ISSN 0258-7122
Bangladesh J. Agril. Res. 35(2) : 331-341, June 2010

IN VITRO SHOOT REGENERATION THROUGH ANTHER CULTURE OF *BRASSICA* SPP.

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Abstract

The experiment was conducted to investigate the performance of three different genotypes (BARI Sarisha-6, BARI Sarisha-8, and BARI Sarisha-11) in two different media viz., MS and B5 with different concentrations of phytohormone (2, 4-D) for callus induction from uninucleate stage anthers of *Brassica* and subsequent plant regeneration in MS media with different concentrations of phytohormone (BAP and NAA). Among the genotypes, BARI Sarisha-8 showed the best performance for all the parameters of callus induction. The performance of BARI Sarisha-6 was poor compared to others. Maximum rate of callus induction (%) was observed in MS + 0.5 mg/L 2, 4-D followed by B5 + 0.5 mg/L 2,4-D. The media combination MS + 1.0 mg/L BAP 0.3 mg/L 2,4-D showed the best performance for maintenance of calli. Significant variations were observed among the genotypes and media composition for shoot regeneration. Among the genotypes, BARI Sarisha-8 showed the best performance for shoot regeneration followed by BARJ Sarisha-l1. The genotype BARI Sarisha-8 produced higher percent of shoots/calli and required minimum days for shoot initiation. Higher percent calli without shoot were produced by the genotype BARI Sarisha-6. The media combination MS + 2.0 mg/L BAP + 0.5 mg/L NAA showed the best performance for shoot regeneration and required maximum days for shoot initiation.

Keywords: Regeneration, BARI Sarisha-6, BARI Sarisha-8, BARI Sarisha-11, anther culture, phytohormone

Introduction

The oleiferous *Brassica* represented by rapeseed and mustard, commonly known as mustard, plays an important role in vegetable oil production of the world. It is the third most important edible oil sources in the world after soybean and palm (Piazza and Foglia, 2001; Walker and Booth, 2001; FAO, 2003). Still today, Bangladesh is facing a huge shortage in edible oils (BBS, 2007). The per capita consumption of edible oil is one of the lowest in the world (11 g/head/day), and one fifth of recommended requirement for a balance diet (Elias, 1998). *Brassica* spp. has consistently proven to be one of the most recalcitrant members of the

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Brassiceae in tissue culture (Hachy *et al.*, 1991). Due to the recalcitrant nature of *Brassica* tissue *in vitro,* it eluded any notable progress in this regard for a long time. Fortunately, constant efforts with more diverse cultural procedures have overcome many of the obstacles (Bhojwani *et al.*, 1988). The regeneration of plants from tissue culture is an important and essential component of biotechnological research. Anther culture has attracted considerable attention as supplementary tools to crop improvement.

Anther culture derived haploids have been used to produce homozygous diploids, which accelerate breeding programmes. Haploid production of *Brassica* spp. through anther culture proved to be an important approach of tissue culture. Traditionally, plant breeders usually achieve homozygosity of the cross products by using the self-fertilization, a time consuming process (Morrison and Evans, 1988). By anther culture, homozygous plant can be produced within a year as compared to the long inbreeding method, which might take 8-10 years. The genotype, culture media, physiological status of donor plant, anther wall factor, stage of pollen development, and effect of temperature and light basically influence callus induction and plant regeneration frequently from immature anther. Anther culture and subsequent plant regeneration offer an alternative and efficient technique to conventional breeding method and enable production of several plants from single anther. Therefore, *in vitro* techniques are considered to be alternative tools of conventional method of *Brassica* improvement.

So, efforts to develop *in vitro* techniques for regeneration of *Brassica* through anther culture is not farely established in Bangladesh. So, there is a need for studying the anther culture technique for improvement of *Brassica.* The present research work has, therefore, been planned and executed for screening genotypes of *Brassica* spp. for their good regeneration potentiality through anther culture; the relative efficiency of different culture media for callus induction and subsequently shoot regeneration and establish a suitable and reproducible protocol for *in vitro* regeneration of planticts.

Materials and Method

Expeimental materials

The experiment was conducted in the Tissue Culture Laboratory of Biotechnology I)ivision, Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur during the period of July 2005 to November 2005. Three *Brassica* genotypes viz., BARI Sarisha-6 *(Brassica rapa* L. AA, 2n =20), BARI Sarisha-8 *(Brassica napus*, L. AACC, 2n = 38), and BARI Sarisha-11 *(Brassicajuncea*, L. AABB, 2n =36) were used for the present study. Seeds were germinated and grown under controlled environment in the green house experimental field. Unopened flower buds were collected from the donor plants

and the microspores at the early to mid uninucleated stage (observed using 1% aceto-carmine under microscope). The selected buds with microspore at the early to mid uninucleate stage were wrapped with polythene bag and kept at 4°C chamber for 7 days. The abnormal anthers of the bud were discarded and those at the appropriate size and age were used in the culture.

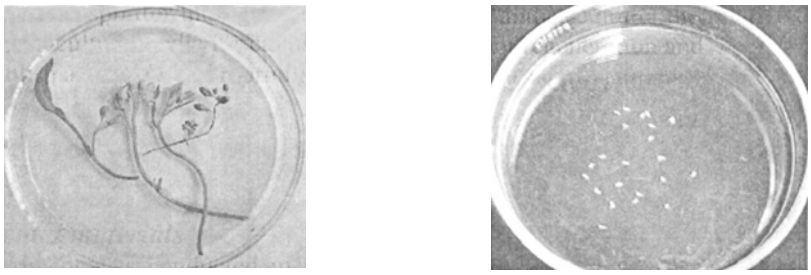


Plate 1. Cluster of buds that were used as explant source (left) and excised anthers that were inoculated as explant (right).

Media used

For callus induction

MS and B5 media with different phytohormonal concentrations

MS + 0.1 mg/L 2,4-D	B5 + 0.1 mg/L 2,4-D
MS + 0.2 mg/L 2,4-D	B5 + 0.2 mg/L 2,4-D
MS + 0.3 mg/L 2,4-D	B5 + 0.3 mg/L 2,4-D
MS + 0.4 mg/L 2,4-D	B5 + 0.4 mg/L 2,4-D
MS + 0.5 mg/L 2,4-D	B5 + 0.5 mg/L 2,4-D

For maintenance of callus

MS media with different phytohormonal combinations

MS+ 0.5 mg/L BAP + 0.3 mg/L 2,4-D

MS+ 1.0 mg/L BAP + 0.3 mg/L 2,4-D

MS+ 1.5 mg/L BAP + 0.3 mg/L 2,4-D

MS+ 2.0 mg/L BAP + 0.3 mg/L 2,4-D

For shoot initiation

MS media with different phytohormonal combinations

MS+ 1.0 mg/L BAP + 0.5 mg/L NAA

MS+ 2.0 mg/L BAP + 0.5 mg/L NAA

MS+ 3.0 mg/L BAP + 0.5 mg/L NAA

MS+ 4.0 mg/L BAP + 0.5 mg/L NAA

Culture techniques

The following culture techniques were employed in the present investigation-

i) Inoculation of anthers.

ii) Subculture or transfer of the callus for regeneration.

i. Inoculation of anthers

Anthers were removed from the sterilized buds using a fine Tweezers (forceps) and inoculated on sterile tubes and petridishes with culture media and incubated at 28°C room temperature for 3-4 weeks in complete dark for callus formation. Fifty anthers of each genotype were inoculated into each treatment.

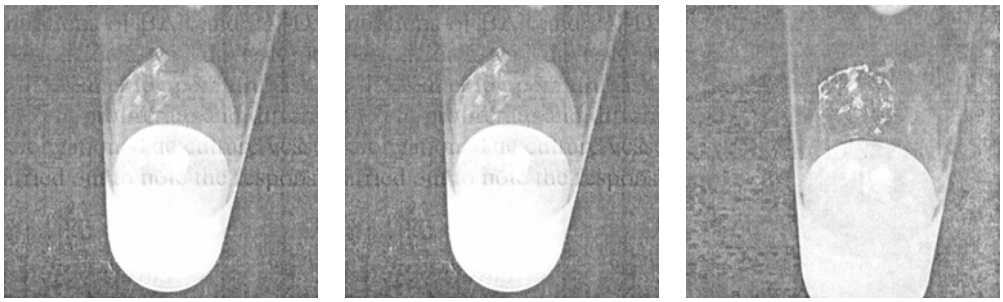
ii. Subculture or transfer of the callus for regeneration

Four to five weeks after inoculation of anthers, the calli attained convenient size. Then they were removed aseptically from the petridish on a sterilized glass plate inside the laminar airflow cabinet and were placed again on freshly prepared sterilized medium containing appropriate hormonal supplements for plant regeneration from the callus. Sub culture was done in the MS media containing different combinations and concentrations of BAY and 2,4-D. The sub cultured petridishes were again incubated at 22 ± 2°C with 16 hrs photoperiod for 5-7 days. Repeated sub cultures were done at an interval of 15 days and incubated under the same temperature as mentioned previously for maintenance of calli. The sub cultured ealli continued to proliferate and differentiated into shoots. After shoot initiation, more light intensity was used for shoot elongation. The culture vessels showing signs of contamination were discarded. Day to day observation was carried out to note the responses.

Results and Discussion

Induction of callus

Assessment on callus induction was studied through three quantitative traits-number of anthers showing callus, percent of callus induction, and days to callus initiation. Among the three genotypes, BARI Sarisha showed the best performance for callus induction (23.47%) followed by BARI Sarisha-11 (20.80°) and BARI Sarisha-6 (16.00%) (Table 1, Plate 2). It was found that BARI Sarisha-8 showed comparatively good potentiality in callus growth than other genotypes. This finding confirmed that of Javed and Hassan (19)2) who noted that callus growth was better in *B. napus* genotype. Callus initiation started from 23 to 29 days after culture. Time required for callus initiation was insignificant among the genotypes.



(a) (b) (c)

Plate 2. (a) Callus initiation of BARI Sarisha-6; (b) Callus initiation of BARI Sarislia-8 and (c) Callus initiation of BARI Sarisha-11.

Among the ten combinations and concentrations of media, average number of anthers showing callus ranged from 2.33 to 18.67 out of 50 anthers (Table 2). The highest number of anthers showing callus was obtained in media containing MS + 0.5 mg/L 2,4-D (37.33%) followed by B5 + 0.5 mg/L 2,4-D (32.67%), and MS 0.4 mg/L 2,4-D (26.67%). On the other hand, the lowest number of anthers showing callus was obtained in MS + 0.1 mg/L 2,4-D (4.67%) followed byB5 + 0.1 mg/L 2,4-D (8.00%) (Table 2). There was no statistical difference between the media containing MS + 0.4 mg/L 2,4-0 (26.67%) and B5 + 0.4 mg/L 2,4-D (24.67%) (Table 2). From the results, it was observed that performance of media with these concentrations of 2,4-D were significantly different, suggesting that callus induction frequency from anther greatly influenced by the concentrations used. The result is correlated with the results of Ockendon and McClcnaghan (1093). They reported that 2,4-D had a large effect on callus induction and increasing 2,4-D was usually beneficial. In respect of time requirement for callus induction, media containing 135 + 0.5 mg/L 2,4-D and MS 0.5 mg/L 2,4-D were statistically identical and required minimum (24.22) time to callus initiation which were significantly different from other composition of the media,. Both MS ± 0.1 mg/L 2,4-D and 135 0.1 mg/L 2,4-D and B5 + 0.1 mg/L 2,4-0 took maximum (28.56) time to callus induction followed by MS 0.4 mg/L 2,4-0 and B5 + 0.4 mg/L 2,4-0 (Table 2), which were also statistically identical. There were no significant differences among the media containing MS + 0.3 mg/L 2,4-D and B5 + 0.3 mg/L 2,4-D (Table 2). This result indicated that days to callus initiation was found to be influenced by different concentrations of 2, 4-D in MS medium for anther culture of *Brassica.*

From the previous discussion, it was found that BARI Sarisha-8 showed better performance among genotypes and MS medium containing 0.5 mg/L 2, 4-D showed the best performance. In case of interaction, it was observed that genotype BARI Sarisha-8 produced the highest percentage of callus on both in

MS 0.5 mg/L 2,4-D and B5 + 0.5 mg/L media. BARI Sarisha-11 also produced the highest percentage of callus in MS medium containing 0.5 mg/L 2, 4-D, which was statistically identical to BARI Sarisha-8. In the previous discussion, it was found that there were no significant differences among the genotypes for days to callus initiation. But the interaction of genotype and media composition for days required for callus initiation indicated that there were significant variations among the genotypes. BARI Sarisha-8 × 135 + 0.5 mg/L 2, 4-D took minimum time for days to callus initiation. On the other hand, BARI Sarisha-6 × MS + 0.1 mg/L 2. 4- 0, BARI Sarisha-11 × MS + 0.1 mg/L 2, 4-0 and BARI Sarisha-6 x B5 ± 0.1 mg/L 2,4-D took the maximum time for callus initiation, which were statistically identical.

Table l. Performance of different genotypes on media with different concentrations of phytohormone for callus induction.

Genotype	No. of anthers showing callus induction	Callus induction (%)	Days to callus induction
BARI Sarisha-6	8.00 b	16.00 b	26.60
BARI Sarisha-8	11.73 a	23.47 a	26.60
BARI Sarisha-11	10.40 ab	20.80 ab	26.33

Table 2. Performance of media with different concentrations of phytohormonc for callus induction *of Brassica* genotype.

Supplement	No. of anthers showing callus induction	Callus induction (%)	Days to callus induction
MS -f 0.1 mg/L 2,4-D (T_1)	2.33 g	4.67 g	28.56 a
MS + 0.2 mg/L 2,4-D (T_2)	6.78 e	13.56 e	27.56 b
MS + 0.3 mg/L 2,4-D (T_3)	9.67 d	19.33 d	26.33 c
MS + 0.4 mg/L 2,4-D (T_4)	13.33 c	26.67 c	25.89 c
MS + 0.5 mg/L 2,4-D (T_5)	18.67 a	37.33 a	24.22 d
B5 + 0.1 mg/L 2,4-D (T_6)	4.00 fg	8.00 fg	28.56 a
B5 ± 0.2 mg/L 2,4-D (T_7)	5.67 ef	11.33 ef	27.56 b
B5 + 0.3 mg/L 2,4-D (T_8)	11.33 cd	22.67 cd	26.33 c
B5 + 0.4 mg/L 2,4-D (T_9)	12.33 c	24.67 c	25.89 c
B5 + 0.5 rng/L 2,4-D (T_{10})	16.33 b	32.67 b	24.22 d

Maintenance of callus

Among the genotypes, there was no conspicuous effect on percent proliferation of callus. The proliferated calli were transferred to regeneration medium for

shoot induction. Among the combinations, best callus proliferation was noted in MS + 1.0 mg/L BAP + 0.3 mg/L 2,4-D (12) followed by MS 1.5 mg/L BAP 0.3 mg/L 2,4-D for all the genotypes (Table 3). The percent of callus proliferation ranged from 46.00 to 90.00 %. From the results, it may be concluded that lower concentration of cytokinin and auxin like 1.0 mg/L BAP + 0.3 mg/L 2,4-D (T_2) is likely to be suitable for callus maintenance (Plate 3). The present finding is correlated with the results of Khan (2002) and Miah *et al.* (1996). The interaction of BARI Sarisha-6 and MS + 1.0 mg/L BAP + 0.3 mg/L 2, 4-D (94.00%) (Table4) showed the best performance for callus proliferation followed by BARI Sarisha-6 x MS + 1.5 mg/L BAP + 0.3 mg/L 2, 4-D.

Table 3. Effect of media combination on maintenance of callus.

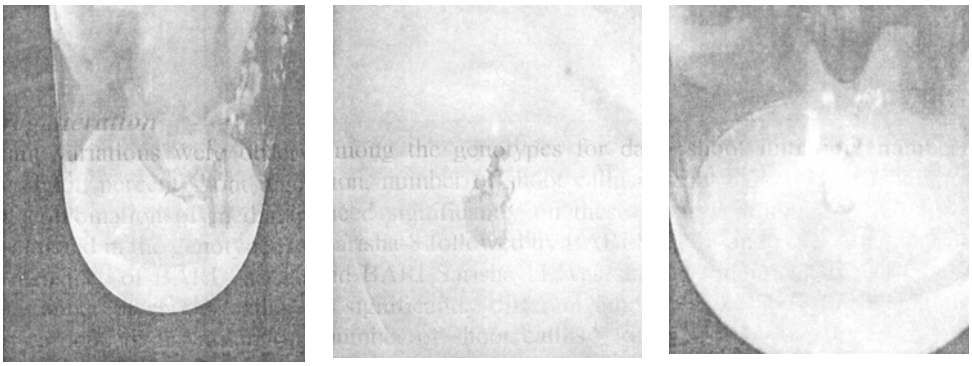
Supplement	No. of explants showing callus proliferation	Callus proliferation (%)
MS+0.5mg/L BAP+0.3 mg/L 2,4-D (T_1)	23.00c	46.00 c
MS + 1.0 mg/L BAP + 0.3 mg/L 2,4-D (T_2)	45.00 a	90.00 a
MS + 1.5 mg/L BAP + 0.3 mg/L 2,4-D (T_3)	43.33 ab	86.67 ab
MS ± 2.0 mg/L BAP + 0.3 mg/L 2,4-D (T_4)	41.33 b	82.66 b

Table 4. Combined effect of genotype and media combination on maintenance of callus.

Supplements	Genotype	No. of explants showing callus proliferation	Callus proliferation (%)	Days to callus subculture
MS + 0.5 mg/L BAP + 0.3 mg/L 2,4-D (T_1)	BARI Sarisha-6	19 f	38 f	15
	BARI Sarisha-8	27 h	54 h	15
	BARI Sarisha-11	23 g	46 g	15
MS + 1.0 mg/L BAP + 0.3 mg/L 2,4-D (T_2)	BARI Sarisha-6	47 a	94 a	15
	BARI Sarisha-8	44 bc	88 bc	15
	BARI Sarisha-11	44 bc	88 bc	15
MS + 1.5 mg/L BAP + 0.3 mg/L 2,4-D (T_3)	BARI Sarisha-6	45 ab	90 ab	15
	BARI Sarisha-8	41 de	82 de	15
	BARI Sarisha-11	44 bc	88 bc	15
MS + 2.0 mg/L BAP + 0.3 mg/L 2,4-D (T_4)	BARI Sarisha-6	43 bcd	86 bcd	15
	BARI Sarisha-8	39 e	78 e	15
	BARI Sarisha-11	42 cd	84 cd	15

Shoot regeneration

Significant variations were observed among the genotypes for days to shoot initiation, number of calli showing shoot, percent shoot regeneration, number of shoots/callus, percent calli without shoot. Similarly different combinations of media influenced significantly on these characters. Maximum number of shoots (20.00) was initiated in the genotype BARI Sarisha-8 followed by BARI Sarisha-11 and BARI Sarisha-6 (Table 5). The performance of BARI Sarisha-6 and BARI Sarisha-11 was statistically identical. It was observed that average number of shoots/callus was significantly different among the genotypes. The genotype BARI Sarisha-8 produced maximum (2.50) number of shoots/callus followed by BARI Sarisha-11 and BARI Sarisha-6 (Table 5). The performance of BARI Sarisha-6 was statistically identical with BARI Sarisha- 11. Significant variation was observed among the genotypes for percent of calli without shoot. Maximum percentage of calli without shoot was found in the genotype BARI Sarisha-6 (71.00%). Minimum percentage of calli without shoot was observed in the genotype BARI Sarisha-8 (60.00%) (Table 5). In respect of time requirement for shoot regeneration, maximum time was required in BARI Sarisha-6 (23.00) followed by BARI Sarisha-8 and BARI Sarisha-11 (Table 5), which were statistically identical.



(a) (b) (c)

Plate 3. (a) Shoot regeneration of BARI Sharisha-6; (b) Shoot regeneration of BARI Sliarisha-8 and (c) Shoot regeneration of BARI Sharisha-11.

Number of calli showing shoot was significantly different in different media combinations. The MS medium containing 2.0 mg/L BAP + 0.5 mg/L NAA showed the highest percent shoot regeneration from calli followed by MS + 3.0 mg/L BAP + 0.5 mg/L NAA (49.33%) (Table 6). Minimum percentage of shoot regeneration was observed on the media combination of MS + 1.0 mg/L BAP + 0.5 mg/L NAA (20.00%) (Tahle 6). It was observed from the present study that

Table 5. Effect of genotype on shoot regeneration.

Genotypes	No. of calli showing shoot regeneration	Shoot regeneration (%)	Shoots/callus	Without shoot (%)	Days to shoot regeneration
BARI Sarisha-6	14.50 b	29.00 b	1.25 b	71.00 a	23.00 a
BARI Sarisha-8	20.00 a	40.00 a	2.50 a	60.00 b	21 .00 b
BARI Sarisha-11	17.25 ab	34.50 ab	1.83 b	65.50 ab	21.75 b

Table 6. Effect of media combination on shoot regeneration.

Media	No. of calli showing shoot regeneration	Shoot regeneration (%)	Shoot callus^{-1}	Without shoot (%	Days to shoot regeneration
MS + 1.0 mg/L BAP + 0.5 mg/L NAA	10.00 d	20.00 d	*1.55* b	80.00 a	21.67 b
MS + 2.0 mg/L BAP + 0.5 mg/L NAA	24.67 a	49.33 a	2.44 a	*50.67* d	23.33 a
MS + 3.0 mg/L BAP + 0.5 mg/L NAA	20.00 b	40.00 b	1.89 ab	60.00 c	21.00 b
MS + 4.0 mg/L BAP + 0.5 mg/L NAA	14.33 c	28.67 c	1.56 b	7l.33 b	21.00 b

use of BAP in the medium helped in shoot regeneration. This result corroborates with the findings of Du *et al.* (2000) and Wang *et al.* (2000), who reported that BAP was the most effective stimulator for shoot regeneration. It was found that the highest number of shoots/callus was found in media containing MS + 2.0 mg/L BAP + 0.5 mg/L NAA (2.44) and the lowest number of shoots/callus was found in media containing MS + 1.0 mg/L BAP + 0.5 mg/L NAA and MS 4.0 mg/L BAP 0.5 mg/L NAA, which were statistically identical (Table 6). It was observed that percent calli without shoot was significantly different among the media with different hormonal concentrations. The media with 1 .0 mg/L BAP + 0.5 mg/L NAA showed higher percentage of calli without shoot and the media with 2.0 mg/L BAP + 0.5 mg/L NAA showed lower percentage ofcalli without shoot (Table 6). The media with 1.0 mg/L BAP + 0.5 mg/L NAA took the maximum time for shoot regeneration. On the other hand, media with 2.0, 3.0

and 4.0 mg/L BAP + 0.5 mg/L NAA took minimum time for shoot regeneration, which were statistically identical (Table6).

In case of interaction, it was observed that both BARI Sarisha-8 and BARI Sarisha-11 produced the highest percent shoot regeneration in MS -t 2.0 mg/L BAP + 0.5 mg/L NAA media. BARI Sarisha-8 x MS + 2.0 mg/L BAP + 0.5 mg/L NAA also produced the highest percentage of shoots/callus followed by BARI Sarisha-8 x MS ± 3.0 mg/L BAP + 0.5 mg/L NAA). The media with 1.0 mg/L BAP + 0.5 mg/L NAA x all genotypes produced the highest percent calli without shoot (Table 6). On the other hand, the lowest percent calli without shoot found in BARI Sarisha-8 x MS + 2.0 mg/L BAP + 0.5 mg/L NAA and BARI Sarisha-l I x MS + 2.0 mg/L BAP + 0.5 mg/L NAA, which wore statistically identical (Table 6). These results indicated that interaction between genotype and media composition played a vital role for shoot regeneration. In respect of time requirement, BARI Sarisha-6 x MS + 2.0 mg/L BAP + 0.5 mg/L NAA and BARI Sarisha 6 x MS + 4.0 mg/L BAP + 0.5 mg/L NAA took the highest time for shoot regeneration, which were statistically identical. Whereas, BARI Sarisha-8 x MS + 3.0 mg/L BAP + 0.5 rngL1 NAA took the minimum time for shoot regeneration (Table 6).

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