezed of rice floret by homogenizer, P_4 = squeezed of excised anther by homogenizer, P_5 = squeezed of rice floret by blender, P_6 = squeezed of excised anther by blender were considered for this study. The percentage of embryo induction was recorded from different isolation methods of rice are shown in Table II. The developmental stages of microspore and subsequent formation of embryoids from different isolation procedures are shown in Figs. 2 (A)-(D) and Figs. 3 (A)-(C).

TABLE II
INDUCTION OF EMBRYOIDS AND REGENERATION EFFICIENCY OF RICE ANTHER CULTURE BY DIFFRENT MICROSPORE ISOLATION PROCEDURE

Procedure	Embryo like structures (ELS) per 10^5 microspores/ 100 anthers	Regenerated embryos (%)	Regeneration (per 100 embryos)		
			TRP (%)	GRP (%)	ARP (%)
Control	28.00	17.00	23.00	16.00	7.00
P_1	65.00	44.00	55.00	47.00	8.00
P_2	123.00	110.00	102.00	91.00	11.00
P_3	103.00	78.00	65.00	53.00	12.00
P_4	177.00	136.00	125.00	109.00	16.00
P_5	99.00	83.00	58.00	43.00	15.00
P_6	137.00	97.00	94.00	69.00	25.00
Mean	104.57	80.71	74.57	61.14	13.43

Control = Excised anthers (100) directly cultured in liquid AMC [24], P = Procedure, P_1 = squeezed of rice floret by glass rod, P_2 = squeezed of excised anthers by glass rod, P_3 = squeezed of rice floret by homogenizer, P_4 = squeezed of excised anther by homogenizer, P_5 = squeezed of rice floret by blender, P_6 = squeezed of excised anther by blender, 10^5 microspores = 100 anthers, TRP= Total regenerated plants, GRP = Green regenerated plants, ARP = Albino regenerated plants.

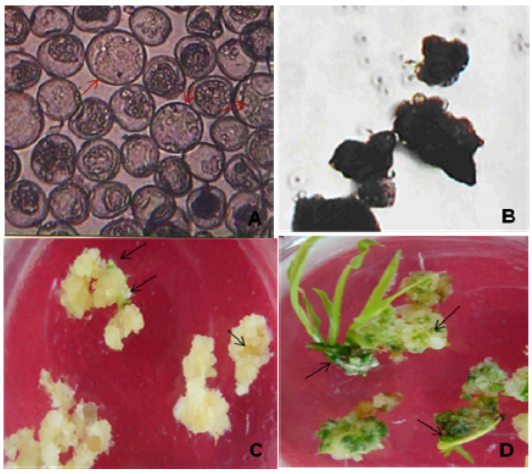

Fig. 2 Stages of isolated microspore culture and its subsequent regeneration of rice (A) Microspore isolation after 3 days of culture initiation (B) Embryoids formation after 2-3 weeks of culture (C) Microspore-derived embryoids transferred to regeneration medium (D) Embryoids showing good regeneration in RRG medium

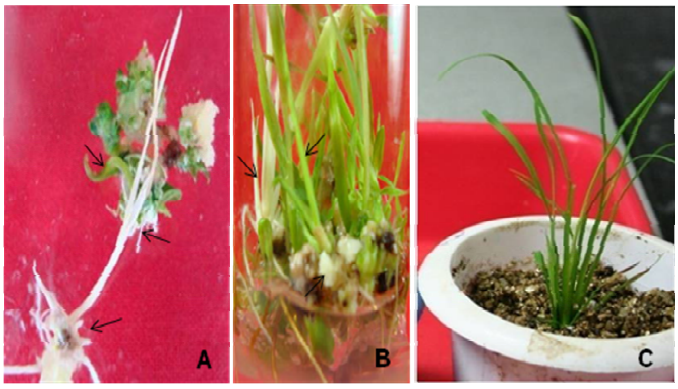

Fig. 3 Green and albino plant regeneration from microspore-derived embryoids in rice (A) Green and albino plants development with good root and shoot (B) Plants were transferred to test shoot elongation and root development (C) Microspore-derived plants were transferred to pot for doubled heploids seed collection

Fully formed embryoids were observed within 4-5 weeks of culture initiation. It was observed that out of three major methods, microspore isolation procedure by glass rod (P_1) was much easier and simplest and less expensive because for that sophisticated equipments are not required. However, lower percentage of embryoids (65% from 100 anthers) were recorded by glass rod isolation procedure where green plants were recorded (47%) and less number of albino plants (8%) in P_1. Highest percentage of embryoids (177%) were recorded in P_4 where anthers were squeezed by homogenizer (Polytron) are shown in Table II. Out of the mentioned microspore isolation procedure the best performance found in P_4 where anther were squeezed by homogenizer and in this case excised anthers were used as explants. The efficiency of isolated microspore culture technique of rice has been studied by major three methods and successfully standardized suitable isolation techniques. It was observed that better embryo yields of microspores isolation by homogenizer showed the best than glass rod and blender isolation. But on that case regeneration was lesser and albino plants obtained little higher that other two procedures of glass and polytron isolation. In *Brassica napus* the technique of pestle maceration by which the anthers are pressed through a sieve with a certain mesh size by using a teflon rod [44]. This technique also successfully adapted for wheat [45], barley [46], [13] and maize [47]. In maize and barley the microblending technique was found to be superior for isolation of microspores compared to the maceration method with respect to yield of microspores [48]. Similar observation was done by Gustafson et al. [49]. They compared four isolation methods of microspore and obtained highest initial microspore viability (75%) with blending isolation technique. Our system for homogenizer (polytron) and blending isolation technique gave a very high number of embryoids per 100 anthers. However, for isolation technique of microspores by homogenizer and blender also gave quite similar results with the system [48]. By using a microblender the rapid isolation of large number of microspores free from tissue and cellular debris is possible. For microspore isolation, a series of sieve were used with different pore sizes in order to separate microspores of different stages (e.g. non-vacuolated early microspores and vacuolated microspores or bi-cellular pollen grains) before centrifugation, or to eliminate the sub-cellular components [50]. Regeneration from microspores obtained by microblending was described for wheat [51], barley [48] and maize [47]. Gustafson et al. [49] compared four microspore isolation methods e.g. blending, stirring, maceration and floating and obtained highest initial microspore viability (75%) with blending isolation technique. They reported that blender isolation without mannitol conditioning and an initial density 2×10^5 microspore ml^{-1} was best for continued microspore viability. Those techniques are as follows: isolation of microspores by sterile glass rod, by homogenizer from anthers and by blender from anthers. Albinisms still have a great problem for mainly in cereals crops for androgenetic study and it has no agronomical value. However, under this study we found that the microspore isolation by sterile glass rod and homogenizer showed quite better performance and reducing albino plants compared with control and other isolation procedures.

IV. Conclusions

Rapid production of doubled haploids through anther and isolated microspore culture is very efficient and useful methods. Under this study six microspore isolation procedures (P_1-P_6) from three major groups e.g. squeezed of rice floret and excised anthers by glass rod, rice floret and excised anther by homogenizer, rice floret and excised anther by blender isolation techniques were used. Microspore isolation procedure by glass rod (P_1) was much easier and simplest methods. Less number of embryoids (65% from 100 anthers) were recorded when rice floret squeezed by glass rod whereas with same procedure but isolation form anthers showed good

embryo yield (123%). For Control, around two to four fold less embryoids (28%) were recorded when anther were directly cultured in the same induction medium (AMC). In this case, all microspore isolation methods showed good performance on embryo yield and total plant regeneration. But higher frequency of embryoids yield (177%) and green plantlets (109%) were recorded when anthers were squeezed by homogenizer and albino plants reduced compared to control. It may conclude that for production of high yield embryos and reducing albinisims microspore isolation procedure is very much important besides other factors of androgenetic study.

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REFERENCES

[1] Khush, G. S. and G. H. Toenniessen, 1991. *Rice Biotechnology*. International Rice Research Institute. 6: VII-VIII.
[2] Sopory, S. K. and M. Munshi. 1996. Anther culture, edited by S M Jain, S K Sopory and R E Veilleux, *In Vitro Haploid Production in Higher Plants* (Kluwer Academic Publishers, Netherlands), pp. 145–176.
[3] Islam, S. M. S. 2010a. The role of drought stress on anther culture of wheat (*Triticum aestivum* L.). *Plant Tissue Cult Biotech*. 20: 55-61.
[4] Rukmini, M., G. J. N. Rao. and R. N. Rao. 2013. Effect of cold pretreatment and phytohormones on anther culture efficiency of two Indica rice (*Oryza Sativa* L.) Hybrids- Ajay and Rajalaxmi. *J. Exp. Biol. Agr. Sci*., (2): 69-76.
[5] Stöger, E., C Fink, M. Pfosser, and E. Heberle-Bors. 1995. Plant transformation by particle bombardment of embryogenesis pollen. *Plant Cell Rep*. 14: 273-278.
[6] Jähne, A. and H. Lörz. 1995. Cereal microspore culture. *Plant Sci.* 109: 1-12.
[7] Guo, Y. D. and S. Pulli. 2000. Isolated microspore culture and plant regeneration in rye (*Secale cereale* L.). *Plant Cell Rep.* 19: 875-880.
[8] Islam, S. M. S. and N. Tuteja (2013) Production of abiotic stress tolerant fertile transgenic plants using androgenesis and genetic transformation methods in cereal crops. *In*: Tuteja N & Gill SS (Eds.). *Crop Improvement Under Adverse Conditions.* Springer New York Heidelberg London pp 213-229.
[9] Coumans, M. P. and D. Zhang. 1995. Doubled haploid sunflower (*Helianthus annuus*) plant production by androgenesis: fact or artifact? Part 2. *In vitro* isolated microspore culture. *Plant Cell, Tiss Org Cult*. 41 (3): 203-209.
[10] Ferrie, A. M. R. and K. L. Caswell. 2011. Isolated microspore culture techniques and recent progress for haploid and doubled haploid plant production. *Plant Cell Tiss Org Cult*. 104: 301-309.
[11] Islam, S. M. S. and N. Tuteja. 2012. Enhancement of androgenesis by abiotic stress and other pretreatments in major crop species. *Plant Science* 182: 134-144.
[12] Islam, S. M. S. 2010b. The effect of colchicine pretreatment on isolated microspore culture of wheat (*Triticum aestivum* L.). *Aus J Crop Sci*. 4(9): 660-665.
[13] Hoekstra, S., M. H Zijderveld, F. Heidekamp. and F. Mark. 1993. Microspore culture of *Hordeum vulgare* L.: The influence of density and osmolality. *Plant Cell Rep*. 12: 661-665.
[14] Shim, Y. S., Pauls K. P. and 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.
[15] Köhler, F. and G. Wenzel. 1985. Regeneration of isolated barley microspores in conditioned media and trials to characterize the responsible factor. *J Plant Physiol.* 121: 181-191.
[16] Wei, Z. M., M. Kyo. and H. Harada. 1986. Callus formation and plant regeneration through direct culture of isolated pollen of *Hordeum vulgare* cv. Sabarlis. *Theor. Appl. Genet*. 72: 252-255.
[17] Cistué, L., A. Ziauddin, E. Simion. and K. J. Kasha. 1995. Effects of culture conditions on isolated microspore response of barley cultivar Igri. *Plant Cell Tiss Org Cult* 42: 163-169.
[18] Cistué L, I. Romgosa, F. Batlle. and B. Echávarri (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.
[19] Datta, S. K., I. Potrykus, M. Bolik. and G. Wenzel. 1990. Culture of isolated pollen of wheat (*Triticum aestivum* L.). In: Bajaj YPS (eds) *Biotechnology in Agriculture and Forestry*, vol. 13, pp 435-447.
[20] Raina, S. K. and S.T. Irfan. 1998. High-frequency embryogenesis and plantlet regeneration from isolated microspores of indica rice. *Plant Cell Rep*. 17: 957-962.
[21] Wenzel, G., F. Hoffmann, I. Potrykus. and E. Thomas. 1975. The separation of viable rye microspores from mixed populations and their development in culture. *Mol. Genet*. 138: 293-297.
[22] Nägeli, M., J. E. Schmid, P. Stamp. and B. Büter. 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.
[23] Obert, B., L.A. Szabo, Juditmityko, Annapretova. and B. Barnabás. 2004. Morphological events in cultures of mechanically isolated maize microspores. *In Vitro Cell Div Biol-Plant* 41: 775-782.
[24] Kunz, C., S. M. S. Islam, J. Berberat, S. O. Peter, B. Büter, P. Stamp. and J. E. Schmid. 2000. Assessment and improvement of wheat microspore derived embryo induction and regeneration. *J. Plant Physiol*. 156: 190-196.
[25] Islam, S. M. S., M. A. Bari, M. N. Amin. and J. E. Schmid. 2001. *In vitro* plant regeneration through anther culture of some Bangladeshi wheat varieties. *Plant Tissue Cult Biotech.* 11(1): 31-39.
[26] Liu, W., Zheng, M. Y., Polle, E. and C. F. Konzak. 2002. Highly efficient doubled-haploid production in wheat (*Triticum aestivum* L.) via induced microspore embryogenesis. *Crop Sci.,* 42: 686-692.
[27] Slama-Ayed, O., De J. Buyser, E. Picard, Y. Trifa. and H. S. Amara. 2010. Effect of pre-treatment on isolated microspores culture ability in durum wheat (*Triticum turgidum* subsp. *Durum* Desf.). *J. Plant Breed Crop Sci*. 2: 30-38.
[28] Ekhveh, M. J., A. Moieni. and M. J. Javaran. 2013. The evaluation of response to isolated microspores culture in some Iranian hexaploid wheat (*Triticum aestivum* L.) cultivars. *J. Agr. Sci*. 5: 206-216.
[29] Weber S, F. Ünker. and W. 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.
[30] Takahira, J., A. Cousin, M. N. Nelson. and W. A. Cowling. 2011. Improvement in efficiency of microspore culture to produce doubled haploid canola (*Brassica napus* L.) by flow cytometry. *Plant Cell, Tiss Org Cult*. 104: 51–59.
[31] Górecka, K., U. Kowalska., D. Krzyżanowska. and W. Kiszezak. 2010. Obtaining carrot (*Daucus carota* L.) plants in isolated microspore cultures. *J Appl Genet.* 51: 141-147.
[32] Grewal, R. K., M. Lulsdorf, J. Crosser, S. Ochatt, A. Vandenberg. and T. D. Warkentin. 2009. Doubled-haploid production in chickpea (*Cicer arietinum* L.): role of stress treatment. *Plant Cell Rep*. 28: 1289-1299.
[33] Ali, M. A. and J. K. Jones. 2000. Microspore culture in *Corchorus olitorius*: effect of growth regulators, temperature and sucrose on callus formation. *Indian J Exp Biol.* 38(6): 593-597.
[34] Dragosavac, D. C., S. Stevovic. and S. Zdravkovic. 2010. Impact of genotype, age of three and environmental temperature on androgenesis induction of *Aesculus hippocastanum* L. *African J Biotech*. 29: 4042-4049.
[35] Ochatt, S., C. Pech, R. Grewal, C. Conreux, M. Lulsdorf. and L. Jacas. 2009. Abiotic stress enhances androgenesis from isolated microspores of some legume species (*Fabaceae*). *J Plant Physiol*. 166: 1314-1328.
[36] Barany, I., M. P. Gonzalez, B. Fadon, J. Mityko, M. C. Risueno. and P. S. Testillano. 2005. Microspore-derived embryogenesis in pepper (*Capsicum annuum* L.): subcelluar rearrangements through development. *Biol Cell* 97(9): 709-22.
[37] Kim, M., I. C. Jang, J. A. Kim, E. J. Park, M. Yoon, and Y. Lee. 2008. Embryogenesis and plant regeneration of hot pepper (*Capsicum annuum* L.) through isolated microspore culture. *Plant Cell Rep*. 27: 425-434.
[38] Kim, M., Park, E. J. and Y. Lee. 2013. High-quality embryo production and plant regeneration using a two-step culture system in isolated microspore cultures of hot pepper (*Capsicum annuum* L.). *Plant Cell Tiss Org. Cult*. 112: 191–201.

[39] Islam, S. M. S. 2010c. Effect of embryoids age, size and shape for improvement of regeneration efficiency from microspore-derived embryos in wheat (*Triticum aestivum* L.) *Plant Omics J.* 3(5): 149-153.
[40] Olmedilla, A. 2010. *Microspore Embryogenesis*. In: Pua EC, Davey MR (eds) Plant Developmental Biology- Biotechnological Perspectives. Springer-Verlag Berlin Heidelberg, vol. 2, pp 27-44.
[41] Talebi, R., M. R, Rahemi, H. Arefi, M. Nourozi. and Bagheri, N. 2007. *In vitro* plant regeneration through anther culture of some Iranian local rice (*Oryza sativa* L.) cultivars. *Pak. J. Bio. Sci.* 10(12): 2056-2060.
[42] Khatun, R., Islam, S. M. S., Ara, I., N. Tuteja. and M. A. Bari. 2012. Effect of cold treatment and different media in improving anther culture response in rice (*Oryza sativa* L) in Bangladesh. *Indian J. Biotech.* 11: 458-463.
[43] Schmid, J. E. 1990. *In vitro* production of haploids in *Triticum spelta.* In: Bajaj YPS (Eds.) Biotechnology in Agriculture and Forestry, vol. 13, pp 363-381.
[44] Lichter, R. 1982. Induction of haploid plants from isolated pollen of *Brassica napus*. Z. Pflanzenphysiol. 105: 427-434.
[45] Tuvesson, I. K. D. and R. C. V. Öhlund. 1993. Plant regeneration through culture of isolated microspores of *Triticum aestivum* L. *Plant Cell, Tiss Org Cult.* 34(2): 163-167.
[46] Joersbo, M., R. B. Jorgensen. and P. Olesen. 1990. Transient electropermeabilization of barley (*Hordeum vulgare* L.) microspores to propidium iodide. *Plant Cell, Tiss Org Cult.* 23: 125-129.
[47] Pescitelli, S. M., C. D. Johnson. and J. F. Petolino. 1990. Isolated microspore of maize: Effect of isolation technique, reduced temperature, and sucrose level. *Plant Cell Rep*. 8: 628-631.
[48] Olsen, F. L. 1991. Isolation and cultivation of embryogenic microspores from barley (*Hodeum vulgare* L.). Hereditas 115: 255-266The effects of ammonium nitrate, glutamine and asparagine as nitrogen sources. *Carlsberg Res. Commun.* 52: 393-404.
[49] Gustafson, V. D., P. S. Baenziger, M. S. Wright, W. W. Stroup. and Y. Yen. 1995. Isolated wheat microspore culture. *Plant Cell, Tiss Org. Cult.* 42: 207-213.
[50] Bedinger, P. A. and M. D. Edgerton. 1990. Developmental staging of maize microspores reveals a transition in developing microspore proteins. *Plant Physiol.* 92: 474-479.
[51] Mejza, S. J., V. Morgant., D. E. DiBona. and J. R. Wong. 1993. Plant regeneration from isolated microspores of *Triticum aestivum*. *Plant Cell Rep.* 12: 149-153.