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IN VITRO REGENERATION AND OVEREXPRESSION OF PEA DNA HELICASE 45 (*PDH45*) GENE THROUGH *AGROBACTERIUM*-MEDIATED GENETIC TRANSFORMATION IN OILSEED *BRASSICA* SPP.

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Key words: Oilseed *Brassica*, *In vitro* regeneration, *PDH45* gene, Overexpression, *Agrobacterium*-mediated transformation

Abstract

An efficient genotype independent method for *in vitro* regeneration and genetic transformation of *Brassica* spp. viz. *Brassica juncea* and *Brassica napus* have been developed for the local varieties using cotyledonary leaf with petioles and hypocotyls as explants. Explants obtained from 6-day old aseptically grown seedlings were inoculated and co-cultivated with *Agrobacterium tumefaciens* strain EHA105 harbouring a binary vector with *PDH45* gene under the regulatory control of 35s promoter and terminator sequences, permitting transformed shoots to be selected on hygromycin containing medium. Well rooted transformed plants were transferred to pots containing soil and after acclimatization the plants were maintained under controlled environmental condition. A total of 9 transformed plants were obtained from BARI Sarisha-8 variety, presumably indicating this protocol is more amenable to genotype independent genetic transformation. Integration and expression of the introduced transgene (*PDH45* and *hptII*) were analysed by polymerase chain reaction (PCR). Factors influencing transformation efficiency include explant age, optical density of *Agrobacterium* culture for infection, duration of infection and co-cultivation with *Agrobacterium* were also assessed. Genetic transformation method developed in this study certainly could be utilized for introducing abiotic stress resistance in the local cultivars of oilseed *Brassica*.

Introduction

Brassica spp. (rapeseed and mustard) is one of the major oil yielding crops of the world. Oilseed *Brassica* crops are also the major source of edible oil in Bangladesh. Both rapeseed and mustard are rich in various nutrients and considered to be good for human health. Both *Brassica juncea* and *Brassica napus* contain 40 - 45% oil and 20 - 25% protein with well- balanced amino acids and minerals [1]. Moreover, lowest amount of saturated fatty acids among the vegetable oils are present in the *Brassica* spp.[2-4]. It covers the

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highest acreage, which is 61.2% of the total oilseed's acreage of Bangladesh and 52.6% of the total production[5]. Improvement of crop quality and productivity significantly pestered due to traditional breeding methods. However, the current plant breeding programs are limited in terms of availability of suitable genes within its available germplasm. The techniques of modern biotechnology i.e., tissue culture and plant genetic engineering could be the ways to achieve substantial improvement of desired crop plants.

It is worth mentioning here that oilseed *Brassica* crops are severely affected by various degrees of biotic and abiotic stresses. Currently soil salinization is the most prominent problem for the cultivation of popular and valuable crops in different parts of the world including Bangladesh. Approximately 7% of the world's land area is affected by salinity [6] and 50% of world cropland are salt affected [7]. In Bangladesh 30% of its total area is cultivable and about 20% covering the coastal area are saline soil [8]. Moreover, salinity affected area has increased to 0.833 from 0.1056 million hectares in the last four decades [9]. It has been reported that in dry season the salinity level is higher than 4 ppt in salinity prone Khulna and Bagerhat districts of Bangladesh [10]. This salinity in the soil is increasing gradually due to the changing climate as well as through the various human activities such as irrigation of water in cultivated land, fertilization in land, industrialization etc. These problems raised much more concern in the production of *Brassica* as they are mainly grown in arid and semi-arid areas and in addition salinity is associated with the reduction of seed germination rate, affecting the pollen viability [11, 12] and in an acute level of salinity plant cannot grow at all. Apart from these, Kauser *et al*. (2006)[13] and Shah (2007)[14] reported that photosynthetic and osmotic pressure rate found to decrease under saline condition. Maggio *et al.* (2004)[15], Badruddin *et al*. (2005)[16], Jamil *et al*. (2005)[17] pointed out that shoot and root length also affected by salt stress.

Bangladesh requires about 1.73 million tons of edible oils per year of which about 1.6 million fully meet by import. Therefore, it is necessary to increase the production of oilseed *Brassica* to fulfil the ever increasing demand for edible oil. Conventional breeding alone is not sufficient to develop abiotic stress tolerance due to unavailability of suitable germplasm also high degree of segregation in *Brassica* spp. upon cross pollination. Under these circumstances, it is essential to improve various economically and agriculturally important traits via genetic engineering techniques, which are powerful tools for gene functional analysis and crop improvement[18]. Genetic transformation techniques have promoted the improvement of crop varieties by integration of novel genes to satisfy the needs for high-yield and good-quality crops, including characters for effective oil production[19], herbicide and disease-resistance[20,21]. This technique has been successfully used to improve some major crops, such as soybean, maize and cotton[22]. However, to our knowledge, the application of genetic transformation technique in oil seed *Brassica* is still relatively inadequate in obtaining desired transformation efficiency. Development of

abiotic stress tolerance particularly the production of salt tolerant lines in *Brassica* is still a quest for the scientists. Therefore, a suitable and effective genetic transformation system accompanied by a highly efficient *in vitro* regeneration method is essential for the improvement of local varieties oilseed *Brassica*.

Recent reports unveiled that integrating salinity tolerant gene i.e., helicase as it provides duplex unwinding function in an ATP-dependent manner could solve the salinity stress tolerant problems. There is an earlier report on pea DNA helicase 45 (*PDH45*), a homolog of translation initiation factor 4A (*eIF4A*) to play important role in salinity stress tolerance in tobacco[23] and rice varieties from India and Bangladesh[24,25]. It was also reported that overexpression of *PDH45* gene via *Agrobacterium*-mediated transformation showed salinity tolerance in the indica rice variety IR64[26].

In this study, we have explored the possibility of developing a genotype independent regeneration protocol as a pre-requisite for *Agrobacterium*-mediated genetic transformation and successfully developed a method for *in vitro* production of plantlets from the explants of cotyledonary leaf with petioles and hypocotyls from local *Brassica* varieties. Based on this highly efficient regeneration protocol, an efficient transformation system by overexpressing *PDH45* gene in *Brassica* has been established.

Materials and Methods

Plant materials: Three varieties *Brassica,* namely BARI Sarisha-8 and BARI Sarisha-13 of *Brassica napus* as well as BARI Sarisha-16 of *Brassica juncea* provided by the Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur (Fig. 1) were used in this study. Seeds were surface sterilized and germinated according to Mollika *et al.* (2011)[1]. Hypocotyls were dissected with sharp scissors to produce 2-3 mm long segments from 6-day-old aseptically grown seedlings. Hypocoltyls and Cotyledonary leaf with petioles was excised from 6-day-old seedlings and were used for transformation experiments (Fig. 2).

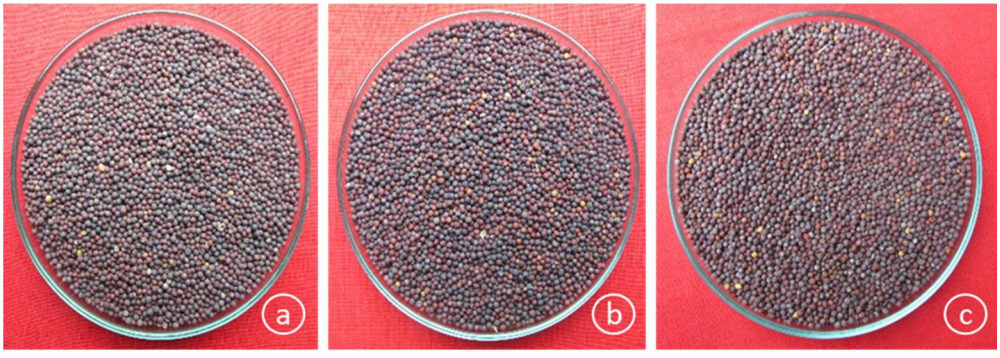

Fig. 1a-c: Seeds of three varieties of *Brassica* spp. used in this investigation. a) *B. napus* var. BARI Sarisha-8, b) *B. napus* var. BARI Sarisha-13, c) *B. juncea* var. BARI Sarisha-16.

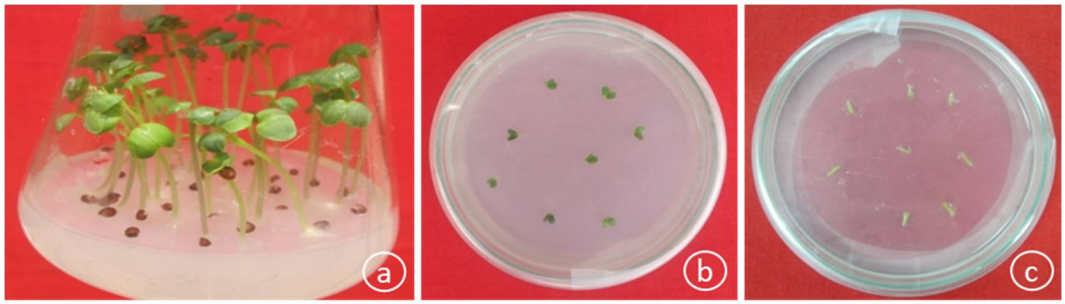

Fig. 2a-c: Explants used in the present study. (a) Explants obtained from seedlings those were used for *in vitro* regeneration and genetic transformation, b) cotyledonary leaf with petiole, and c) hypocotyl of BARI Sarisha-8.

In vitro regeneration of plantlets: Explants isolated from 6-days old seedlings were cultured on MS medium[27] containing BAP, NAA and GA_3 (mg/l) singly or in combinations for regeneration of shoots. *In vitro* regenerated shoots were sub-cultured 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 MS with different concentrations of NAA and IBA. The plantlets with sufficient root system were then transplanted to small plastic pots containing sterilized soil.

Agrobacterium tumefaciens strain and vector: Agrobacterium tumefaciens strains *EHA105* carrying binary vector *pCAMBIA1301-PDH45* was used for transformation studies. The T-DNA region of *pCAMBIA1301-PDH45* contains the selectable hygromycin phosphotransferase gene (*hptII*) for hygromycin (*Hyg*) resistance, *GUS* reporter gene and *PDH45* as gene of interest (Fig. 3).

Agrobacterium-mediated genetic transformation: The overnight grown *Agrobacterium* culture was centrifuged for suspension culture and was used for infection of explants. Prior to this "Optical Density" (OD) of the bacterial suspension was determined at 600 nm of 0.3 - 0.4 using spectrophotometer (Shimadzu, Japan). Pre-incubated explants (on MS basal shoot induction medium kept for 2 days in darkness) were immersed in the *Agrobacterium* suspension for 5 min (for hypocotyls) or 10 min (for cotyledonary leaf with petioles) with gentle shaking.

The explants were then placed on the co-cultivation medium which was covered with the sterile filter paper at 25 ± 2°C. After co-cultivation for 2 days in darkness, the explants were washed with distilled water for three to four times until no opaque suspension was seen, then washed for 15 min with distilled water containing 300 mg/l ticarcillin (Duchefa, The Netherlands). Then the explants were transferred onto the selection medium supplemented with 5 mg/l hygromycin and 400 mg/l ticarcillin. In the shoot induction stage, the explants were sub-cultured every 2 weeks on the same

medium. When the putative transgenic shoots emerged, they were transferred to the root induction medium. All antibiotics (hygromycin, rifampicin and ticarcillin) used in this study were filter sterilized. The culture conditions for selection and regeneration were the same as those for *in vitro* plantlet regeneration described above. Transformation efficiency was calculated as the percentage of the number of regenerated plantlets resistant to hygromycin over the total number of explants infected by *Agrobacterium.* Putative transgenic shoots were transferred to the root induction medium when developed sufficiently (Table 1).

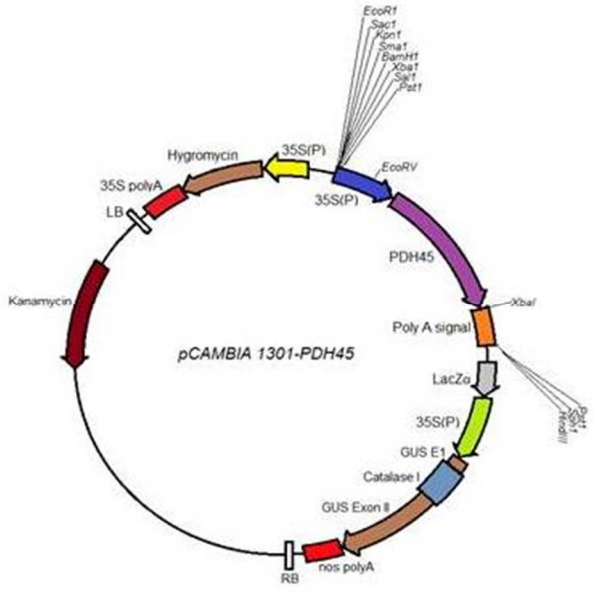

Fig. 3. Schematic representation of the vector construct *pCAMBIA1301-PDH45* gene used in *Agrobacterium*-mediated genetic transformation.

Histochemical analysis of stable GUS expression: Leaves and roots regenerated from 5 untransformed and 9 transformed plantlets were used for histochemical analysis of stable GUS gene expression. Histochemical GUS assays were conducted according to Cervera *et al*. (2005)[28]. The tissue was rinsed once with sodium phosphate buffer (pH 7.0) and then stained in 1 mM 5-bromo-4 chloro-3-indolyl-b-D-gulcuronide (X-gluc) buffer containing 0.5 mM potassium ferricyanide, 0.1% Triton X-100 and 10mM EDTA, pH 7.0. Samples were incubated overnight at 37°C and, if necessary, for one further day at 25°C. The tissues were cleared after staining by soaking in 95% ethanol.

Molecular analysis: Genomic DNA was isolated from the transformed *Brassica* shoots and stable integration of *PHD45* gene was examined by PCR (Eppendorf Master Cycler Gradient) analysis. For this purpose, DNA was isolated from transformed and non-

transformed shoots using the CTAB method. The primers that used for amplification were Fwd: 5'-ATGGCGACAACTTCTGTG-3' and Rev: 3'-GAGTCTAGATTATAT AAG ATCACCAATATC-5'. The plasmid isolated from *Agrobacterium* strain EHA105/ *pCAMBIA1305-PDH45* was used as positive control. The PCR analysis and electrophoresis of the PCR products was followed by the protocol described by Das *et al.* (2012)[29]. The amplified DNA was run on 0.80% agarose gel and stained with ethidium bromide (0.05 $\mu l/ml$). Gel electrophoresis was accomplished at 60 volts.

Results and Discussion

For initiation and development of shoots, MS medium[27] supplemented with different concentrations of BAP, NAA and GA_3 were used. Many researchers used 2, 4-D with BAP and supplements of $AgNO_3$ in MS medium for indirect initiation of shoots through callus culture[30,31]. Cotyledonary leaf with petiole and hypocotyl were used as explants in this study. These were also used as explants by Noman *et al.* (2008)[32] and Mollika *et al*. (2011)[1] in a separate set of experiments. Transformation compatible high frequency uniform *in vitro* regeneration of shoots (88%) was achieved on MS medium supplemented with 13.5 µM BAP + 1.3 µM GA_3 + 1.07 µM NAA in BARI Sarisha-8 from the explants of cotyledonary leaf with petiole (Fig. 4a). Best regeneration responses for shoots induction from hypocotyl explants (Fig. 4c) were obtained on MS medium with hormonal combination of MS + 15.5 µM BAP + 2.0 µM GA_3 + 1.07 µM NAA for BARI Sarisha-8 (86%) (Table 1).

Table 1. Effects of hormone on shoot regeneration and root induction of three varieties of *Brassica*.

Varieties	Explants	Hormonal combination for shoot initiation			Regeneration of shoots (%)	Shoot no./ explant	Combination of hormones with half MS media for root initiation		% of responsive shoots on root induction media
		BAP	GA_3	NAA (µ M)			NAA (µ M)	IBA	
BARI Sarisha-8	Hypocotyls	15.5	2.0	1.07	86	3.2	13.4	2.0	75
	Cotyledonary leaf with petioles	13.5	1.3	1.07	88	3.4			
BARI Sarisha-13	Hypocotyls	13.5	1.3	1.07	81	3.30	13.4	2.0	80
	Cotyledonary leaf with petioles	13.5	1.3	1.07	83	3.2			
BARI Sarisha-16	Hypocotyls	8.88	2.0	1.07	77	3.19	-	2.0	73.33
	Cotyledonary leaf with petioles	8.88	1.3	1.07	83	3.2			

Nearly identical results were reported earlier by Gowshami *et al*. 2020[33] in case of different *Brassica* genotypes. Development of shoots from cotyledonary leaf with petiole explants from the variety BARI Sarisha-8 has been presented in Fig. 4b. Initiation of

regeneration (Fig. 4a,c) from these explants occurred within 3 - 5 days of culture and 16 - 20 days were required for the elongation and multiplication of shoots (Fig. 4b,d) applicable for both the explants of cotyledonary leaf with petioles and hypocotyls. In BARI Sarisha-8 percentage of regenerated shoots were 88 and 86% in case of cotyledonary leaf with petioles and hypocotyl explants, respectively and mean number of shoots per explants were 3.4 and 3.2, respectively in this combination of hormonal supplements (MS + 15.5 µM BAP + 2.0 µM GA_3 + 1.07 µM NAA) using these explants (Table 1). Almost identical responses (77 - 83% of shoot generation) towards regeneration of shoot were observed in case of varieties BARI Sarisha-13 and BARI Sarisha - 16. In this case the further elongation and proliferation of multiple shoots were successfully carried out in hormone free MS medium.

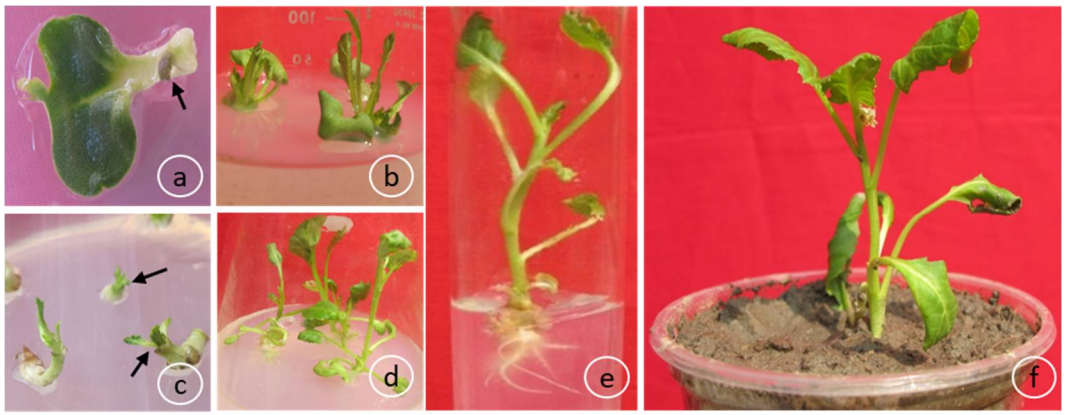

Fig. 4. Different stages of *in vitro* regeneration in case of BARI Sarisha-8. (a - d) Initiation and elongation of shoots (arrow) from cotyledonary leaf with petiole and hypocotyl explants (e) formation of *in vitro* roots (f) *In vitro* regenerated plantlets established in soil.

Various morphogenic responses were recorded during *in vitro* morphogenesis of shoot from specific explants. In case of BARI Sarisha-8, microscopic observation revealed the initiation of shoot bud (Fig. 5a) from the developing callus tissue. Development of shoot primordia (Fig. 5b), formation of vascular tissue within the meristematic dome (Fig. 5c), and development of leaf primordia (Fig. 5d) were visualized at an early stage of shoot development. Stereomicroscopic view of shoot formation from hypocotyl and cotyledonary leaf with petiole has been presented in Fig. 5e, f, respectively. Similarly, these observations were conducted in case of BARI Sarisha-13 and BARI Sarisha-16 to monitor the morphogenic responses during regeneration of shoots.

Several reports are available where IBA was used for adventitious root formation from excised regenerated shoots of *Brassica*[34,35]. In this study maximum root induction was achieved on half strength of MS supplemented with 13.4 µM NAA + 2 µM IBA in

case of BARI Sarisha-8 (75%) and BARI Sarisha-13 (80%) whereas only 2.0 µM IBA was effective for root formation in BARI Sarisha-16 (73.33%) (Table 1). Same hormonal supplement with half strength were used in rooting as reported by Tang *et al*. (2010)[36] and Ali *et al.* (2007)[31]. *In vitro* rooted plantlets were hardened and acclimatized within four weeks (Figs. 4e, f). After sufficient development of roots plantlets were transferred to large earthen pots containing soil and organic manure (5 : 1). This protocol described here for *in vitro* regeneration for *Brassica* varieties were utilized for transformation experiments.

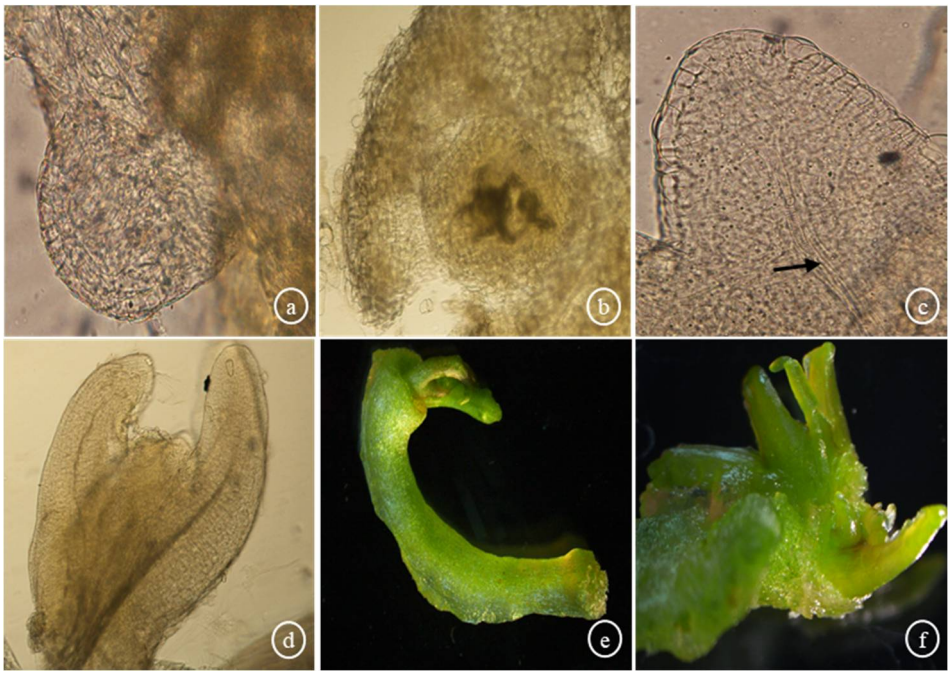

Fig. 5a-f: Microscopic view of various stages of initiation and development of shoot in BARI Sarisha-8 (a) initiation of shoot bud (x 40), (b) formation of apical meristem (x 30), (c) micrograph of developing apical meristem showing the formation of vascular tissue (arrows) within the meristematic dome (x 40), (d) development of leaf primordia (x 20), (e) & (f) shoot formation from hypocotyl and cotyledonary leaf with petiole, respectively.

To develop an effective transformation system for *Brassica*, the variety BARI Sarisha-8 was used during this study. Several parameters required for successful transformation such as optical density of the *Agrobacterium* suspension, incubation period, co-cultivation period of explants were optimized. All the parameters of transformation were optimized through GUS histochemical assay. Transformation efficiency of explants was gradually increased with the increased optical density (0.3, 0.5 and 0.8). During optimization of regulatory factors, it was found that maximum transformation was obtained with bacterial suspension having an OD of 0.5 with 30 minutes of incubation and 3 days of co-

cultivation period for transformation of cotyledonary leaf with petiole and hypocotyl of BARI Sarisha-8. As it was reported that co-cultivation is genotype dependent[37], thus effect of co-cultivation depends on the species. 30 min of incubation period followed by 72 hours of co-cultivation period were found to be the most effective towards transformation. Kamboj *et al*. (2016)[38] reported maximum transient GUS expressions in both hypocotyl (80%) and cotyledon (75%) explants in *B. juncea* when 2-days-precultured explants were inoculated with *Agrobacterium* suspension for 30 min and co-cultivated for 72 hrs. In chickpea, Krishnamurthy *et al*. (2000)[39] were able to obtain transgenic plants when mature embryo explants were incubated for 20 minutes followed by co-cultured of the explants for 3 days. Tewari-Singh *et al.* (2004)[40] employed the same co-cultivation period of 3 days for chickpea but incubated the explants in bacterial suspension for 1 - 2 hrs and thus obtained transgenic plants.

In the present study it was found that, longer co-cultivation period (over 3 days) leads to over-growth of bacteria in culture medium thus hampering the proper growth of infected explants. After co-cultivation, the explants of cotyledonary leaf were cultured on MS medium containing 13.5 µM BAP, 1.3 µM GA_3, 1.07 µM NAA, 6 mg/l hygromycin and 100 mg/l carbenicillin for regeneration of shoots. In this media the initiation of regeneration was found to occur after six to seven weeks from co-cultivation. Hygromycin (6 mg/l) was found to be effective in selecting the transformed shoots about 63% of co-cultured explants were survived and showed initiation of regeneration at the cut ends of explants.

Due to hygromycin selection the non-transformed explants as well as a part of several explants gradually turned yellow to brown in colour (Fig. 6) and such tissue was discarded during the subculture. In this experiment the effect of hygromycin selection was adequate when it was applied immediately after co-cultivation of infected explants. Using hypocotyl explant of *B. napus,* Liu *et al.* (2011)[41] developed transgenic plant in 3 mg/l Hyg selection pressure. From cotyledonary nodes transgenic plant were developed by Kong *et al.* (2009)[42] in 10 mg/l hygromycin selection pressure. 30 mg/l Hyg selection pressure was used to find out transgenic plants of *B. juncea* developed from hypocotyl explant. Following subculture of survived healthy explants, initiation of shoots was observed after 12 - 14 weeks of co-cultivation of explants. It was found that all the responsive explants didn't produce shoots and regeneration efficiency was 19% with efficiency of 2 - 3 shoots per explant. For proliferation and elongation of shoots, small green shoots along with the shoot buds were then transferred to fresh MS containing 6 mg/l hygromycin. Following elongation, the shoots (2 - 3 cm long) were subjected to *in vitro* rooting for three to four weeks. After four weeks the rooted and acclimatized putatively transformed plantlets were transplanted to large earthen pots.

Fig. 6a-c: Selection of transformed shoots of BARI Sarisha - 8 using selectable medium containing 6.0 mg/l hygromycin. (a) shoots survived on selection media, (b) non transformed shoots became albino, and (c) showing the control shoots failed to survive.

GUS histochemical assay was carried out at every step of shoot development during the transformation to monitor the efficiency of transformation. Stable expression of *GUS* gene was visualized through histochemical staining of the transformed rooted shoots. Different plant parts exhibited the characteristic blue colour indicating the efficient insertion of *GUS* gene in the transformed plants. Histochemical localization as well as transient GUS activity of cotyledonary leaf with petiole and hypocotyl explants following *Agrobacterium* infection is presented in Fig. 7.

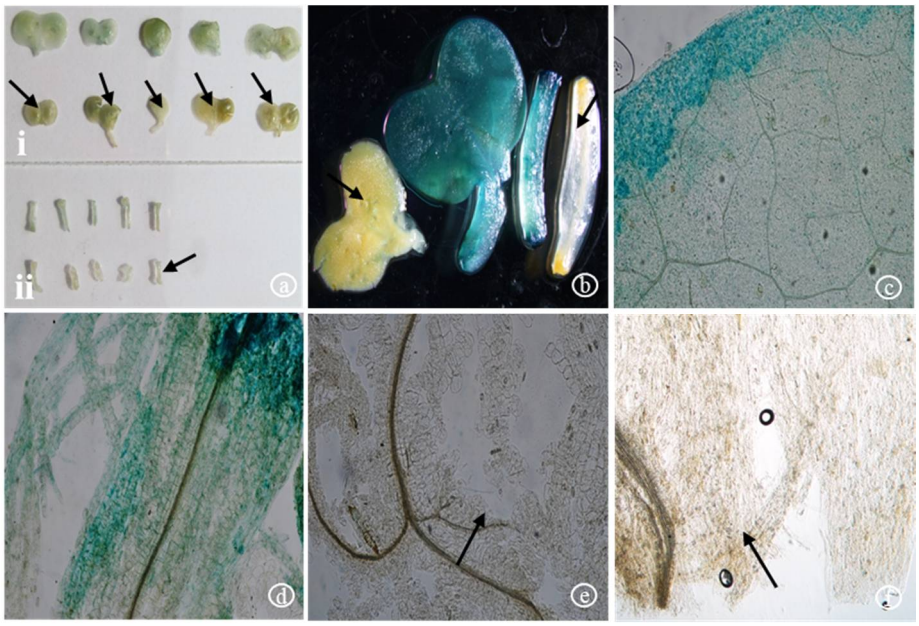

Fig. 7a-f: Histochemical localization of GUS activity of cotyledonary leaf with petiole and hypocotyl explants infected with *Agrobacterium* strain EHA105 containing plasmid *pCAMBIA1301-PDH45*. (a) cotyledonary leaf with petiole (i) and hypocotyl (ii), transformed explants showing blue color but control explants did not show blue color (arrow indicates control explants), (b) stereomicroscopic view of cotyledonary leaf with petiole and hypocotyl showing expression of blue color while control explants (arrow) did not produce characteristic blue color, (c) &(d) photomicrographs of cotyledon leaf and hypocotyl tissue showing in the expression of *GUS* gene developed blue color, and (e) & (f) tissues from control explants showing the absence blue color.

The transgenic nature of the transformed plants was confirmed through PCR analysis to monitor the insertion of the *PDH45* and *hptII* genes within the genomic DNA (Fig. 8a,b) of transformed plants. For this purpose, genomic DNA from 11 putatively transformed plants was used. Specific primers for *PDH45* and *hptII* genes were used for this purpose. From the gel it was evident that both *PDH45* and *hptII* genes were inserted in the genomic DNA of transformed plantlets exhibiting very clear and prominent band identical to that found in the amplified DNA of *Agrobacterium* strain EHA105/ *pCAMBIA1301_PDH45* (positive control). Non transformed plant was used as negative control.

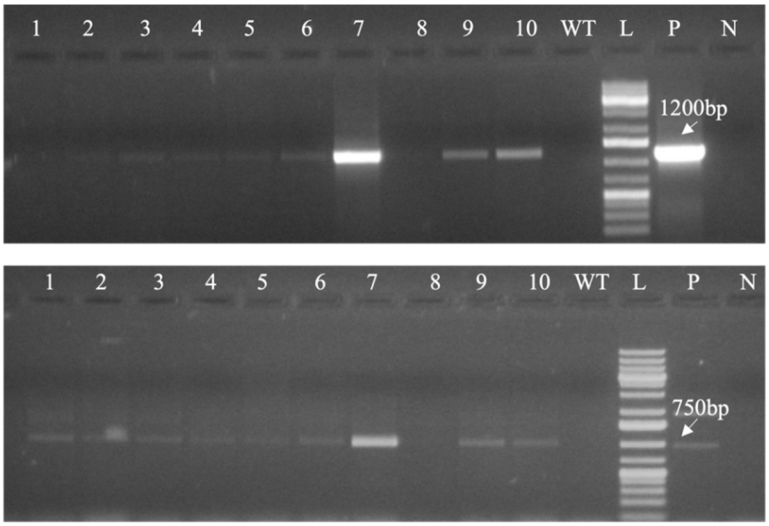

Fig 8a-b: Molecular confirmation of the putative transformed plants. a) PCR amplification of *PHD45* gene from transformed shoots of BARI Sarisha-8. Lane L - 1kb ladder; lane P positive control; lane N - negative control, lane WT - wild type. b) Same as (a) but PCR amplification for *hptII* gene with gene specific primers. Arrow indicates the position of 1200 bp in case of *PDH45* gene and 750 bp in case of *hptII* gene in Fig. (a) and (b).

During this study method for *in vitro* regeneration was established for the three local varieties of oil seed *Brassica* using the explants of cotyledonary leaf with petioles and hypocotyls. This regeneration protocol was found to be compatible to genetic transformation. Therefore, an efficient and reproducible *Agrobacterium*- mediated transformation protocol was also established for BARI Sarisha-8 of Bangladesh using the same explants of cotyledonary leaf with petiole and hypocotyl. Factors influencing transformation efficiency were also established with the help of this explants from BARI Sarisha-8. Utilizing this standardized protocol agronomically important gene/genes can be transferred to the locally grown *Brassica* varieties. Particularly this technique of transformation can be exploited for the development of salinity and drought resistant *Brassica* varieties which will certainly contribute significantly to future agriculture of Bangladesh.

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