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Research Article

IN VITRO PLANT REGENERATION FROM CALLUS OF HYPOCOTYLS AND COTYLEDONARY EXPLANTS OF SOME INDIGENOUS MUSTARD VARIETIES

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ABSTRACT
Genus *Brassica* belongs to the family *Brassicaceae*, which is one of the 10 most economically important plant families. Oilseeds have the highest economic value among the *Brassica* crops. Oil crop breeding is more complex than breeding of cereals or legumes, as most oil crops are dual or multi-purpose crops, which requires the simultaneous manipulation of different quality characters. In the present investigation, the procedure for *in vitro* callogenesis and plant regeneration system were established for the oilseed *Brassicas* (*Brassica napus, Brassica juncea*). The hypocotyls and cotyledonary leaves with petioles of the *in vitro* grown seedlings were used as explants to develop callus in different callus induction media. Different combinations of hormones (2,4-D, BAP, IAA, IBA) and additives ($AgNO_3$, Casein Hydrolysate, Proline) were used for callus production. About 95%-100% formation of callus was obtained in medium supplemented with 0.5 mg l^{-1} 2, 4-D + 1 mg l^{-1} BAP. To induce regeneration of plants from the calli, MS medium (Murashige and Skoog, 1962) supplemented with various concentrations of different auxins and cytokinins were used. The medium supplemented with 2 mg l^{-1} BAP+ 3 mg l^{-1} $AgNO_3$ + 0.1 mg l^{-1} NAA was the best medium of regeneration for all the varieties. Efficient rooting was found in the medium with half-strength of MS plus 1.0 mg l^{-1} of IBA. Among the varieties, BARI-16 and BARI-8 showed better responses in almost all the media tested.

Keywords: *Brassica, Callogenesis, In Vitro Regeneration, Hypocotyls, Cotyledons*

INTRODUCTION
Genus *Brassica* belongs to the family *Brassicaceae*. The family *Brassicaceae*, includes about 3500 genera, is one of the 10 most economically important plant families (Warwick *et al.*, 2000). Oilseeds have the highest economic value among the *Brassica* crops. *Brassica* oilseeds are found within *Brassica junceae, Brassica napus, Brassica rapa* (syn *Brassica campestris*) and *Brassica carinata* collectively and are commonly called oilseed rape. Rapeseed and mustard occupy more or less leading rank among the oilseed crops of Bangladesh. About 70% of the total cultivated mustard oil in Bangladesh is covered by oilseed *Brassica*. Average yield from local varieties are 600-1000 kg/ha and high yielding varieties are 1400-2000 kg/ha, which contributes 71.3% of the total oilseed production of Bangladesh (BBS, 2005). The oilseed (*Brassica* sp.) cultivation has increased tremendously from last few years and by now it is the second largest contributor to the world supply of vegetative oil.

Oil crop breeding is more complex than breeding of cereals or legumes, as most oil crops are dual or multi-purpose crops, which requires the simultaneous manipulation of different quality characters. The advent of biotechnology has provided the plant breeders with new and more accurate tools that have the ability to compress the time taken in directed evolution of crop species. Tissue culture and transformation is an initial step in this aspect. Considerable research has been conducted in tissue culture, transformation and molecular breeding of the *Brassica*. Regeneration in *Brassica* is highly dependent on genotype and age and has been reported in several species (Guo *et al.*, 2005). These genotype and explant types of the *in vitro* culture are a limiting factor for the induction of genetic engineering technique to a wide number of genotypes. As a result, it is important to identify highly regenerated varieties (genotypes) that can be used in transformation via *Agrobacterium tumufaciens*. There are several reports on oilseed transformation with respect to the introduction of various new traits such as modified oil composition

(Knutzon *et al.*, 1992), herbicide tolerance (De Block *et al.*, 1989), altered protein composition (Altenbach *et al.*, 1992) and insect resistance (Stewart *et al.*, 1996). In this research, comparative studies on the *in vitro* regeneration protocols of some indigenous varieties (BARI-8, BARI-11 and BARI-16) have been carried out, so that, in future these varieties can be used to modify necessary traits by genetic manipulation.

MATERIALS AND METHODS

The research work was carried out in the Tissue Culture Lab, Department of Genetic Engineering and Biotechnology, University of Chittagong, to evaluate the hormonal and genotypic effects on callus induction and efficient plant regeneration from variety BARI-8, BARI-11 and BARI-16. Seeds of these varieties were collected from Bangladesh Agricultural Research Institute (BARI), Joydebpur, Dhaka, Bangladesh.

Sterilization of Seeds

At first, the collected seeds were surface sterilized by 70% ethanol (3-5 times), followed by 0.1% $HgCl_2$ (3 times) under the laminar air flow chamber. After using each type of reagents, the seeds had to be washed with sterile distilled water for five times or more. The seeds were then placed on filter paper in the petri plate to soak the water. It was then ready for inoculation in the germination medium.

Explants Preparation

The sterilized seeds are inoculated in half strength of MS medium for germination. Usually, half strength of MS with 2% sucrose and 0.8% (w/v) agar under light conditions were optimum for maximum seed germination. When the seedlings were 7-8 cm in length after about 6-7 days, these were excised into 0.5-1 cm pieces under laminar air flow cabinet. Cotyledonary leaves with petioles and hypocotyls segments of the excised pieces were transferred separately in callus induction media.

Callus Induction

Different concentrations of hormones and additives were used to evaluate the effects of supplements in callogenesis (Table 1). At the same time, the effects of same media on different varieties were also observed. The data of callus formation was collected on the basis of number of callus produced from number of inoculated explants. The supplements added in the callus induction medium in different concentrations were 2,4-D, BAP, $AgNO_3$, IAA, IBA, Proline and Casein Hydrolysate (CH). Both the hypocotyls and cotyledonary leaves with petioles were inoculated in each type of medium and their responses also recorded separately. The initiation of callus from the segments of seedlings was observed after 10-14 days. The calli had to be subcultured at this stage. It took about 28 days for complete development of embryogenic callus.

Shoot Induction

About 28-32 days after culture, calli were taken onto a sterile petri plate under laminar air flow chamber and were cut into convenient size by a sterile scalpel. The pieces of calli were then transferred on to the flasks containing freshly prepared media supplemented with BAP, NAA, $AgNO_3$, Kinetin and Proline at various concentrations (Table 1). Each flask was sealed with parafilm and maintained in a growth room under16 hour light and 8 hour dark cycle at 25 ± 2^0C. It took about 45 days for well shooting of plants. The plants were to be transferred after every 15 days in the respective media.

Root Induction

The well produced shoot was transferred in different rooting media (Table 1). Each of the regenerated shoot was cultured separately in MS medium containing 2% sucrose and solidified with 0.6% agar. The rooting media were supplemented with NAA and IBA. Both of these supplements in concentration of 1 mg l^{-1} produced better root formation in almost all the varieties. pH of the medium was adjusted at 5.8 and autoclaved at 15 psi at 121^0C for 15 min. Root formation was observed after 20 days in most of the cases.

Plants in Soil

After the plantlet regeneration in *in vitro* condition, the plants were placed in soil to adapt them in environment. For this purpose, plants were first planted in small plastic pot in the same temperature and

light condition as these were in the culture room. Usually, plantlets at the height of 4.5-7 cm, with well developed roots were selected for hardening. The soil was also mixed with composed fertilizer before use. The plants that survive were then transferred in small bucket under natural environmental conditions.

Table 1: List of Media Used for Callogenesis, Shooting and Rooting in the Whole Experiment

Media Type	Medium Code and Compositions
Callus Induction Media	CL 1 : MS + 0.5 mgl^{-1} 2, 4-D
	CL 2 : MS + 1.0 mgl^{-1} 2, 4-D + 1.0 mg l^{-1} IBA
	CL 3 : MS + 1.5 mgl^{-1} 2, 4-D + 0.5 mgl^{-1}Proline
	CL 4 : MS + 0.5 mgl^{-1} 2, 4-D + 1.0 mgl^{-1} BAP
	CL 5 : MS + 1.0 mgl^{-1} 2, 4-D + 0.5 mgl^{-1} BAP
	CL 6: MS+1.0 mgl^{-1} 2,4-D + 1.0 mg l^{-1} BAP `
	CL 7: MS+0.5 mgl^{-1} IAA + 0.5 mg l^{-1} IBA
	CL 8: MS+0.5 mgl^{-1} 2,4-D + 0.5 mg l^{-1} $AgNO_3$
	CL 9: MS+0.5 mgl^{-1} 2,4-D + 1.0 mg l^{-1} $AgNO_3$
	CL 10: MS+0.5 mgl^{-1} 2,4-D + 0.1% Casein hydrolysate (CH)
Shoot induction media	SH1: MS + 3.0 mg l^{-1} BAP
	SH2: MS + 2.0 mg l^{-1} BAP +3.0 mg l^{-1} $AgNO_3$+0.1 mg l^{-1} NAA
	SH3: MS + 2.5 mg l^{-1} BAP + 0.5 mg l^{-1} NAA
	SH4: MS + 2.0 mg l^{-1} BAP + 4.0 mg l^{-1} $AgNO_3$
	SH5: MS + 2.0 mg l^{-1} BAP + 6.0 mg l^{-1} $AgNO_3$
	SH6: MS + 2.0 mg l^{-1} BAP + 0.5 mg l^{-1} Kinetin
Root induction media	RT1: MS + 1.0 mg l^{-1} NAA
	RT2: MS + 3.0 mg l^{-1} NAA
	RT3: MS + 1.0 mg l^{-1} IBA
	RT4: 1/2 MS media (1/2 MS salt without hormones)

RESULTS AND DISCUSSION

Results

Three varieties namely, BARI-8, BARI-11 and BARI-16 of *Brassicas* were used for callus induction leading to regeneration of plants. In the first phase, the hypocotyls and cotyledonary segments of the seedlings were inoculated in the respective media to produce efficient callus. During the second phase, the embryogenic calli were placed in different regeneration media (shooting media, rooting media as necessary, Table 1) to establish plantlets.

Callus Induction

For callogenesis, ten callus induction media were tested as afore-mentioned hormonal combinations (Table 1).

The data obtained indicate that, 2,4-D was the most important ingredient for callus formation. The medium supplemented with 0.5 mgl^{-1} 2,4-D + 1mgl^{-1} BAP, (CL4 medium) was the best medium for both the hypocotyls and cotyledons for callus induction. In this medium, the responses of varieties were BARI-8 (87%, 76%), BARI-11 (93.40%, 88.5%) and BARI-16 (90.20%, 81.3%) respectively (Table 2). In case of callus formation, the combination of 2,4-D, $AgNO_3$ and BAP was found to produce vigorous growth and green calli.

Better growth of callus also observed in CL8 and CL9 media supplemented with 2,4-D + $AgNO_3$ (Table 2). Absence of $AgNO_3$ or BAP reduced the size of the callus and callus induction frequency. Among the varieties, BARI-8 and BARI-16 showed better results than BARI-11 in all the media tested, [Figure 1(A-F)].

The percentage of response of the hypocotyls and cotyledonary explants on different callus induction media of BARI-8, BARI-11 and BARI-16 in the entire media are given in (Table 2).

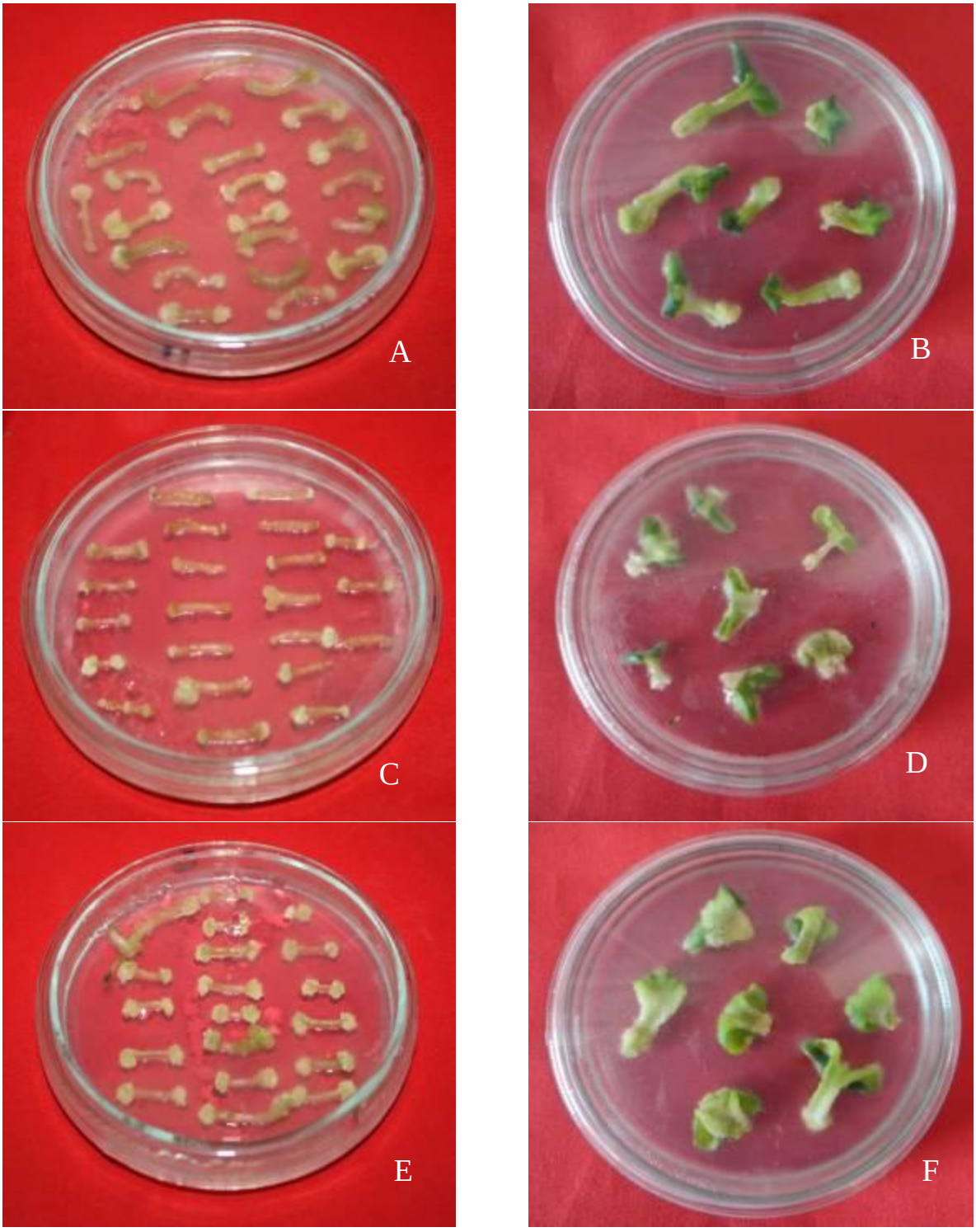

Figure 1: The Responses of the Varieties in Callogenesis on Different Callus Induction Media; A. BARI-8 Hypocotyls; B. BARI-8 Cotyledons; C. BARI-11 Hypocotyls; D. BARI-11 Cotyledons; E. BARI-16 Hypocotyls and F. BARI-16 Cotyledons

The variety BARI-8 show better results in CL9 and CL10 media. In case of BARI-11, the hypocotyls of this variety showed best response (93%) in the media containing 0.5 mg l^{-1} of 2,4-D + 1.0 mg l^{-1} BAP

(CL4 Medium). The percentage of callus formation also increased with increasing concentration of 2,4-D. In cotyledonary leaves with petioles, the callus was highly responsive in the media supplemented with 2,4-D+ BAP + $AgNO_3$, in CL5 and CL9 media (Table 2).

The BARI-16 variety gave better responses in almost all the media (CL1-CL10), both in case of hypocotyls and cotyledons. However, the highest percentage of callus was produced in CL2 (1.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} IBA) medium. Better response of callus production also observed in CL5 and CL7 media (Table 2).

Table 2: Responses of the Varieties (BARI-8, BARI-11 and BARI-16) in Various Callus Induction Media

Supplements	% of Responsive Explants			Callus Type and Color
	BARI-8	BARI-11	BARI-16	
	Hypocotyls Cotyledons	Hypocotyls Cotyledons	Hypocotyls Cotyledons	
CL 1 : MS + 0.5 mgl^{-1} 2, 4-D	73 78	78.5 83	75 79	Small, greenish
CL2 : MS + 1.0 mgl^{-1} 2, 4-D + 1.0 mg l^{-1} IBA	81.5 88	88.6 83.5	85 100	Yellowish
CL3 : MS + 1.5 mgl^{-1} 2, 4-D + 0.5 mgl^{-1}Proline	73 65	92 84.5	78.5 83.5	Vigorous growth, green
CL 4 : MS + 0.5 mgl^{-1} 2, 4-D + 1.0 mgl^{-1}BAP	87 76	93.4 88.5	90.2 81.3	Round shape callus at both ends
CL 5 : MS + 1.0 mgl^{-1} 2, 4-D + 0.5 mgl^{-1} BAP	78 100	87.5 93.5	92.3 95	Dark green
CL 6: MS+1.0 mgl^{-1} 2,4-D + 1.0 mg l^{-1} BAP	81.5 85	85 90.5	76.4 81.5	Moderate size, yellow
CL 7: MS+0.5 mgl^{-1}IAA + 0.5 mg l^{-1}IBA	77 63.75	60.2 70.5	82 89	Whitish green
CL 8: MS+0.5 mgl^{-1} 2,4-D + 0.5 mg l^{-1} $AgNO_3$	75.9 58.2	53 65	77.5 87	Compact, greenish
CL 9: MS+0.5 mgl^{-1} 2,4-D + 1.0 mg l^{-1} $AgNO_3$	90.5 100	73 86.5	75.3 92.69	Large, yellowish
CL 10: MS+0.5 mgl^{-1} 2,4-D + 0.1% Casein hydrolysate (CH)	95 89	71.5 80.2	64.3 95	Yellowish, vigorous growth

Shoot Formation

The well produced calli were transferred in shooting media for regeneration. Shoot regeneration was conducted from previously used two explants, cotyledonary leaf with petioles and hypocotyls. BAP was the most crucial supplements for initiation and elongation of shoot. Medium without BAP, could not produce any shoot.

The optimum concentration of BAP was 2-3 mg l^{-1} (Figures 2-5). The addition of NAA and $AgNO_3$ with BAP increased the efficiency of shoot formation. Multiple shoot also produced in this medium. It was found in the experiment that, the medium supplemented with 2 mg l^{-1} of BAP + 3 mg l^{-1} of $AgNO_3$ + 0.1 mg l^{-1} NAA produced good percentage of shoots in all the varieties (Figure 2).

The highest percentage of shooting was found in the variety BARI-16 (83.40% and 87.24%), [Figure 6(A)] while BARI-11 variety showed the least response (66.33% and 71.45%).

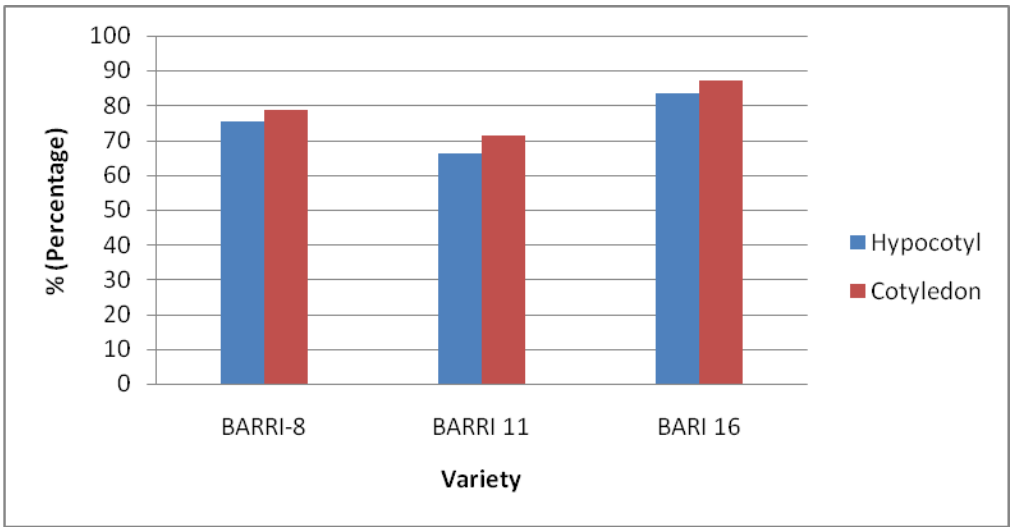

Figure 2: Percentage of Shoot Formation from Calli Derived from Hypocotyls and Cotyledons of Different Varieties on MS Medium Supplemented with 2.0 mg l^{-1} BAP + 0.1 mg l^{-1} NAA + 3.0 mg l^{-1} $AgNO_3$

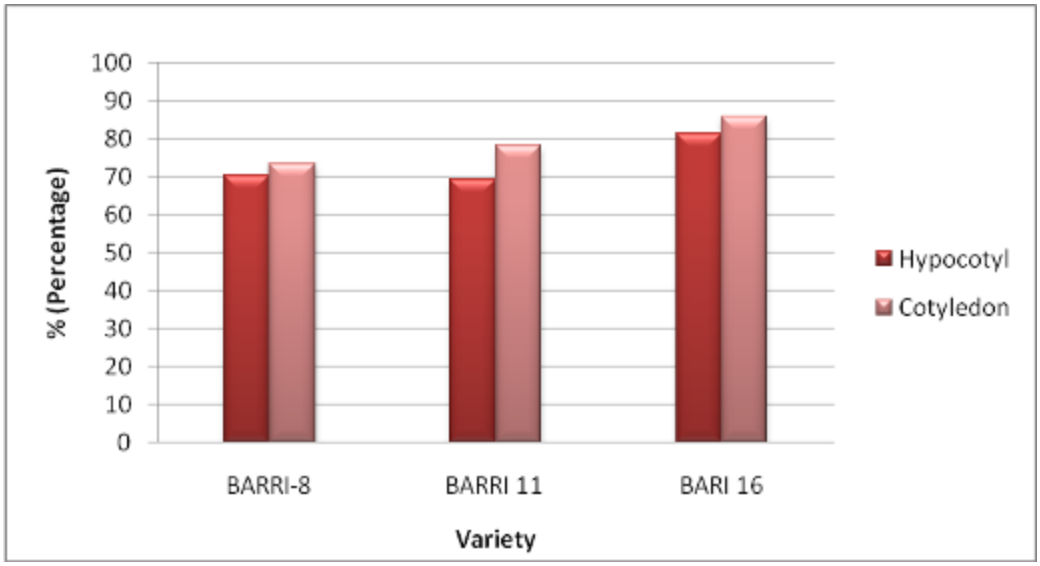

Figure 3: Percentage of Shoot Formation from Calli Derived from Hypocotyls and Cotyledons on MS Medium Supplemented with 2.5 mg l^{-1} BAP + 0.5 mg l^{-1} NAA

When BAP was used in combination of NAA, the medium produced better percentage of shoots, than BAP or NAA used independently. The medium supplemented with 0.5 mg l^{-1} BAP and 2.5 mg l^{-1} NAA produced good percentage of shoots in all the varieties tested such as, in varieties BARI-8 (70.20% and 73.50%), BARI-11 (69.35 % and 78.21%) and BARI-16 (81.30% and 85.75%) shoots were produced for hypocotyls and cotyledonary explants (Figure 3).

$AgNO_3$ was added in concentration of 3-6 mg l^{-1}. When it was used above 7 mg l^{-1}, the regeneration frequency was decreased. At the concentration of 4.0 mg l^{-1} $AgNO_3$ in association with BAP (2 mg l^{-1}), the percentage of shoot formation was better from both hypocotyls and cotyledonary explants of all the

varieties (Figure 4). With increasing concentration of $AgNO_3$, shoot production from all the varieties decreased (Figure 5).

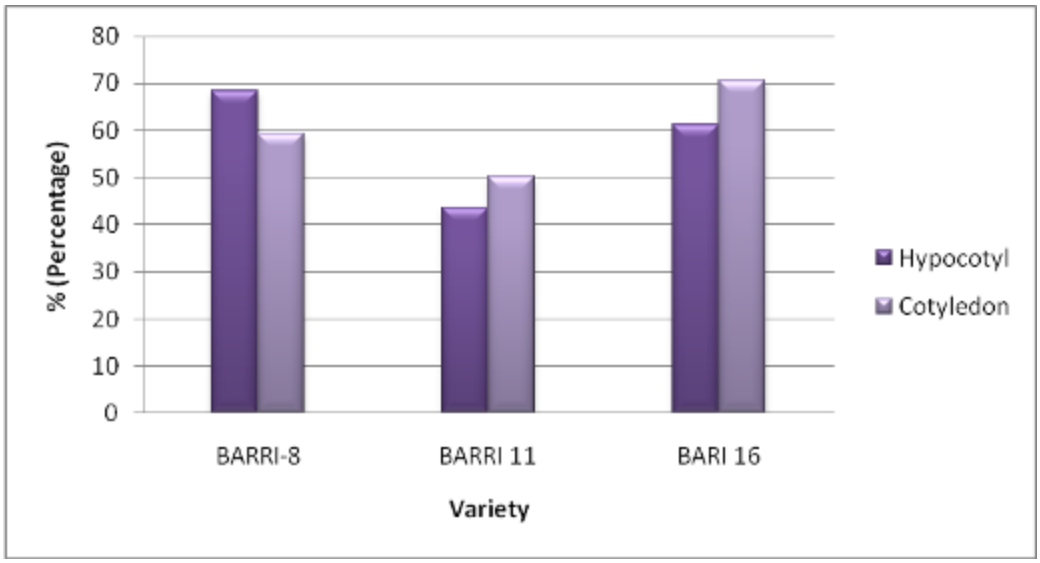

Figure 4: Percentage of Shoot Formation from Calli Derived from Hypocotyls and Cotyledons on MS Medium Supplemented with Only 2.0 mg l^{-1} BAP + 4.0 mg l^{-1} $AgNO_3$

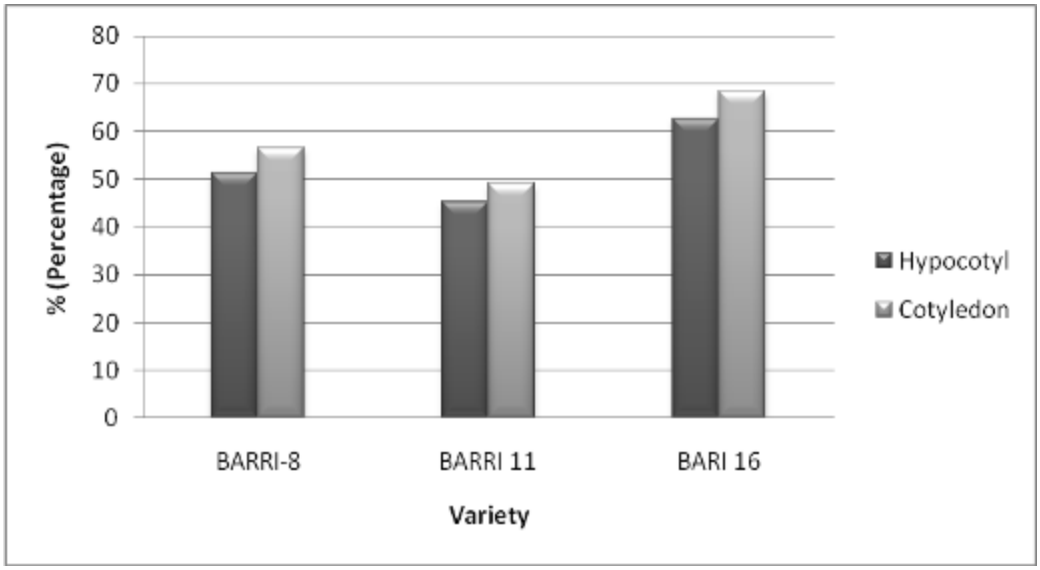

Figure 5: Percentage of Shoot Formation from Calli Derived from Hypocotyls and Cotyledons on MS Medium Supplemented with Only 2.0 mg l^{-1} BAP + 6.0 mg l^{-1} $AgNO_3$

Root Formation and Transfer in Soil

The well produced and elongated shoots were transferred in different rooting media. The medium supplemented with MS + 1 mg l^{-1} of IBA was the best medium for rooting in which root formation was observed from all the varieties within 20-24 days. NAA had an adverse effect on root induction in higher concentrations. When it was added in less amount (1 mg l^{-1}) it produce shoot in some varieties. However, the addition of NAA in 3 mg l^{-1} turned the plants yellow (Table 3).

Somewhat better roots also produced in half MS medium without any hormonal supplements, but at that case it was observed that the shooting medium for the respective plants supplemented with NAA in

association with BAP. Better root formation was observed in variety BARI-16 [Figure 6(B)]. The well rooted plants were then transferred in soil to establish them in natural conditions.

Figure 6: Shooting, Rooting and Hardening of the Variety BARI-16; A: Multiple Shooting in the Variety; B: Root Formation and *In Vitro* Flowering was Observed in the Plant and C: The Well Rooted Plant was Transferred in the Soil

Table 3: Effects of Different Concentrations of Additives on Root Induction from the *In Vitro* Regenerated Shoots of Varieties BARI-8, BARI-11 and BARI-16

	Supplements (mg l^{-1})	Days to Root Induction			Remarks
		BARI-8	BARI-11	BARI-16	
Effect of IBA	0.0	17-18	-	-	Root was initiated in some varieties
	1.0	20	20	17	Good responses in all cases
Effect of NAA	1.0	22	-	19	Root was developed in most of the varieties.
	3.0	-	-	-	All the plants became yellowish

Discussion

In this study, three varieties, one *B. napus* BARI-8; two *B. juncae* (BARI-11 and BARI-16) were used. Although, at first, the investigation was carried out, taking six varieties. In the later cases, three varieties were used as their response was well in callogenesis. In the first phase of the experiment, all the varieties were induced for *in vitro* development of callus in different callus induction media. The hypocotyls and cotyledonary leaves with petioles of the seedlings were used as explants to induce callogenesis. Usually,

the explants were excised from 4 days old seedlings and cut into 0.5-1.0 cm pieces under laminar flow hood. The explants excised from 3-4 days-old seedlings gave optimal regeneration rates which collaborate with the results obtained in Canola by Cardoza and Stewart (2003).

The excised pieces of explants were placed on MS medium supplemented with various concentrations of 2,4-D in association with other hormones (BAP, IAA, IBA) and additives ($AgNO_3$, Proline). In this experiment, 2,4-D in concentration of 0.5 and 1.0 mg l^{-1} in combination with BAP or $AgNO_3$ gave the best percentage of callus with better quality (embryogenic). Green callus with vigorous growth was also produced in these media. Absence of $AgNO_3$ or BAP reduced the size of the callus and callus induction frequency. Similar reports were provided by Ali *et al.,* (2007) and Khan *et al.,* (2002) that supports the present investigation.

The induced calli were transferred to the shoot regeneration medium containing different concentrations of BAP, NAA, $AgNO_3$ and Kinetin. The medium supplemented with BAP, in combination of NAA showed the best results in all instances. When BAP concentration was decreased from 4 mg l^{-1} to 2mg l^{-1}, shoot regeneration increased upto 5%. It was found that BAP at a concentration of 2 mg l^{-1} was optimum for shoot regeneration. The most critical factor for enhancement of shoot regeneration was the inclusion of NAA in the medium. With NAA, regeneration frequency increased to an optimum of 70% at 1 mg l^{-1} NAA and 2 mg l^{-1} BAP.

The addition of $AgNO_3$ also induced shoot formation in some cases. Mukhopadhyay *et al.,* (1992) also observed that silver nitrate enhanced shoot regeneration from both cotyledon and hypocotyl explants, especially from the latter. In medium supplemented with 2 mg l^{-1}(BAP), 0.1 mg l^{-1} (NAA) and 3 mg l^{-1} ($AgNO_3$), the shoot regeneration frequency was: 71.20% (BARI-8) and 86.50% (BARI-16) respectively. However, the variety BARI-11 (55.00%) showed somewhat poor responses. It was also observed in the present experiment that, increasing $AgNO_3$ concentration above 7 mg l^{-1} decreased the rate of regeneration.

To induce rooting, green and healthy shoots were taken and were placed on medium for root formation. The rooting media were supplemented with different concentrations of hormones (NAA, IBA). Ali *et al.*, (2007) reported that, best result of rooting was obtained on media having 0.3 mg l^{-1} IBA in half strength MS medium. In this study, it also found that, good percentage of root formation occur in medium supplemented with 1 mg l^{-1} of IBA and variety BARI-16 showed increased root formation within very short time.

When NAA was added in association of BAP in shooting media, some root formation was also observed. In such cases, transfer of the plant to a medium free of growth regulators allowed elongation of both roots and shoots to occur. The rooting of *in vitro* grown shoots using half strength of MS medium has been reported by several authors (Samantaray *et al.,* 1995; Upreti and Dhar 1996; Kooi *et al.,* 1999).

On the basis of observation and results obtained, the varieties BARI-8 and BARI-16 showed a very good regeneration potentiality in *in vitro* conditions. Although, BARI-11 showed shoot formation in some cases, but it did not show the same response in all the cases. Thus, based on the results obtained and discussed it can be concluded that the present regeneration protocol can be used reliably for indigenous varieties BARI-8 and BARI-16 for *in vito* culture which is the prerequisite for any kind of genetic manipulation.

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