

***In vitro* Plant Regeneration in *Brassica* spp.**

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Abstract

MS with different concentrations and combinations of hormones were used for *in vitro* multiple shoot regeneration in two varieties of *Brassica juncea*, namely, BARI Sarisha-11 and BARI Sarisha-16 and one variety of *Brassica campestris*, Tori-7. The highest percentage of responsive explants towards the regeneration of shoots was obtained on MS with 2.0 mg/l BAP, 0.2 mg/l NAA and 0.5 mg/l Kn in case of BARI Sarisha-11 and BARI Sarisha-16. For Tori-7, 3.0 mg/l BAP and 0.2 mg/l NAA was best for obtaining maximum number of shoots per explant. Among three varieties, BARI Sarisha-11 showed best response in terms of shoot regeneration as well as number of shoot per explant. Days required for induction of shoots was also recorded to be lowest in BARI Sarisha-11. Best root induction in BARI Sarisha-11 and BARI Sarisha-16 was achieved on hormone free MS. After proper hardening the *in vitro* regenerated plantlets were successfully transplanted into soil. Interestingly some of the *in vitro* regenerated shoots produced *in vitro* flowers on regeneration media as well as hormone free MS.

Introduction

The genus *Brassica* comprises many commercially important vegetable and oil seed crops. Oil seed *Braasica* is one of the most important sources of edible vegetable oil, industrial used oil and protein-rich product in the world. Oil seed *Brassica* ranks third after soybean and palm oil in the global production (Canola Council of Canada 2006). In this subcontinent three species of *Brassica*, namely, rapeseed, *Brassica rapa* (Syn. *Brassica campestris* L.), mustard, *B. juncea* (L.) Czern and Coss and *B. napus* L. belonging to Brassicaceae (Cruciferae) are cultivated for the production of oil.

In Bangladesh mustard oil is used for cooking purposes, for salad dressing and to marinate a number of food stuffs before cooking. It is also used as preservative for pickling purposes and for chutneys. The oil possesses a number of properties which has some significant human health benefit. Species of *Brassica* contains 40 - 45% oil and 20 - 25% protein. It is one of the best cooking oils particularly for heart patient because it has omega 3 and omega 6 fatty acid

compositions (linolic and alpha linolic acid, respectively). There are a number of other fatty acids, namely palmitic acid, stearic acid, arachidic acid, behenic acid, lignoceric acid, oleic acid, eicosenic acid, erucic acid and linolenic acid present in mustard oil. It is rich in natural antioxidants and Vitamin E. It has various medicinal properties too and is antibacterial in nature and fights against infections. Mustard oil has antifungal properties, due to the presence of substance like glucosinolate. The oil meal, which left over product of the seeds after extracting oil, is used as a protein supplement in dairy, beef, poultry ration and is recognized for its consistent high quality and value. Besides these, some cultivars of *Brassica campestris* are grown for their high erucic acid level. This oil has also industrial application in plastics, lubricants, lacquers and detergents.

The productivity and quality of these crops are affected by various biotic and abiotic stresses. Biotic stresses that reduce the production of crops include infestation by insects, damage due to bacterial and fungal pathogens. In Bangladesh rapeseed and mustard suffer from 14 different diseases. Among these leaf blight, downy mildew and parasitic plant are important. The most important disease of *Brassica* in Bangladesh is leaf blight disease, caused by *Alternaria brassicae* (Ahmed 1952). The disease causes leaf blight, pod blight and seed abnormalities (Meah 1986). Crop loss between 30 and 100% due to this disease has been reported (Meah et al. 1988). The most important pest of this crop is aphid (*Lipaphis erysimi*).

Although a number of new varieties have been released from different research institutes of Bangladesh, none has come up to the expectation of breeders, because the released varieties are not resistant to above diseases and pests. Plant breeders in the past for several decades have used interspecific hybridization to transfer genes between species. Cross incompatibility barriers severely limit the possibility of gene transfer between species, although some of the *Brassica* species can be easily crossed conventionally (Puddephat et al. 1996). Conventional breeding programmes alone were not successful enough in *Brassica* due to high degree of segregation upon cross pollination and unavailability of suitable germplasm. Conventional breeding of *Brassica* is labor and resource intensive and time consuming; it takes eight to ten generations to develop a new variety (Cardoza and Stewart 2006). On the other hand, in recent years genetic transformation technique has been used to develop biotic and abiotic stress tolerant crop plants. However, before embarking upon such a programme on genetic transformation it is necessary to establish *in vitro* plant regeneration system of that particular plant.

Brassicaceae are generally considered to be recalcitrant in tissue culture (Zhang et al. 1998). However, there are reports on the use of *in vitro* techniques like

organogenesis and somatic embryogenesis for the regeneration of different *Brassica* spp. (Antonio et al. 1987, Jain et al. 1988, Ono et al. 1994, Koh and Loh 2000, Khan et al. 2002). Considering the importance of *Brassica* spp. in the present investigation attempts were made to establish a genotype independent regeneration protocol for locally available oilseed *Brassica* spp.

Materials and Methods

Three varieties of *Brassica* spp., namely *Brassica juncea* var. BARI Sarisha-11 and BARI Sarisha-16 and *Brassica campestris* var. Tori-7 were collected from the Oil Seed Division of Bangladesh Agricultural Research Institute (BARI) and used in the present investigation.

The surface sterilized seeds were inoculated on to half strength of MS with 2% sucrose and 0.8% agar for germination and seedling development. The cultured seeds were kept in dark condition till the germination and then transferred to 16 hrs light condition at $25 \pm 2^\circ\text{C}$ in growth room. Normally germination took place within 2 - 3 days of seed inoculation. Explants like cotyledonary leaf with or without petiole, petiole without cotyledon, cotyledonary node, hypocotyl, epicotyl and leaf segment were excised from germinating seeds. Four to five days old seedlings were used as the source of explant. Isolated explants were cultured on MS containing BAP, NAA and Kn singly or in combinations for regeneration. *In vitro* regenerated shoots were subcultured regularly to fresh medium at an interval of 12 - 15 days for further multiplication. Elongated shoots were separated and cultured on rooting medium for root formation. About 2 - 3 cm long shoots were separated and cultured on rooting medium containing full and half strengths of MS without hormonal supplement or with different concentrations and combinations of IBA and NAA. The plantlets with sufficient root system were then transplanted to small pots containing sterilized soil. Pots were covered with transparent perforated polythene bags. After proper hardening, plantlets were transferred to natural environment.

Results and Discussion

Different concentrations of BAP, NAA and Kn were used in MS to determine the optimum media composition for initiation and development of multiple shoots from three varieties of *Brassica* used in this investigation. Among the different explants used, cotyledonary leaf with petiole and hypocotyl were found to be the most responsive in terms of percentage of shoot regeneration as well as the number of shoots per explant in all the varieties tested. In case of BARI Sarisha-11 and BARI Sarisha-16, MS with 2.0 mg/l BAP, 0.2 mg/l NAA and 0.5 mg/l Kn

showed best response for callus induction and multiple shoot regeneration. Percentage of shoot regeneration as well as the number of shoots per explant were maximum on this medium composition (Table 1). In this case, 83.33% explants of BARI Sarisha-11 and 81.00% explants of BARI Sarisha-16 showed shoot regeneration. The number of shoots per explant was 3 - 7 in BARI Sarisha-11 (Fig. 1A) and 3 - 5 shoots in BARI Sarisha-16. On the other hand, Tori-7 showed best multiple shoot regeneration on MS with 3.0 mg/l BAP and 0.2 mg/l NAA. Shoot regeneration percentage in this variety was 46.66 and the number of shoots per explant was 2 - 4 (Table 1) in this variety.

Shoot primordia were originated from the cut end of the petiole of cotyledonary leaf explant. A small number of shoot regeneration (1.6 - 10.0%) was observed when cotyledonary leaf without petiole used as explant. So, the presence of petiole was found to be critical for shoot regeneration when cotyledon was used as explant.

Next to cotyledonary leaf with petiole, hypocotyl showed second highest response regarding percentage of shoot development (Fig. 1B). In case of BARI Sarisha-11, the percentage of shoot regeneration from hypocotyl was 68.33 (Table 1) and in case of BARI Sarisha-16 it was 66.03 (Table 1). In case of Tori-7, after inoculation creamy white coloured callus was formed at both cut edges of hypocotyl and with the increase of time most of the explants became brown and finally died. It may be mentioned that hypocotyl as a suitable explant for regeneration of shoots was reported Tang et al. (2003), Zhang et al. (2006) and Khan et al. (2010).

In the present investigation a considerable variation in shoot regeneration from cotyledon explants was observed in all the varieties used. Among the three varieties used, *B. juncea* var. BARI Sarisha-11 was most responsive based on the above mentioned observation. It was also noticed that *B. juncea* showed higher response towards *in vitro* regeneration than that of *B. campestris* (Tori-7). It showed maximum percentage of response in terms of shoot regeneration as well as highest number of shoots per explants (Fig. 1C). Moreover, the days required for shoot regeneration was less than other varieties. *Brassica campestris*, in contrast to other species of *Brassica*, has consistently been proved more difficult to regenerate *in vitro*. Tang et al. (2003) observed similar response towards shoot regeneration in both between and within *Brassica* species. George and Rao (1980) observed maximum regeneration from cotyledon explants in *B. juncea* with BAP and NAA rather than BAP alone. Hachey et al. (1991) had also reported efficient regeneration in *B. campestris* with BAP in combination with NAA. Cardoza and Stewart (2004) observed that regeneration in *Brassica* is highly genotype dependent and genotype specificity is a limiting factor in *Brassica* tissue culture

and regeneration, which severely limits the germplasm that can be manipulated or improved.

Table 1. Effect of BAP, NAA and Kn on shoot regeneration from cotyledonary leaf with petiole (CP+) and hypocotyl (H) explants of three varieties of *Brassica**

Varieties	Hormonal supplements (mg/l)			Explants	No. of explants inoculated	% of responsive explants	Days to shoots initiation	Mean no. of shoots/explant
	BAP	NAA	Kn					
BARI Sarisha -11	0.5	0.2	0.5	CP+	60	50.00	14-15	3.68
				H	60	-	-	-
	1.0	0.2	0.5	CP+	60	70.00	8-10	2.96
				H	60	-	-	-
	2.0	0.2	0.5	CP+	60	83.33	9-12	7.20
				H	60	68.33	30-35	4.01
	2.0	0.2	1.0	CP+	60	66.66	9-16	4.36
				H	60	64.00	30-32	5.55
	5.0	0.2	1.0	CP+	60	15.00	15-17	1.30
				H	60	16.66	35-36	1.02
BARI Sarisha -16	0.5	0.2	0.5	CP+	60	36.66	14-15	2.16
				H	60	-	-	-
	1.0	0.2	0.5	CP+	60	50.00	16-18	2.95
				H	60	-	-	-
	2.0	0.2	0.5	CP+	60	81.00	17-20	5.30
				H	60	66.03	30-32	3.20
	2.0	0.2	1.0	CP+	60	71.66	9-14	2.19
				H	60	63.66	30-35	2.20
	5.0	0.2	1.0	CP+	60	10.00	15-18	1.20
				H	60	13.63	30-34	1.02
Tori-7	0.5	0.2	-	CP+	60	1.06	18-20	1.22
				H	60	-	-	-
	1.0	0.2	-	CP+	60	3.33	18-20	1.36
				H	60	-	-	-
	2.0	0.2	-	CP+	60	6.66	15-18	3.06
				H	60	-	-	-
	2.0	0.5	-	CP+	60	7.00	18-20	1.04
				H	60	-	-	-
	3.0	0.2	-	CP+	60	46.66	18-20	4.11
				H	60	-	-	-

* MS was used in all media combinations.

In most *Brassica* species, regeneration is dependent on the age of the explants (Ovesna et al. 1993). It was also reported that young explants have been shown to give better results than older explants. Most researchers have found that explants excised from 4 - 5 days old seedlings gave optimal regeneration rates in different *Brassica* spp. (Sharma et al. 1990, Hachey et al. 1991). During the present investigation, cotyledonary leaf with petiole and hypocotyl from 4 - 5 days old

seedlings also showed best response towards both direct and indirect shoot regeneration.

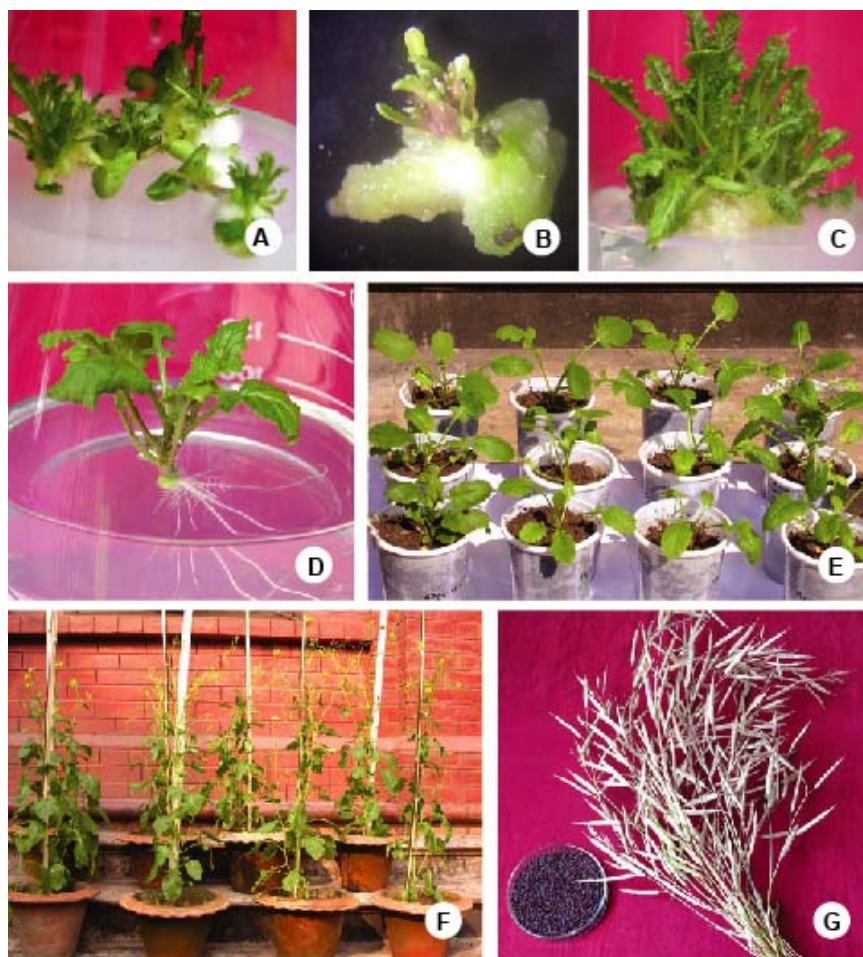


Fig. 1. *In vitro* regeneration of *Brassica juncea* var. BARI Sarisha-11 on MS with 2.0 mg/l BAP, 0.2 mg/l NAA and 0.5 mg/l Kn. A. Initiation of shoots from cotyledonary leaf with petiole. B. Multiple shoot formation from callus of hypocotyl. C. Elongated shoots derived from cotyledonary leaf with petiole. D. Induction of roots on hormone free MS. E. *In vitro* regenerated plantlets transferred to soil in small plastic pots. F. Flowers and pod formation in *in vitro* regenerated plants. G. Harvested pods developed from *in vitro* raised plants.

Root induction at the base of the *in vitro* regenerated shoot is essential to establish the regenerated shoots in soil. In varieties BARI Sarisha-11 and BARI Sarisha-16 root induction was possible on MS without any hormonal supplement. About 90% shoots of BARI Sarisha-11 showed profuse root induction (Fig. 1D). On the other hand 80% shoots of var. BARI Sarisha-16 produced root on the same medium. However, Tori-7 did not produce any root

on hormone free MS but only 20% of the regenerated shoots produced root on half strength of MS with 0.3 mg/l IBA.

Interestingly it was recorded that the *in vitro* regenerated shoots of BARI Sarisha-11, BARI Sarisha-16 and Tori-7 produced *in vitro* flowers on regeneration media. These *in vitro* flowers were relatively smaller than these of *in vivo* produced flowers. This result revealed that regenerated shoots may synthesize flower inducing hormone in their body and induced flowering spontaneously without any external flowering hormonal supplement. The similar result on *in vitro* flowering of *B. campestris* and in cauliflower was also reported by Verma and Singh (2007) and Vandana et al. (1995), respectively.

After sufficient development of roots, plantlets of varieties BARI Sarisha-11 and BARI Sarisha-16 were successfully transplanted into small plastic pots. Using this method the survival rate of the transplanted plantlets was 100%. The plantlets survived in the pots containing soil (Fig. 1E). The plantlets of BARI Sarisha-11 and BARI Sarisha-16 flowered within 2.5 months (Fig. 1F) and they flowered throughout the season. Seeds were harvested from the mature plants. Plants of BARI Sarisha-11 produced by culture also established in the field. The harvested pods developed from the *in vitro* raised plant BARI Sarisha-11 (Fig. 1G).

Based on the results it may be concluded that regeneration protocol developed in the present investigation for BARI Sarisha-11 and BARI Sarisha-16 is reliable and be effectively utilized for genetic transformation of *Brassica* species.

References

- Ahmed QA (1952) Fungi of East Bengal. Pak. J. For. 2: 91-115.
- Antonio BA, Namai H and Kikuchi F (1987) Tissue culture ability of vegetative organs from different cultivars of *Brassica*. SABRAO Journal 19(2): 73-79.
- Canola Council of Canada (2006) <http://www.canola council.ca>.
- Cardoza V and Stewart CN Jr (2004) *Brassica* biotechnology: progress in cellular and molecular biology. *In Vitro Cell Dev. Biol-Plant*. 40: 542-551.
- Cardoza V and Stewart NC (2006) Canola (*Brassica napus* L.). Methods Mol. Biol. (ed. Wang, K.). Human Press Inc., Totowa, New Jersey 343: 257-266.
- George L and Rao PS (1980) *In vitro* regeneration of mustard plants (*Brassica juncea* var. RAI-5) on cotyledon explants from non-irradiated, irradiated and mutagen-treated seed. Ann. Bot. 46: 107-112.
- Hachey JE, Sharma KK and Moloney MM (1991) Efficient shoot regeneration of *Brassica campestris* using cotyledon explants cultured *in vitro*. Plant Cell Rep. 9: 549-554.

- Jain RK, Chawdhury JB, Sharma DR and Friedt W** (1988) Genotypic and media effects on plant regeneration from cotyledon explant cultures of some *Brassica* species. *Plant Cell, Tiss. Org. Cult.* **14**(3): 197-200.
- Khan MR, Rashid H and Quraishi A** (2002) Effect of various growth regulators on callus formation and regeneration in *Brassica napus* cv. Oscar. *Pak. J. Biol. Sci.* **5**: 693-695.
- Khan MA, Arif Hasan Khan Robin ABM, Nazim-Ud-Dowla MANS, Talukder SK and Hasan L** (2010) *In vitro* regeneration potentiality of oil seed *Brassica* genotypes in differential growth regulators. *Bangladesh J. Agril. Res.* **33**(2): 189-191.
- Koh WL and Loh CS** (2000) Direct somatic embryogenesis, plant regeneration and *in vitro* flowering in rapid-cycling *Brassica napus*. *Plant Cell Rep.* **19**: 1177-1183.
- Meah MB** (1986) Management of diseases of oilseeds; mustard, groundnut, sesame. *In: BAU Research Progress. Workshop held on 4-5 October, 1986.* **1**: 82-100.
- Meah MB, Howlider MAR and Alam MK** (1988) Effect of fungicide spray at different time and frequencies on *Alternaria* blight of mustard. *Thai. J. Agric. Sci.* **21**: 101-107.
- Ono Y, Takalata Y and Kaizuma N** (1994) Effect of genotype on shoot regeneration from cotyledonary explants of rapeseed (*B. napus* L.). *Plant Cell Rep.* **14**: 13-17.
- Ovesna J, Ptacek L and Opatrny Z** (1993) Factors influencing the regeneration capacity of oilseed rape and cauliflower in transformation experiments. *Biol. Plant* **35**: 107-112.
- Puddephat IJ, Riggs TJ and Fenning TM** (1996) Transformation of *Brassica oleracea*: a critical review. *Mol. Breed.* **2**: 185-210.
- Sharma KK, Bhojwani SS and Thorpe TA** (1990) Factors affecting high frequency differentiation of shoots and roots from cotyledon explants of *Brassica juncea* (L.) Czern. *Plant Sci.* **66**: 247-253.
- Tang GXW, Zhou JHZLi, Mao BZ, He ZH and Yoneyama K** (2003) Medium, explant and genotype factors influencing shoot regeneration in oilseed *Brassica* spp. *J. Agro. and Crop Sci.* **189**: 351-358.
- Vandana AK, Kumar A and Kumar J** (1995) *In vitro* flowering and pod formation in cauliflower (*B. oleracea* var. botrytis). *Curr. Sci.* **69**(6): 543-545.
- Verma R and Singh RR** (2007) Regeneration and *in vitro* flowering in *Brassica campestris* (L.) var. Bhavani. *Nature* **5**: 21-24.
- Zhang FL, Takahata Y and Xu JB** (1998) Medium and genotype factors influencing shoot regeneration from cotyledonary explant of Chinese cabbage (*Brassica campestris* L. spp. pekinensis). *Plant Cell Rep.* **17**: 780-786
- Zhang Y, Xu J, Han L, Wei W, Guan Z, Cong L and Chai T** (2006) Efficient shoot regeneration and *Agrobacterium*-mediated transformation of *Brassica juncea*. *Plant Mol. Bio. Rep.* **24**: 255a-255i.