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AGROBACTERIUM-MEDIATED GENETIC TRANSFORMATION OF TWO VARIETIES OF *BRASSICA:* OPTIMIZATION OF PROTOCOL

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Abstract

Two rapeseed varieties, namely Tori-7 and BARI Sarisha-8, respectively, from *Brassica rapa* and *Brassica napus* were selected to observe the transformation ability. Petioles were inoculated in *Agrobacterium tumefaciens* strain LBA 4404 carrying a binary vector pBl2l with *GUS* (reporter) and *nptII* (kanamycin resistant) gene. The transformation experiment was performed by optimizing two important factors: preculture time and co-cultivation time and also selected out the best variety. Infection was most effective when explants were pre-cultured for 72 hours (80% GUS positive). and co-cultivated for 72 hours (72% *GUS* positive). The variety Tori-7 showed the best response to *GUS* assay (65% *GUS* positive). Callus induction was the highest in Tori-7, which were 6% with 72 hours of preculture period and 9% in 48 hours of co-cultivation. Number of putative transformed plantlets were highest in Tori-7 (7 plants) followed by BARI Sarisha-8 (3 plants).

Key words: Transformation, *Brassica*, *GUS*, *Agrobacterium.*

Introduction

Rapeseed *(B. rapa* and *B. napus)* oil has many advantages, while many of the other edible oils lack one of the two essential fatty acids; rapeseed-mustard provides both the essential fatty acids to the human body-linoleic acid and linolenic acid. Significantly, it contains the lowest amount of saturated fatty acids among the vegetable oils, higher amounts of which form deposits in the arteries, causing attendant heart problems. The de-oiled cake serves as animal feed and fertilizer. It is a good source of protein with well-balanced amino acids and minerals (Pachauri, 2001). Rapeseed, *Brassica rapa* L. and *Brassica napus* are important oil-yielding crops in Bangladesh. Approximately 70% of the total cultivated mustard in Bangladesh is *Brasstca rapa* and *Brasstca napus* varieties. The average yield of local varieties and HYV (High Yielding Varieties) are 600-1000 kg/ha and 1400-2000 kg/ha, respectively, which contributes 52% to the total production and 61.2% to the oil seed production of Bangladesh (BBS, 1999).

By introducing new gene responsible for quality traits through genetic engineering, many breeding needs could be overcome. It is fact that in the last two decades, significant developments in plant transformation technology was observed, and many transgenic crop plants have now been released for

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commercial production. In some countries, the highest percentage of the acreage of some economically important crops are transgenic; an increasing number of these transgenic varieties are or will soon be generated by *Agrobacterium-mediated* transformation (Gelvin, 2003). Due to the wide host range *Agrobacterium* mediated genetic transformation is very popular method for introducing gene of interest into plant. *Agrobacterium tumefaciens* as a gene vector is limited to soybean (Hinchee *et al.,* 1988, broad bean (Jelenic *et al.,* 2000), sesame (George *et al.,*1987) and sunflower (Weber *et al.,* 2003). *Brassica* is also a suitable host for *Agrohacterium* spp. (Godwin *et al.,* l991; Toriyama *et.al.,* 1991). So, the non-oncogenic *Agrobacterium* strain as a vector (Lin dsey, 1992) can make possible to transfer desired gene in *Brassicu.* Still now, a little success in genetic transformation has been reported in oil crop due to their recalcitrant *in vitro* condition (Nisbet and Webb, 1990). The main problem about the *Brassica* is that, the transformed tissues (callus) are not regenerable and the regenerable tissues (meristematic tissues) arc not transformable.

However, the result shown by Hachey *et al.* (l991) was quite optimistic, he got a successful regeneration of transformed tissues. Cotyledonary petioles from the shoots growing *in vitro* showed positive expression for the presence of foreign gene, such as β-glucuronidase (GUS) Cardoza and Stewart (2003) also obtained transgenic *Brassica napus* L. from hypocotyl segments following *Agrobacterium* mediated transformation.

Considering all issues, the objectives of the present study was to develop a reproducible any efficient protocol for the insertion of molecular genes into *Brassica* through *Agrobacterium tumefaciens* vectors and to standardize the periods of preculture and co-cultivation required for transformation and to analyze the putative transgenic plants using histochemical *GUS* assay and PCR technique.

Materials and Method

The experiment was conducted in the Tissue Culture Laboratory and Genetic Engineering & Biotechnology Laboratory of the Department of Genetics and Plant Breeding, Bangladesh Agricultural University, Mymensingh during the period from July 2004 to April 2005. Two cultivars one from *Brassica rapa* and the other from *Brassica napus* were taken to standardize different parameters of plant transformation. The varieties were Tori-7 and BARI Sarisha-8, respectively.

Strain and plasmid of vector *Agrobacterium*

Genetically engineered *Agrobacterium tumefaciens* strain LBA4404 was used as a vector for infection in the transformation experiment, which contains plasmid

pBIl2l of 14 KDa (binary vector) (Fig. 1). This binary vector contains following genes within the right border (RB) and left border (LB) region of the construct:

The udiA gene (Jefferson, 1986) predetermining *GUS* (β-gIucuronidase), driven by CaMV promoter and NOS terminator. This reporter gene can be used to assess the efficiency of transformation. The nptII gene (Herrera-Estrella *et al.*, 1983) encoding neomycin phosphotransferase II (nptll) conferring kanamycin resistance, driven by NOS promoter and NOS terminator. The bacterium also contains plasmid pAL4404 which is a disarmed 1 plasmid (132 KDa) containing the virulence genes.

Plasmid pBI 121

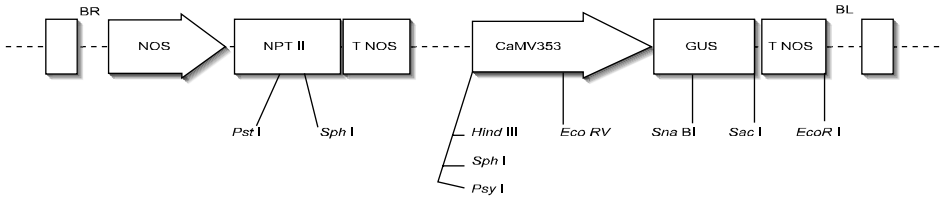


Fig. 1. Region between left (BL) and right (BR) border of pBI 121 from *Agrobacterium tumefaciens* strain LBA4404.

Media used

For seed germination

Half strength MS (Murashige and Skoog, 1962) medium supplemented with 20 gL^{-1} sucrose.

For preculture

MS (Murashige and Skoog, 1962) medium supplemented with 2 mgL^{-1} BAP and 0.1 mgL^{-1} NAA.

For Agrobacterium culture and inoculation

Two different types of culture media, namely YMB (Yeast extract Mannitol Broth) medium. LB (Luria Broth) medium were used with kanamycin as antibiotic to grow the strain of genetically engineered *Agrobacterium tumefaciens.* Here two sorts of media were used, such as *Agrobacterium* maintenance medium and *Agrobacterium* working culture medium for transformation.

For co-cultivation

MS medium supplemented with 2 mgL^{-1} BAP and 0.1 mgL^{-1} NAA

For washing of explants after co-cultivation

MS liquid medium supplemented with 500 mgL^{-1} cefotaxime

For post-cultivation and callus induction

MS medium supplemented with 2 mgL^{-1} BAP, 0.1 mgL^{-1} NAA and 200 mgL^{-1} cefotaxime.

For low selection

MS medium supplemented with 2 mgL^{-1} BAP, 0.1 $rngL^{-1}$ NAA, 20 mgL^{-1} kanamycin and l0 $rngL^{-1}$ cefotaxime.

For selection and regeneration

MS medium supplemented with 2.5 mgL^{-1} BAP, 2 mgL^{-1} NAA, 30 mgL^{-1} kanamycin and 75 mgL^{-1} cefotaxime.

For root initiation

Half strength MS medium + 50 mgL^{-1} kanarnycin + 50 mgL^{-1} cefotaxime

Half strength MS medium + 0.5 mgL^{-1} IBA + 50 mgL^{-1} kanamycin + 50 $rngL^{-1}$ cefotaxime

Half strength MS medium + 1.0 mgL^{-1} IBA + 50 mgL^{-1} kanamycin + 50 mgL^{-1} cefotaxime

For transplanting of plantlets from culture vessel to pot in growth chamber

Soil containing 25% garden soil + 50% sand + 25% cowdung

For watering the plantlets in the growth chamber

Hoagland's solution

For transplanting of plantlets from growth chamber's pot to earthen pot

Soil containing 25% garden soil + 50% sand + 25% cowdung

The following culture techniques were employed in the present investigation

Explant preparation and preculture

The germinated seedlings raised in axenic culture were used as the source of explants. Petiole segments were used as explants. After four days, petioles were excised into 3-4 mm pieces from the seedlings. This was carried out by gently holding the base of the petioles with forceps, and slicing through the stem just below the shoot tip using sterilized surgical blades. Ten pieces of petiole segments were arranged horizontally on each petridish and gently pressed into the surface of the sterilized pre-culture medium. The culture plates containing explants were placed under fluorescent light in a room with controlled temperature ($25\pm2^{0}C$) using 16 hours photoperiod. All the explants were kept in preculture media for 1-3 days. The petridishes were checked daily to note the response and the development of contamination.

Agrobacterium culture

Generally two kinds of culture media were needed for the *Agrobacterium* strain. One br maintaining the *Agrobacterium* stock and the other for the infection of explants. For maintenance, one single colony from previously maintained *Agrobacterium* stocks vas streaked into freshly prepared petridish containing YMB medium having kanamycin. The petridish was sealed with parafilm and kept in room temperature for atleast 48 hours. This was then kept at 40°C to check over growth. Such culture of the *Agrohacteriurn* strain was thus ready to use for liquid culture. The cultures were subcultured regularly at each week in freshly prepared media to maintain the stock. For infection, from this *Agrobacterium* stock, single streak was taken in an inoculation loop and was inoculated in a conical flask containing liquid LB medium with 50 mgL^{-1} kanamycin. The culture was then allowed to grow at 28°C to get optimum population of *Agrohacteriurn* for infection and co-cultivation of explants.

Infection and incubation

The *Agrobacteria* grown in liquid LB media were used for infection and incubation. Prior to this "Optical Density" of the bacterial suspension was determined at 600 nm with the help of a spectrophotometer. Following the determination of density, to get suitable and sufficient infection of the explants, freshly excised and precultured explants were dipped into bacterial suspension (0D600=0.6) for 10 minutes before transferring them to co-cultivation medium.

Co-cultivation

Following infection and incubation, the explants were co-cultured on co-cultivation medium. Prior to transfer of all explants to co-cultivation media, they were blotted dry with sterile filter papers for a short period of time to remove excess bacterial suspension. All the explants were maintained in co-cultivation media for 12 hrs-72hrs. Co-cultured petridishes containing explants were placed under fluorescent illumination with 16/8 hours light/dark cycle at 25±2°C. The intensity of light was maintained at 1500 lux. The culture vessels were checked daily to discard the contaminated vessels and to note the behaviour of the explants.

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. 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°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* infection. After X-

glue treatment, explants were transferred to 70% alcohol for degreening. Following degreening explants were observed under stereomicroscope.

Washing and post-cultivation

After co-cultivation for required periods, the infected petiole explants were ashed twice with sterile distilled water and once with liquid MS media supplemented with 500 mgL^{-1} cefotaxime. Then the explants were transferred onto post-cultivation medium.

Transfer to the selection medium

Following one week of post-cultivation, the explants were transferred onto low selective medium (LSM) consisting of MS medium with 2.0 mgL' BAP, 0.1 mgL^{-1} NAA, *2* mgL^{-1} $AgNO_3$, 20 mgL^{-1} kanamycin, and 100 mgL^{-1} cefotaxime. After culture for eight days, the petioles with calli were transferred onto selection and regeneration medium (SRM) consisting of MS medium supplemented with 2.5 mgL^{-1} BAP, 2.0 mgL^{-1} NAA, 2.0 mgL^{-1} $AgNO_3$, 25 mgL^{-1} Kanamycin, and 75 mgL^{-1} cefotaxime for further selection and shoot regeneration. After three weeks, the calli were subculturcd once on selection and regeneration media.

Transfer of the transgenic shoots for root initiation

Subcultured 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 vials with freshly prepared rooting medium to induce root. The conical flasks/vials containing plantlets were incubated at 22±2°C with 16 hours photoperiod. Day to day observations were carried out to note the responses.

Preparation of pot and transplantation

Potting mixture containing garden soil, sand, and cowdung in the ratio of 1:2:1 was mixed properly and autoclaved for one hour in 121°C at 1.16 $kgcm^{-2}$. After cooling, the soil mixture was taken into 10 cm plastic pots for growing the plantlets at *in vivo* condition. When the plantlets become 5-8 cm in length with sufficient root system, they were taken out from the vials. Medium attached to the roots was gently washed out with running tap water. The plantlets were then transplanted to pots containing potting mixture mentioned above. Immediately after transplantation, the plants along with the pots were covered with moist polythene bag to prevent desiccation. To reduce sudden shock, the pots were kept in a growth room for seven to fifteen days under controlled environment. The interior of the polythene bags was sprayed with distilled water at every 24 hours

to maintain higher humidity around the plantlets. At the same time, plantlets were also nourished with Hoagland's solution. After two to three days, the polythene bags were gradually perforated to expose the plants to natural environment. The polythene bags were completely removed after ten to fifteen days when the plantlets appeared to be self-sustainable. At this stage, the plantlets were placed in natural environment for 3-10 hours daily. Finally, after fifteen to twenty days, they were transferred to the field condition.

To investigate the effect of different treatments of the experiment, data were collected on the following parameters: percent *GUS* positive, days to callus initiation, percent callus induction, days to shoot initiation, total number of shoots per petridish, number of transgenic plantlets, and number of transgenic shoots with roots. The data for the parameters under present experiments were statistically analyzed wherever applicable. The analyses of variances for different parameters were performed and means were compared by the Duncan's Multiple Range Test (DMRT).

Results and Discussion

Histochemical GUS (β-gIucuronidase) assay

After infection of the petiole explants in *Agrobacterium* suspension culture, the were transferred to co-cultivation medium. Following incubation and co-cultivation with *Agrobacterium*, transformation ability was monitored through histochemical assay of GUS reporter gene in explant tissue. Transient *GUS* assay was done at the end of co-cultivation with randomly selected 20% inoculated explant tissue. In the *GUS* assay done according to Jefferson *et al.* (1987), conspicuous *GUS* positive (blue colour) regions were detected at the entire surface of the petiole explants. The detailed results of this investigation are presented in the Table 1.

Effect of genotypes

After the *GUS* histochemical assay, it was found that both Tori-7 and BARI Sarisha-8 showed positive responses towards transformation. Between the varieties, Tori-7 showed the better response (65% *GUS* positive) than BARI Sharisa-8 which showed poor response (50% *GUS* positive) to *GUS* assay (plate I). Control explants did not show any response (plate 2).Long time in preculture periods was found significant to influence transformation process. Positive GUS activity in explant increased with the hours of preculture. Therefore, the highest percentage of *GUS* activity (80% in preculture) was found in preculture period of 72 hours.

Table 1. Response of two varieties of *Brassica* towards *GUS* histochemical assay over different preculture and co-cultivation periods.

Variety	Preculture period (hours)	Co-cultivation period (hours)	No. of explants inoculated	No. of explants assayed for *GUS*	No. of explants + ve for GUS	GUS + ve explants (%)
Tori-7	00	24	50	10	0	00
		48	50	10	4	40
		72	50	10	4	40
	24	24	50	10	0	00
		48	50	10	7	70
		72	50	10	8	80
	48	24	50	10	1	10
		48	50	10	8	80
		72	50	10	9	90
	72	24	50	10	6	60
		48	50	10	8	80
		72	50	10	10	100
BARI Sarisha-8	00	24	50	10	2	20
		48	50	10	0	00
		72	50	10	1	10
	24	24	50	10	2	20
		48	50	10	3	30
		72	50	10	5	50
	48	24	50	10	4	40
		48	50	10	6	60
		72	50	10	7	70
	72	24	50	10	5	50
		48	50	10	7	70
		72	50	10	8	80

Another factor co-cultivation period of the explants with *Agrobacteriurn* was also found influencing genetic transformation. For this purpose, different co-cultivation periods were followed using bacterial suspension having constant optical density (0D600 = 0.6). Long time in co-cultivation period affected the callus induction and survivability. The percentage of *GUS* positive explants increased with the increase of co-cultivation period, if preculture period remains

constant. The highest percentage (72%) of *GUS* positive explants was in co-cultivation period of three days.

Table 2. Response of *Brassica rapa* and *Brossica napus* varieties towards callus induction and towares plant transformation over different preculture and co-cultivation periods..

Variety	Preculture period (hours)	Co-cultivation period (hours)	No. of explants inoculated	No. of explants producing callus	Callus induction (%)	No. of viable callus after 30 days co-cultivation
Tori-7	00	24	50	2	4	1
		48	50	3	6	2
		72	50	2	4	1
	24	24	50	0	0	0
		48	50	5	10	3
		72	50	4	8	2
	48	24	50	1	2	0
		48	50	6	12	3
		72	50	4	8	2
	72	24	50	3	6	0
		48	50	7	14	2
		72	50	4	8	1
BARI Sarisha-8	00	24	50	0	0	0
		48	50	1	2	1
		72	50	1	2	1
	24	24	50	2	4	1
		48	50	3	6	2
		72	50	4	8	2
	48	24	50	1	2	0
		48	50	5	10	3
		72	50	3	6	2
	72	24	50	2	4	0
		48	50	6	12	1
		72	50	3	6	0

Callits induction

The explant started to initiate callus in 12-15 days after inoculation by swelling the cut ends (Plate 3). Number of callus/petridish showed variation depending on

different preculture and co-cultivation periods. The results are shown in the Table 2. Tori-7 showed better performance in callus induction than BAR Sharisa-8 when the explants were pre-cultured in 72 hours and co-cultivated in 48 hours. The average percentage of callusing/petridish was the highest (3.14) in Tori-7 and the lowest (2.58) in BARI Sharisa-8. Average callus induction was also highest in seventy two hours period of preculture (6 %) and forty eight hours of cocultivation (9%) (Data not presented). But with the increase in co-cultivation period survivability of callus after 30 days decreased dramatically, as a result, the number of viable callus per petridish decreased after 30 days of co-cultivation with 72 hours (1.37).

Mean values for number of callus/petridish of different preculture and co-cultivation periods were found statistically significant. indicating significant differences among the periods. Preculture period of seventy two hours showed highest callusing/petridish (4) with the 24 hours co-cultivation. Twenty-four hours of co-cultivation period showed best performance in callus induction than any other times (1.5) as shown in the Table 3.

Tbale 3. Performance of different preculture and co-cultivation period on number of callus/petridish of *Brassica*.

	Time (hours)	Number of callus/petridish
Preculture Periods	00	1.80D
	12	3.00C
	24	4.00 B
	72	4.90A
Co-cultivation Periods	24	0.50C
	48	1.50A
	72	1.04B

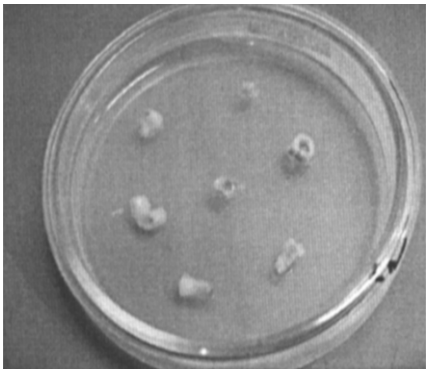
Initiation of shoot

After culturing on low selection media, calli were transferred to selection and regeneration media for shoot initiation. Calli started to initiate shoot buds after 50-60 days of incubation. The percentage of putative plant transformation showed variation among two varieties, precuhure and co-cultivation periods. The detailed results of this investigation are presented in the Table 5. Presence of kanamycin in the selection media greatly influenced the emergence of transgenic shoot from the transformed callus. Most of the calli failed to produce shoot and died in course of time. A few of the calli continued to grow and differentiated

into shoots (Plate 4). Long time in the co-cultivation period results no shoot regeneration from the callus. These results are in concordance with the findings of Kumria *et al*. (2001), who stated that prolonged infection time adversely affects the callus growth and subsequent regeneration. From the detailed result, it is clear that both Tori-7 (Plate 5) and BARI Sarisha-8 were able to produce putative transformed plantlet under the different levels of kanamycin. Transgenic shoot regenerated from *Agrohacterium* infected calli transferred to root induction medium. Numbers of rooted shoots are presented in Table 4.

Plate 1. Histochemical GUS activity (Blue zone) of three-day-precultured expalnts.

Plate 1. Histochemical GUS activity (No Blue zone) of without infected explants.



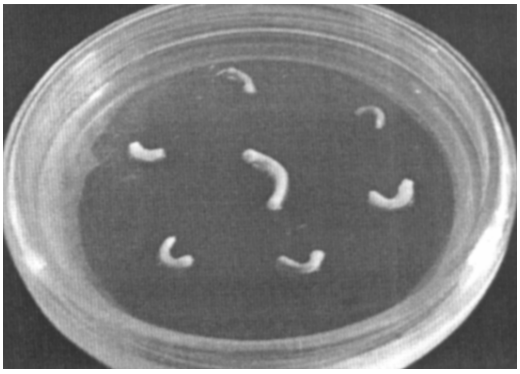


Plate 3. Callus initiation on selection medium from *Agrobacterium* infected petioles (left) and non-infected perioles (right) of Tori-7.

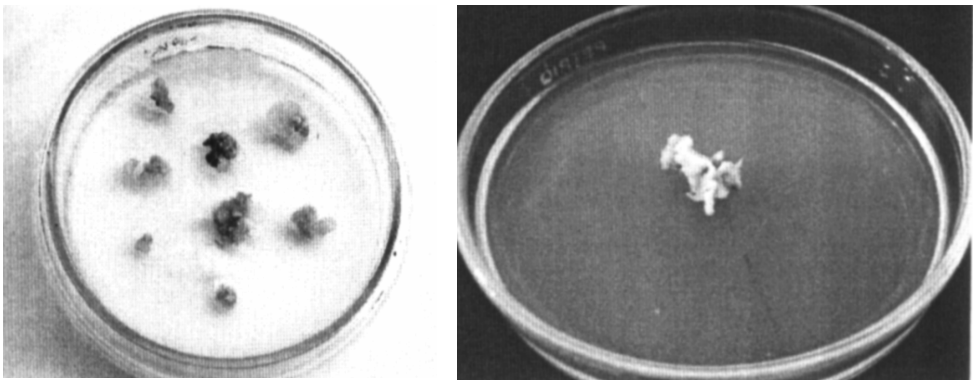


Plate 4. Initiation of shoot from putative transformed calli on selection and regeneration medium (left), regenerated albino plant (right).

Plate 5. Plantlet derived from *Agrobacterium* infected petioles of Tori-7.

In this piece of study, higher preculture period and co-cultivation time was found to bring higher success of bacterial infection to the explants as proved by *GUS* test. But most importantly, this initial success was found unsafe since the survivable of the calli after 3 days of co-cultivation were greatly reduced. So it is an important issue arises for the further investigation to sort out the possible reasons behind the screen for the future progress of transformation studies in *Brassica.* Another interesting finding of the study was negative relation between the increased co-cultivation period and regeneration success. Since longer co-cultivation period brings eventual death of the regenerating shoots so it could he speculated that excessive bacterial growth within the regenerating cells is one of the possible reason of cell death. But this sort of speculation is in need of further study to prove after explaining the possible biochemical reasons.

Table 4. Response of two *Brassica* varieties towards plant transformation over different preculture and co-cultivation period.

Variety	Preculture period (hours)	Co-cultivation period (hours)	No. of explants inoculated	No. of callus produced shoot	No. of regenerated shoot (transformed)	No. of shoot showing root
Tori-7	00	24	50	0	0	0
		48	50	0	0	0
		72	50	0	0	0
	24	24	50	0	0	0
		48	50	2	2	0
		72	50	0	0	0
	48	24	50	0	0	0
		48	50	3	5	3
		72	50	0	0	0
	72	24	50	0	0	0
		48	50	0	0	0
		72	50	0	0	0
BARI Sarisha-8	00	24	50	0	0	0
		48	50	0	0	0
		72	50	0	0	0
	24	24	50	0	0	0
		48	50	1	2	1
		72	50	0	0	0
	48	24	50	0	0	0
		48	50	1	1	0
		72	50	0	0	0
	72	24	50	0	0	0
		48	50	0	0	0
		72	50	0	0	0

Conclusion

We have got a simple protocol for *Brassica* transformation hut to make it more precise and reproducible, further research considering many other factors with more laboratory facilities should be carried out.

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