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Agrobacterium-mediated genetic transformation in *Brassica* species

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ABSTRACT

An efficient and reproducible protocol for the production of transgenic Brassica plants was developed by inoculating hypocotyl explants with Agrobacterium tumefaciens strain LBA4404 carrying a binary vector pBI121, which contains selectable marker gene nptII conferring resistance to kanamycin and the GUS reporter gene. The transformation experiment was performed by optimizing two important parameters: preculture period and co-cultivation period. Infection was most effective when explants were precultured for three days (68.75% GUS positive) and co-cultivated for three days (82.92% GUS positive) with Agrobacterium. Among the varieties, BARI sarisa-8 showed the highest response to GUS assay (64.38% GUS positive). Callus induction was highest in Rai-5 (10%) and three day period of preculture and co-cultivation (12.33 and 13.33%, respectively). Transformation percentage was also highest in Rai-5 (4.75%), and three day period of preculture and co-cultivation (7.50 and 5.42%, respectively). Highest percentage (33.33) of root induction from transgenic shoots was observed in ½ MS + 0.5 mgL^{-1} NAA + 50 mgL^{-1} kanamycin + 50 mgL^{-1} cefotaxime. Among the varieties, BARI sarisa-8 produced the highest percentage (37.78) of rooted shoots.

Key Words: *Agrobacterium tumefaciens, Brassica, Co-cultivation, Preculture and Transformation*

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I. Introduction

The oleiferous *Brassica* represented by rapeseed and mustard plays an important role in vegetable oil production of the world. It is the third most important edible oil source in the world after soybean and palm (Walker and Booth 2001). About 13.2% of the world's edible oil supply comes from this crop (Downey and Robbelen, 1989). It is the top ranking oilseed crop of Bangladesh which covers about 60% of the total production (BBS, 2009). Bangladesh has been facing a huge shortage in edible oils for the time being. Undoubtedly, lower yield of edible oil seeds is one of the major causes of such deficiency in our country. There are also a number of other reasons behind this low yield such as, infection of several diseases and insect pests (Meah *et al.*, 1985).Conventional breeding methods were employed for the improvement of *Brassica* but they were not very successful due to high degree of segregation upon cross-pollination and unavailability of suitable wild germplasm of *Brassica*. Moreover, conventional breeding programme is time consuming, extending over seven to eight years

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involving crossing and wise selection of desirable traits. Besides, *Brassica spp*. have consistently proven to be one of the most recalcitrant members of the *Brassicaceae* in tissue culture that eluded any notable progress in *Brassica* improvement through *in vitro* techniques (Hachey *et al*. 1991). *Agrobacterium*-mediated genetic transformation technique offers the potential for the introduction of specific genes from any source (related or unrelated plant species or even from animal) into the existing elite plant lines without altering of its existing traits (Gardner, 1993). It is considered as an alternative way to introduce desirable traits into plant species.

Although *Brassica* had been considered as a host of *Agrobacterium tumefaciens* (Godwin *et al*. 1991), no evidence of T-DNA integration was described until 1989. De Block *et al*. (1989) showed first successful integration of foreign genes in *Brassica* but failed to produce transgenic plants. There are several reports on *Brassica* 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) and insect resistance (Stewart *et al*., 1996). Transformation has been carried out using various explants, such as stem internodes (Fry *et al*., 1987), stem segments (Pua *et al*., 1987), cotyledonary petioles (Moloney *et al*., 1989) and hypocotyl segments (Radke *et al*. 1988, De Block *et al*. 1989, Stewart *et al*. 1996). However, the transformation efficiency is not satisfactory and no such report is available in Bangladesh. An improvement in the transformation and regeneration of transgenic is desirable in order to decrease the amount of resources needed to produce transgenic plants, and to potentially provide a higher baseline for subsequent transformation of different *Brassica* varieties. Two important factors that govern the efficiency of transgenic plant recovery are preculture of the explants before inoculation with *Agrobacterium* and co-cultivation period after inoculation. In this paper, we describe the conditions that enhanced the *Agrobacterium* infection frequency of hypocotyl segments and a reproducible and genotype-independent *Agrobacterium*-mediated transformation system for *Brassica*.

II. Materials and Methods

Plant materials

The experiment was conducted in the Tissue Culture Laboratory of the Department of Genetics and Plant Breeding, Bangladesh Agricultural University, Mymensingh during the period from January 2003 to April 2004. Six varieties of *Brassica* were used to standardize different parameters influencing plant transformation. The varieties were Tori-7, Kallyania, Rai-5, Daulat, BARI sarisa-7 and BARI sarisa-8 under three different species (*Brassicacampestris*, *Brassicajuncea* and *Brassicanapus*), two from each.

Agrobacterium strain and plasmid

Genetically engineered *Agrobacterium tumefaciens* strain LBA4404 was used for infection in the experiment, which contains plasmid pBI121 of 14 KDa (binary vector) (Figure 01). This binary vector contains following genes within the right border (BR) and left border (BL) region of the construct:

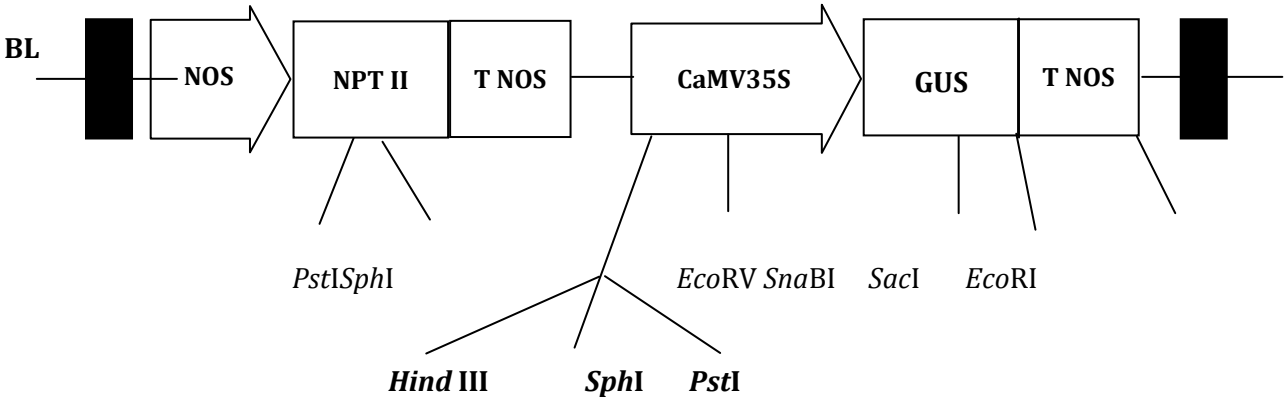

Figure 01. Region between left (BL) and right (BR) border of pBI121 from *Agrobacterium tumefaciens* strain LBA4404

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Transformation, selection and plant regeneration

Seeds were germinated on half-strength MS (Murashige and Skoog, 1962) medium with 20 g/l sucrose at 25±2^{0}C under a 16/8-h (day/night) photoperiod. Hypocotyl segments (5-7 mm) from four day old seedlings were precultured for 1-3 days on MS medium supplemented with 1 mgL^{-1} BAP and 0.1 mgL^{-1} NAA. Bacterial cultures were shaken overnight at 180 rpm in liquid LB medium supplemented with 50 mgL^{-1} kanamycin at 28^{0}C until the OD_{600} was 0.6. The precultured explants were inoculated with the *Agrobacterium* suspension for 10 min, returned to the preculture medium and incubated for a further 1-3 days at 25±2^{0}C. Following co-cultivation, the explants were washed twice with sterile distilled water and once with liquid MS media supplemented with 500 mgL^{-1} cefotaxime. Then the explants were transferred onto the post-cultivation medium (MS + 1 mgL^{-1} BAP + 0.1 mgL^{-1} NAA + 200 mgL^{-1} cefotaxime).

Following one week of post-cultivation, the explants were transferred onto low selective medium (MS + 1.0 mgL^{-1} BAP + 0.1 mgL^{-1} NAA + 2 mgL^{-1} $AgNO_3$ + 20 mgL^{-1} kanamycin + 100 mgL^{-1} cefotaxime). After culture for seven days, the hypocotyls with calli were transferred onto selection and regeneration medium (MS medium supplemented + 4.0 mgL^{-1} BAP + 2.0 mgL^{-1} NAA + 2.0 mgL^{-1} $AgNO_3$ + 30 mgL^{-1} Kanamycin + 75 mgL^{-1} cefotaxime) for further selection and shoot regeneration. After three weeks, the calli were subcultured once on selection and regeneration media.Sub cultured calli continued to proliferate and differentiated into green shoots. When these shoots grew about 2-3 cm in length, they were rescued aseptically from the cultured petridishes and were separated from each other and again were cultured on conical flasks/vials with freshly prepared rooting medium to induce root. The conical flasks/vials containing plantlets were incubated at 22±2^{0}C with 16 hrs. photoperiod. Day to day observations were carried out to note the responses.

GUS (β-glucuronidase) histochemical assay

From each batch of explants following each transformation experiment, randomly selected co-cultured tissues were examined for GUS histochemical assay (Jefferson *et al*. 1987). For this experiment, co-cultured explant tissues were immersed in X-gluc (5-bromo-4-chloro-3-indolyl glucuronide) solution and were incubated at 37^{0}C overnight. A characteristic blue colour would be the expression of *GUS (β-glucuronidase)* gene in the plant tissue.Proper control for GUS histochemical assay was done with the explants having no *Agrobacterium* inoculation. After X-gluc treatment, explants were transferred to 70% ethyl alcohol for degreening. Following degreening explants were observed under stereomicroscope.

Statistical analysis of data

The experiment was arranged in Completely Randomized Design. The ANOVA for different parameters were performed and means were compared by the Duncan's Multiple Range Test.

III. Results and Discussion

Histochemical GUS (*β*-glucuronidase) assay

Conspicuous GUS positive (blue colour) regions were detected at the entire cut surface of the hypocotyl explants. Among the varieties, BARI sarisa-8 showed highest response (64.38% GUS positive) and BARI sarisa-7 showed lowest response (38.13% GUS positive) to GUS assay (Figure 01). Both the preculture and co-cultivation period of three days were most effective for producing best response (68.75 and 82.92% GUS positive, respectively) in GUS assay. Control explants did not show any response (Plate 01 &02) towards GUS assay.

Callus induction

The explants started to induce callus by swelling the cut ends within 12 days of inoculation. The percentage of callusing was highest (10) in Rai-5 (Figure 02) and lowest (4.44) in BARI sarisa-7. Callus induction was also highest in three day period of preculture (12.33%) and co-cultivation (13.33%).

The ANOVA for number of callus/petridish, effects of different varieties, and different periods of preculture and co-cultivation on this character are summarized in Table 01. Rai-5 showed highest

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(1.000) callusing/petridish (Plate 03) followed by Kallyania (0.925) (Table 02). Callusing was highest in both three days period of preculture (1.233) and co-cultivation (1.333) (Table 03 & 04). Three day preculture × Rai-5 was the best (1.55) for callusing/petridish (Table 05). Control explants failed to produce callus and remained albino (Plate 03 &04). .

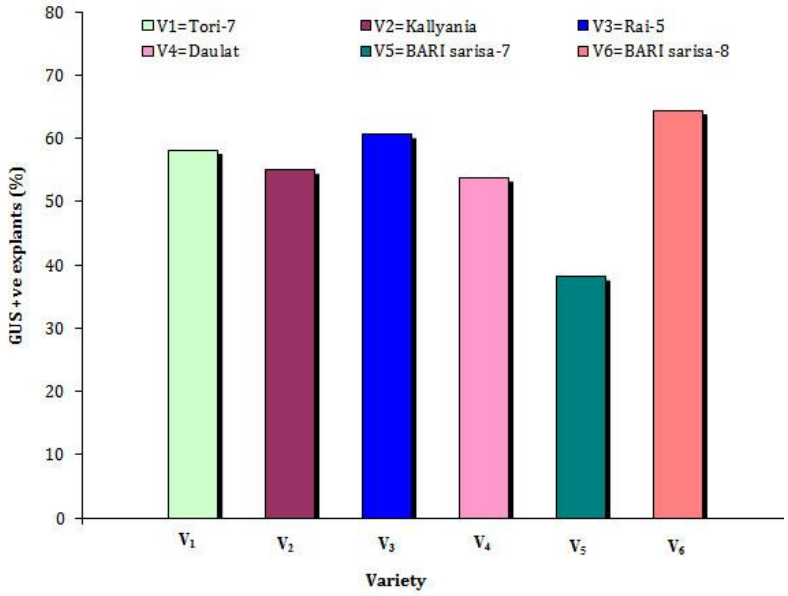

Fig. 1. Response of six varieties of *Brassica* towards GUS histochemical assay

Plate 01. Histochemical localization of GUS activity (blue zone) at the entire cut surface of hypocotyl segment of Rai-5 (left) with control explant (right).

Plate 02. Histochemical localization of GUS activity (blue zone) at the entire cut surface of hypocotyl segment of Tori-7 (left) with control explant (right).

Table 01. Mean square of values for total number of calli/petridish

Parameter	Sources of variation							
	Var. (with 5 df)	Precul. (with 3 df)	Var. × Precul. (with 15 df)	Co-cul. (with 3 df)	Var. × Co-cul. (with 15 df)	Precul. × Co-cul. (with 9 df)	Var. × Precul. × co-cul. (with 45 df)	Error (with 384 df)
Total number of calli/pertridish	*1.098***	*12.950***	*0.502***	*18.844***	*0.163 NS*	*0.261 NS*	*0.095 NS*	*0.214*

** 1% level of significance; NS = Non significant

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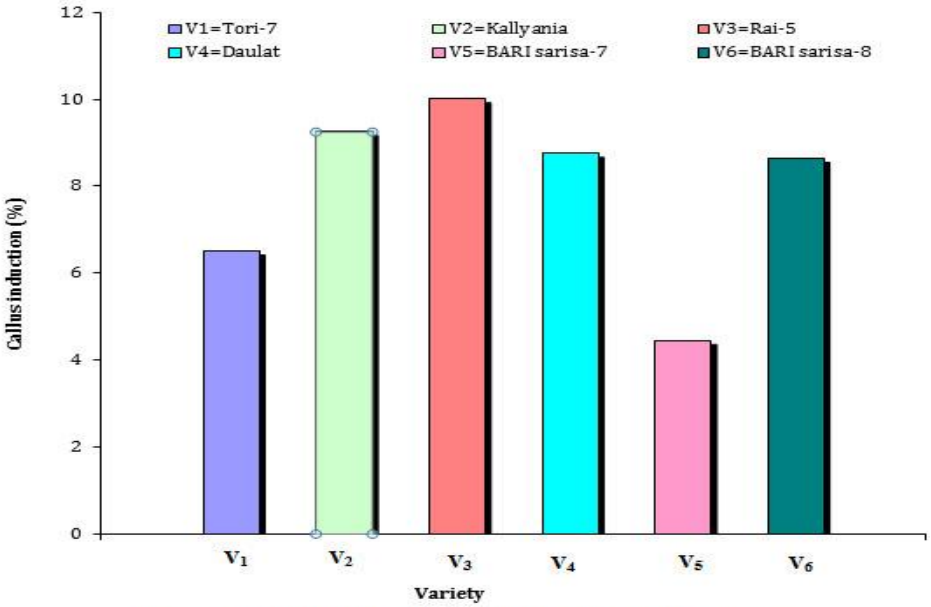

Fig. 2. Response of different varieties towards callus induction

Table 02. Performance of different varieties of *Brassica* on number of callus/petridish

Parameter	Variety					
	Tori-7	Kallyania	Rai-5	Daulat	BARI sarisa-7	BARI sarisa-8
Number of callus/petridish having ten explants	0.650 b	0.925 a	1.000 a	0.875 a	0.444 c	0.863 a

Table 03. Performance of different preculture periods on number of callus/petridish

Parameter	Preculture period (days)			
	0	1	2	3
Number of callus/petridish having ten explants	0.500 d	0.708 c	0.950 b	1.233 a

Table 04. Performance of different co-cultivation periods on number of callus/petridish

Parameter	Co-cultivation period (days)			
	0	1	2	3
Number of callus/petridish having ten explants	0.400 d	0.741 c	1.000 b	1.333 a

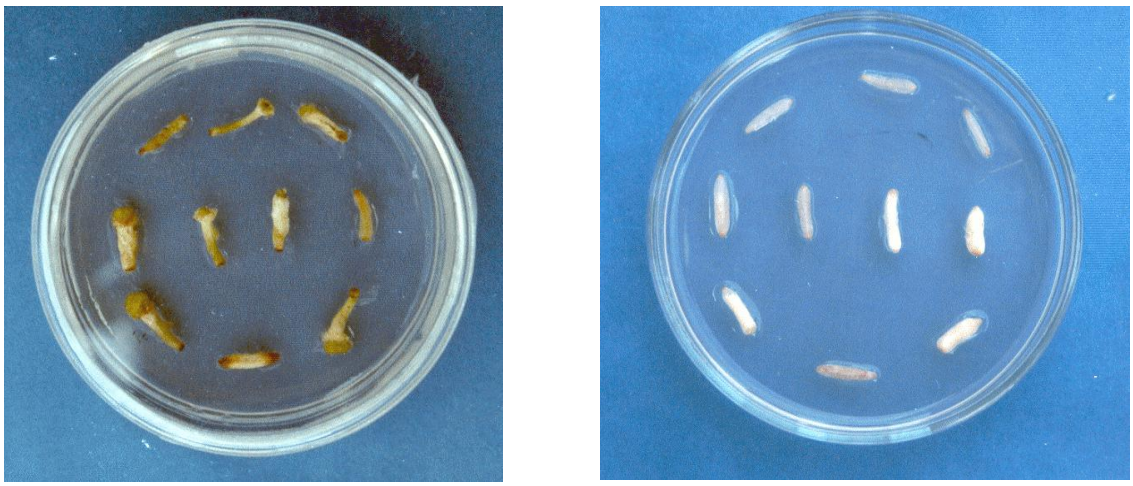

Plate 03. Callus initiation from *Agrobacterium* inoculated hypocotyl of Rai-5 (left) on selection medium, control explants failed to initiate callus (right.)

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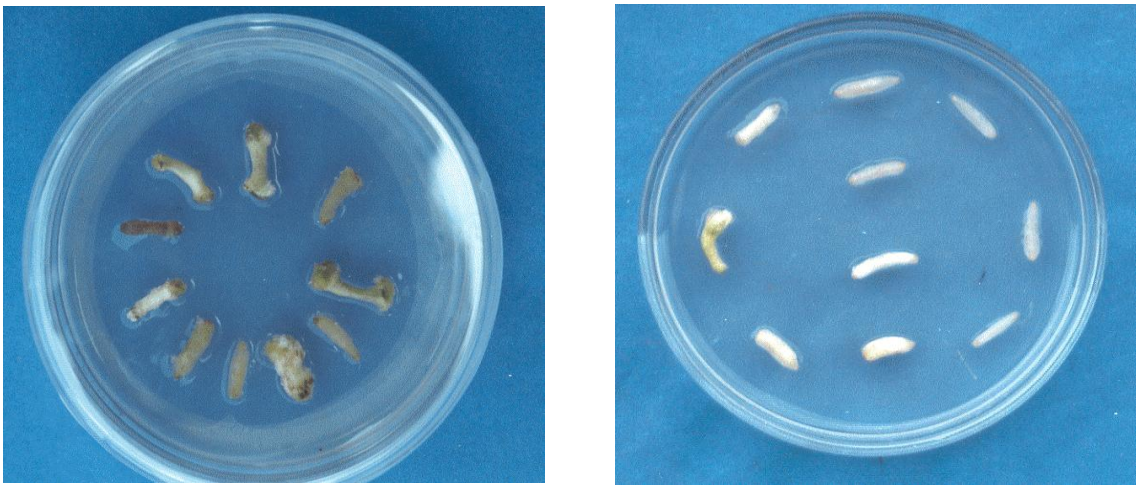

Plate 04. Callus initiation from *Agrobacterium* inoculated hypocotyl of Tori-7 (left) on selection medium, control explants failed to initiate callus (right).

Table 05. Effects of variety × preculture periods on callus formation

Variety × Co-cultivation peried (days)		Total number of calli/petridish
Tori-7	0	0.40 hi
	1	0.45 g-i
	2	0.55 g-i
	3	1.20 bc
Kallyania	0	0.40 h-i
	1	0.90 c-f
	2	1.10 bc
	3	1.30 ab
Rai-5	0	0.55 g-i
	1	0.75 d-g
	2	1.15 bc
	3	1.55 a
Daulat	0	0.60 f-i
	1	0.65 f-i
	2	1.00 b-e
	3	1.25 ab
BARI sarisa-7	0	0.35 i
	1	1.05 b-d
	2	0.90 c-f
	3	1.15 bc
BARI sarisa-8	0	0.70 e-h
	1	0.65 f-i
	2	1.00 b-e
	3	1.20 bc

Initiation of shoot

Calli started to initiate shoot buds after 50-60 days of incubation. Among the varieties, Rai-5 showed highest rate of plant transformation (4.75%) while BARI sarisa-7 in showed 1% transformation (Figure 03). Transformation rate was highest in both three days period of preculture (7.50%) and co-cultivation (5.42%). On the other hand, no transgenic shoot was regenerated in case of zero day of preculture and co-cultivation.

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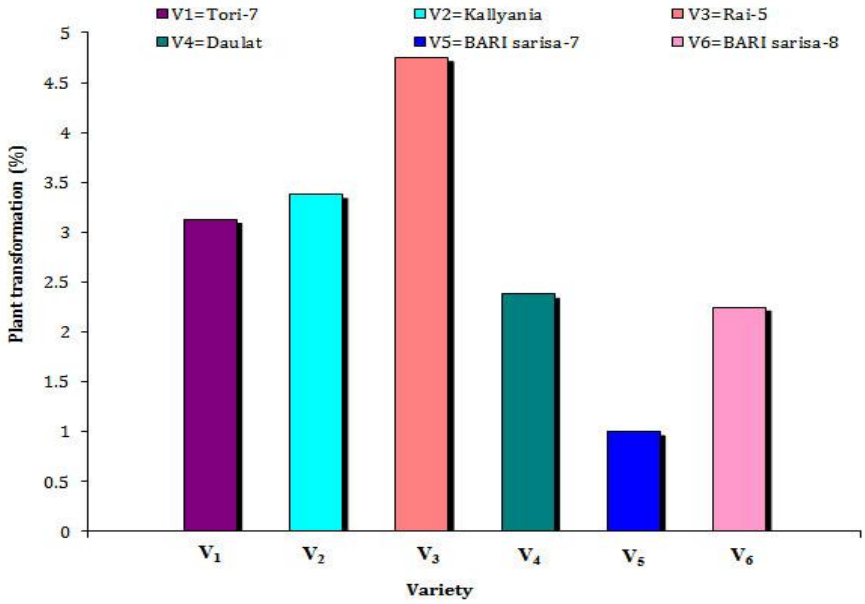

Fig. 3. Response of different varieties of *Brassica* towards plant transformation

The ANOVA for total number of shoot/petridish, effects of different varieties, and different periods of preculture and co-cultivation on this character are summarized in Table 06. Kallyania performed best (0.363) for total number of shoots/petridish (Table 07). Total number of shoots/petridish was higher in three day period of preculture (0.742) compared to 0, 1 or 2 days (Table 08) and three day period of co-cultivation (0.542) compared to 0, 1 or 2 days (Table 09). No shoot was regenerated from explants without preculture. Control regenerated plantlets failed to grow on selection and regeneration media and remained albino (Plate 05 and 06). Total number of shoots/petridish was highest (1.400) in 3 day preculture × 3 day co-cultivation (Table 10).From the above discussion, it is evident that three day period of preculture and co-cultivation is the best for recovery of transgenic shoot. These results showed similarities with Cardoza and Stewart (2003), Tsukazaki *et al*. (2002), Mahmoudin *et al*. (2002), Zhang *et al*. (2000) and Ding *et al*. (1998).

Table 06. Mean square of values for total number of shoots/petridish

Parameter	Sources of variation							
	Var. (with 5 df)	Precul. (with 3 df)	Var. × Precul. (with 15 df)	Co-cul. (with 3 df)	Var. × Co-cul. (with 15 df)	Precul. × Co-cul. (with 9 df)	Var. × Precul. × co-cul. (with 45 df)	Error (with 384 df)
Total no. of shoot/petridsh	0.745*	13492**	0.428NS	6.747**	0.277NS	2.216**	0.239NS	0.240

** 1% level of significance; * 5% level of significance; NS = Non significant

Table 07. Performance of different varieties of *Brassica* on total number of shoots/petridish

Parameter	Variety					
	Tori-7	Kallyania	Rai-5	Daulat	BARI sarisa-7	BARI sarisa-8
Total number of shoots/petridish	0.313 a	0.363 a	0.338 a	0.238 ab	0.225 ab	0.100 b

Table 08. Performance of different preculture periods on total number of shoots/petridish

Parameter	Preculture period (days)			
	0	1	2	3
Total number of shoots/petridish	0.000 c	0.067 c	0.242 b	0.742 a

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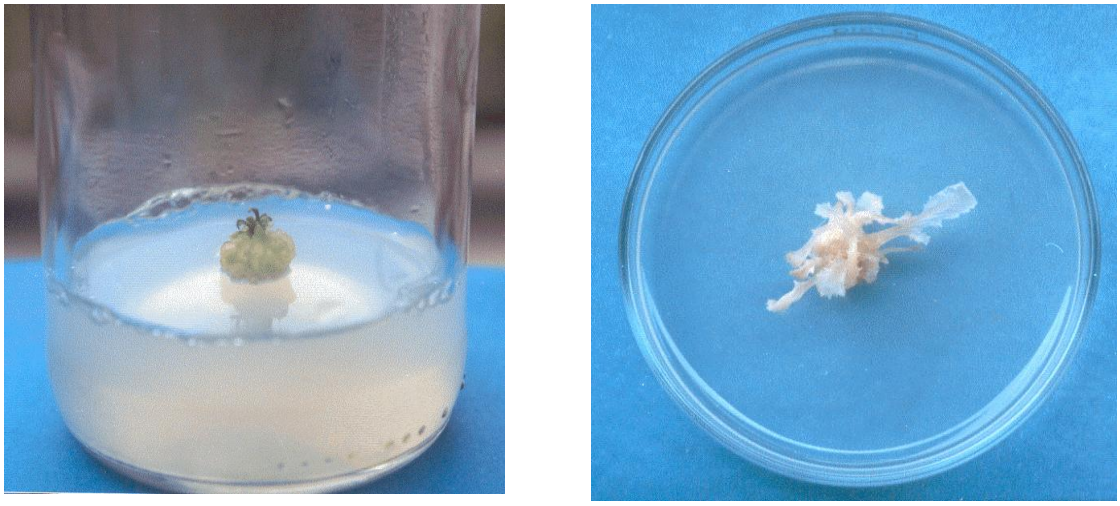

Plate 05. Initiation of shoot from transformed calli of Rai-5 on selection and regeneration medium (left), regenerated albino plant (right).

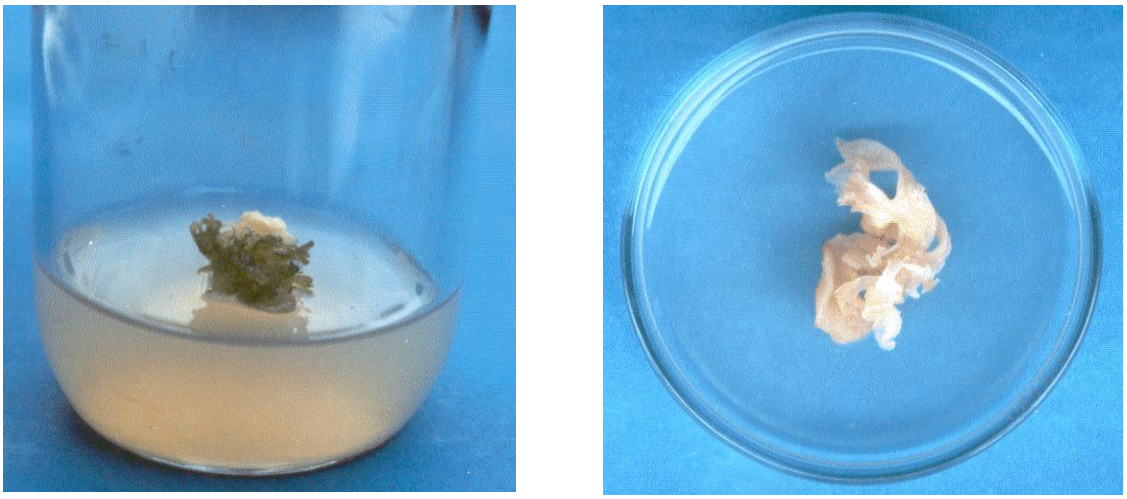

Plate 06. Initiation of shoot from transformed calli of Tori-7 on selection and regeneration medium (left), regenerated albino plant (right).

Table 09. Performance of different co-cultivation periods on total number of shoots/petridish of *Brassica*

Parameter	Co-cultivation period (days)			
	0	1	2	3
Total number of shoots/petridish	0.000 d	0.150 c	0.358 b	0.542 a

Rooting in transgenic shoot

Half strength MS medium without hormone, and half strength MS medium supplemented with two concentrations of NAA (0.5, 1.0 mgL^{-1}) with constant concentration of cefotaxime (50 mgL^{-1}) and kanamycin (50 mgL^{-1}) were used to observe the rooting response of transgenic shoots. Root formation was found to be the best (33.33%) on the ½ MS + 0.5 mgL^{-1} NAA + 50 mgL^{-1} cefotaxime + 50 mgL^{-1} kanamycin. Among the varieties, BARI sarisa-8 produced the highest percentage (37.78) of rooted shoot (Figure 04).

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Table 10. Effects of preculture × co-cultivation periods on shoot regeneration of *Brassica*

Preculture × Co-cultivation (days)		Total number of shoots/petridish
0	0	0 e
	1	0 e
	2	0 e
	3	0 e
1	0	0 e
	1	0 e
	2	0.330 e
	3	0.233 de
2	0	0 e
	1	0.067 e
	2	0.367 cd
	3	0.533 c
3	0	0 e
	1	0.533 c
	2	1.033 b
	3	1.400 a

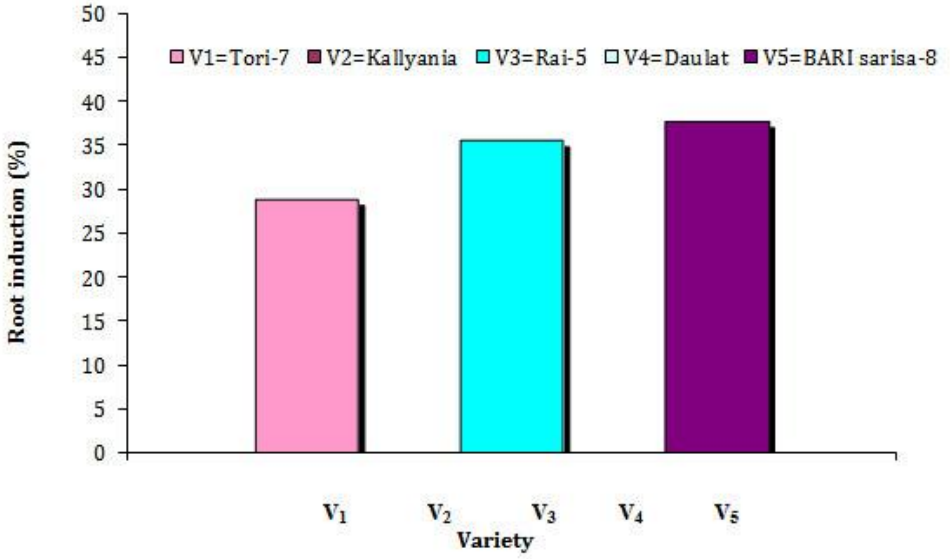

Fig. 4. Response of different varieties towards root induction in transgenic shoot of *Brassica*

The results of the analysis of variance (mean squares) for number of transgenic shoots producing root and effects of different concentration of NAA (media) on this character are summarized in Table 11.

Table 11. Analysis of variance (mean squares) for number of transgenic shoots with root

Parameter	Source of variation			
	Variety (with 4 df)	Media (with 2 df)	Variety × Media (with 8 df)	Error (with 60 df)
No. of transgenic shoots with root	4.847**	2.893**	0.527*	0.207

** 1% level of significance, * 5% level of significance

BARI sarisa-8 showed the highest number of shoots with root (1.133) (Plate 07) followed by Rai-5 (1.067) and Tori-7 (0.867). Kallyania and Daulat failed to produce root (Table 12). Highest number of shoots with root (1.00) was in ½ MS + 0.5 mgL^{-1}NAA + 50 mgL^{-1} cefotaxime + 50 mgL^{-1} kanamycin (Table 13).

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Table 12. Performance of different varieties of *Brassica* on number of transgenic shoot with root

Parameter	Variety				
	Tori-7	Kallyania	Rai-5	Daulat	BARI sarisa-8
Number of shoot with root	0.867 a	0.000 b	1.067 a	0.000 b	1.133 a

Table 13. Performance of different combinations of phytohormone on number of transgenic shoot with root in *Brassica*

Phytohormone combinations	Number of shoot with root
½ MS + 50 mgL-1 cefotaxime + 50 mgL-1 kanamycin	0.360 b
½ MS + 0.5 mgL-1 NAA + 50 mgL-1 cefotaxime + 50 mgL-1 kanamycin	1.000 a
½ MS + 1.0 mgL-1 NAA + 50 mgL-1 cefotaxime + 50 mgL-1 kanamycin	0.480 b

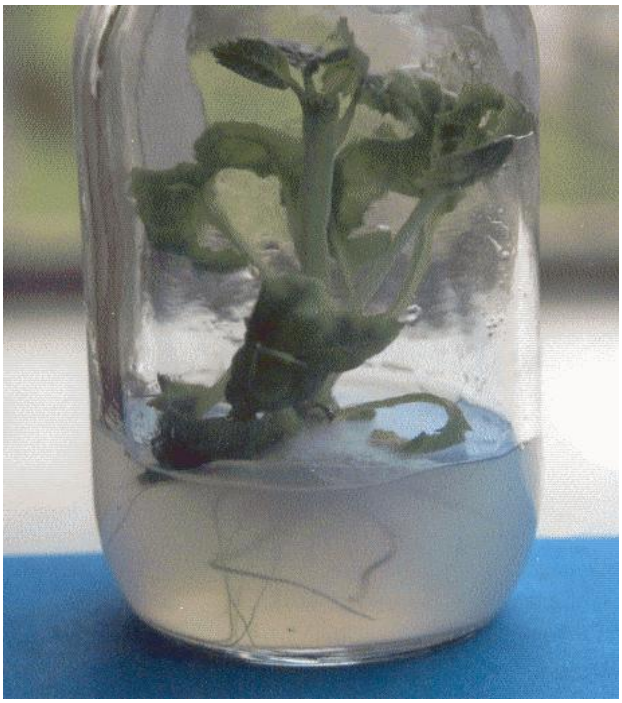

Plate 07. Initiation of root in transgenic shoot of BARI sarisa-8.

Hypocotyl explants were found very sensitive to co-cultivation with *Agrobacterium* and turn necrotic very easily. Necrosis can be overcome by preculturing of the explants on callus induction medium. This preculturing of the explants before co-cultivation helps in inhibiting necrosis and increases the transformation efficiency (Cardoza and Stewart, 2003). Improvement in transformation frequency upon preculturing of the explants has been reported in *Arabidopsisthalina* (Sangwan *et al.* 1992), sugarbeet (Jacq *et al.*, 1993), tobacco (Sunilkumar *et al.*, 1999), watermelon (Choi *et al.*, 1994) and *Populusnigra* (Confalonieri *et al.*, 1994). The pre-culturing of explants has been reported in *B. napus* by Ovesna *et al.* (1993), who precultured the explants for seven days on induction medium supplemented with 2, 4-D.

The improvement in transformation efficiency as the result of preculturing can be attributed to the initiation of active cell division upon wounding (Sangwan *et al.*, 1992), the improved binding of *Agrobacterium* to the newly synthesized cell wall at the wound sites (Binns, 1991) and the production of *vir*-inducing compounds by the metabolically active cells (Spencer and Towers, 1991). Sunilkumar *et al.* (1999) reported that the production of *vir* gene inducers by the explant during the preculturing period is an important factor that contributes to increased transformation efficiency.Another interesting result of this investigation was that the time of co-cultivation also made a significant difference in the transformation efficiency. A co-cultivation time of 3 day gave the best transformation efficiency. Transformation rate decreased with the decreasing period of co-cultivation. No transgenic

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shoot was recovered from explant without any co-cultivation.Rooting of transformed shoots is one of the problems encountered in *Brassica* transformation studies (Cardoza and Stewart, 2003). The rooting of *in vitro* shoots using half-strength medium has been proven to be beneficial for root induction. Similar results were obtained by Figueiredo *et al*. (2001). Use of kanamycin helped in the selection of transgenic plants.

IV. Conclusion

This protocol for the transformation of *Brassica* varieties needs to be confirmed for the integration of two marker genes (*GUS* and *nptII*) by stable GUS assay of the regenerants and PCR based tests like southern blot and SSR. In the future programme agronomically important gene(s) can be transferred to the locally grown *Brassica* varieties using this protocol. Specially for the development of disease resistant *Brassica* variety, this technique of transformation may be utilized successfully.

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