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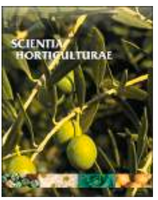

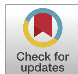

An improved *Agrobacterium* mediated transformation and regeneration protocol for successful genetic engineering and genome editing in eggplant

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ABSTRACT

Successful genetic manipulation largely depends on effective transformation and regeneration methods. The *Agrobacterium* mediated transformation has always been the method of choice for crop improvement through genetic engineering and the recently developed CRISPR/Cas9 based genome editing approaches. In this study, we have developed an improved regeneration and *Agrobacterium* mediated transformation protocols for three high yielding eggplant varieties BARI begun 2, 4 and 6. Several key parameters were intensively studied which include culture media composition, concentration of growth regulators, compatible antibiotic selection, hyperhydricity and rooting process. For the three different explants used in the study, it was found that MS+2.5 mg/l BAP alone is optimal as a hormone supplement for maximum shoot regeneration. Significant reduction (16.67 ± 0.11%) in hyperhydricity was observed when casein hydrolysate along with sorbitol was used at a concentration of 0.2 mg/l and 0.1 mg/l respectively in MS+BAP 2.5 mg/l supplemented medium. Transformation efficiency was found to be highest with an *Agrobacterium* concentration of 0.6 at OD 600 nm, 10 min of infection and two days of co-cultivation. We found 100 mg/l concentration of kanamycin to be suitable as a selection pressure for screening eggplant transformation events. Rooting media composition standardized in this protocol provides a higher rate of root formation (85%) in both *in vitro* and *ex vitro* conditions. Using this protocol, existing problems in eggplant genetic engineering can easily be overcome with higher transformation efficiency.

1. Introduction

Agrobacterium mediated transformation is the most widely used method of genetic engineering for crop improvement. It is the preferred choice for delivery of a host or foreign gene overexpression construct into the crop genome, knock-down of a negative regulatory gene expression by RNAi or precise genetic manipulation by gene knock-out or targeted nucleotide substitution by gene editing tools. *Agrobacterium*-based genetic transformation has been the widely used in several crops for introducing useful traits such as resistance genes (R-genes) against insects and pathogens obtained from different germplasm, or for the introduction of entirely new beneficial traits such as those used for parthenocarpy and biofortified rich fruits, *etc.* Till date, conventional breeding approaches have been successfully applied in crop improvement programs to develop high-yielding varieties; however, these entail a lot of time and man-power. Such limitations can now easily be overcome with the availability of cutting-edge genetic engineering technologies for important agronomic trait manipulation. Therefore, *Agrobacterium* mediated genome engineering is a powerful alternative to traditional methods of breeding for eggplant (*Solanum*

Abbreviations: BAP, 6-benzylminopurine; NAA, Naphthalene acetic acid; IBA, Indole-3-butyric acid; IAA, Indole-3-acetic acid; hptII, Hygromycin-B-phosphotransferase II; nptII, Neomycin phosphotransferase II; CaMV 35S, Cauliflower mosaic virus promoter; NosT, Nopaline synthase terminator; GUS, β-glucuronidase; GFP, Green fluorescent protein; t/ha, Tonnes per hactor; mg/l, Miligram per litre; h, Hour; YEM, Yeast extract-mannitol.

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melongena L.) improvement. Also, eggplant draft genome has recently been available in public database which provides the basic requirements for the advancement of eggplant functional genomics study (Hirakawa et al., 2014), genome wide association analysis for identification of novel candidate genes of agronomic importance (Cericola et al., 2014; Portis et al., 2015). Furthermore, the study of functional genomics, crucial for genetic improvement of any crop can easily be achieved with the help of CRISPR-based gene knock-out/-in or transgenic approaches such as RNA interference (RNAi).Eggplant is a popular household vegetable crop in India and Bangladesh, cultivated mainly for cash and as a source of nutrition by resource-poor small and marginal farmers (Kumar et al., 2011). Even though this crop occupies about 25% of Bangladesh's total vegetable cultivation area, its productivity (9.2 t/ha) is found to lag much behind the global average of 26.1 t/ha (BBS, 2020). Incidences of viral, bacterial and fungal diseases coupled with abiotic stresses are major reasons behind the reduction of eggplant production in Bangladesh. An earlier report showed that fruit and shoot borer pest alone causes about 30%–60% worldwide yield losses in eggplant even after repeated insecticidal sprays (Shelton et al., 2018). Therefore, it is necessary to look for alternative approaches to overcome such serious agronomic impediments in eggplant cultivation. *Agrobacterium* mediated transformation for transgenesis and CRISPR-based gene editing has been successfully applied for the improvement of many crops. Similar strategies could also be implemented for genetic improvement in the commercially important eggplant varieties available in Bangladesh.

In order to apply transgenic and genome editing applications to develop improved eggplant varieties, an easy, effective and reproducible *Agrobacterium* mediated transformation protocol is a pre-requisite. To date, few studies have reported and documented the possibilities of genetic transformation in eggplant (Jadhav et al., 2015; Pratap et al., 2011). However, these earlier experiments were not particularly focused on the development of an efficient and reproducible *Agrobacterium* mediated transformation protocol. Most of these studies were actually devoted to gene characterization and developing transgenic eggplants with a few were focused on medium composition (Fári et al., 1995; Foo et al., 2018), explant types (Sharma and Rajam 1995; Magioli et al., 1998; Franklin and Sita 2003) or studied the effects of growth regulators and antibiotic (Billings et al., 1997) and delivery vectors (Rotino and Gleddie, 1990) on eggplant transformation. However, it is well known that the success rate of genetic transformation is affected by a wide range of factors such as explants sources, genotype or genetic background, pH of media, temperature of growth condition, *Agrobacterium* cell density on liquid suspension culture and co-cultivation medium, time duration of co-cultivation, antibiotic selection pressure, hormonal composition of shoot elongation or regeneration medium *etc*. Therefore, a comprehensive study of eggplant transformation and regeneration is important for understanding these crucial factors affecting genetic transformation efficiency.

Genetic background of different cultivated varieties and source of explant is an important factor that affects the different response to eggplant regeneration and transformation efficiency (Sharma and Rajam, 1995). The response of growth hormones in the culture media is also variable within genotype and explant for somatic embryogenesis and organogenesis in eggplant (Slater et al., 2003). Although, the first successful genetic transformation in eggplant in was achieved in way back to 1988 (Guri and Sink, 1988) to date, the transformation efficiency has been recorded very low with a range from 8 to 23% (Rotino and Gleddie, 1990; Magioli et al., 2000). Also, this low range of transformation has been reported mostly with cotyledonary leaves as an explants source in different eggplant cultivars. Lack of efficient transformation has already limited the eggplant functional gene study, and also increases bottleneck as to become a model organism for functional genetics within *solanaceae* family as well as trait improvements. Also, problems like tissue hyperhydricity and low rooting frequency were found to severely affect the efficiency of eggplant regeneration. The seriousness of hyperhydricity and low rooting frequency problems have been reported in earlier studies (Calvo-Asensio et al., 2014; Picoli et al., 2001); however, no study has reported the solutions to these critical issues. In the present study, we have first attempted to develop an easy, quick, reproducible and highly efficient *in vitro* regeneration protocol addressing some of the important parameters such as genotype, explants sources, media and hormone composition and pH, and also focused on tissue hyperhydricity and rooting frequency. Based on these observations we have further undertaken various experiments to established an improved protocol for *Agrobacterium* mediated transformation. The objective of the current study was to present a comprehensive transformation and regeneration protocol for popular eggplant varieties cultivated in Bangladesh. This newly developed protocol with all the modifications could be combined to attain a maximum number of transformation and efficient regeneration events in eggplant which will further help us to study the structural and functional genomics of eggplant. Not only that an efficient genetic transformation of eggplant varieties of Bangladesh will also speed up the development of new stress resilient varieties through molecular breeding.

2. Materials and methods

2.1. Plant material, surface sterilization and growth condition

Three high yielding eggplant (*Solanum melongena* L.) varieties namely, BARI begun 2, 4 and 6 were collected from the Bangladesh Agricultural Research Institute, Joydebpur, Gazipur, Bangladesh. Seeds were washed twice with sterile distilled water to discard non-viable floating seeds. All the viable seeds were then sterilized with 1% (w/v) bavistin and 0.1% $HgCl_2$ solution for 5 min followed by four to five washes with distilled water. The surface-sterilized seeds were air-dried on sterile filter paper for 5 min. For germination, the seeds were then inoculated on agar-solidified MS medium (Murashige and Skoog, 1962) with 3% sucrose. *In vitro* cultures were maintained in a growth room under fluorescent illumination on a 16 h photoperiod at 25 ± 2 °C. Cotyledon, hypocotyl and root from *in vitro* seedlings of 12–14 day-old were used as explant for the regeneration study and *Agrobacterium* transfection. Each of the cotyledon, hypocotyl and root was cut into two or three segments and each segment was used as an explant for optimizing *in vitro* regeneration and subsequent *Agrobacterium* mediated transformation.

2.2. Standardization of media composition for in vitro regeneration

Two to three-slices of cotyledon, hypocotyl or root explants were cultured on MS medium supplemented with different concentrations of cytokinin alone (6-benzylminopurine, BAP; zeatin) and also in combination with auxin (naphthalene acetic acid, NAA) for shoot regeneration. The concentrations of BAP and zeatin used were range from 1.0 to 3 mg/l (*i.e.* 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l) whereas 0.2 and 0.5 mg/l concentration of NAA was applied with BAP and zeatin at a concentration of 0.2 and 0.5 mg/l respectively (Supplementary Table 1). Similarly, a range of different concentration of casein hydrolysate (0.1–0.3 mg/l) and sorbitol (0.05–0.2 mg/l) were added to the shoot regeneration medium to test their effects on reducing hyperhydricity. For the root induction media, approximately, 3,4 cm long shoots were excised individually and cultured on full or half strength MS medium supplemented with different concentrations of IBA (indole-3-butyric acid), IAA (indole-3-acetic acid) and NAA (each with 0.2, 0.5, 1.0, 1.5 mg/l concentration) (Supplementary Table 2). The pH of the media was adjusted to 5.8 before adding gelrite. All media were solidified with 0.4% gelrite. The cultures were incubated at a controlled temperature of 25±2 °C with a 16 h photoperiod. Sub-culturing was done at intervals of 12–14 days. The well-rooted plantlets were then taken out from the test tubes and gently washed with water to discard the adhered medium. The washed plantlets were then transplanted to earthen pots containing a mixture of soil and compost (2:1). All pots were covered with perforated

polythene bags for two weeks and maintained inside the green house. After two weeks of acclimatization in the growth room, plantlets were transferred to big earthen pots for further growth and development.

2.3. Construction of expression vector(s) used for eggplant transformation

Agrobacterium tumefaciens strain EHA105 was used for the genetic transformation of eggplant. Different expression vectors namely pMDC100 and pCAMBIA1302 were used in this study. The vector pMDC100 contains a *neomycin phosphotransferase II (nptII)* gene which provides kanamycin resistance and pCAMBIA1302 contains a *hygromycin-B-phosphotransferase II* gene (*hptII*) responsible for hygromycin B resistance. Both these plant selection markers were present under the control of a cauliflower mosaic virus (CaMV35S) promoter and CaMV35S poly-A tail terminator of the respective vector backbone. Four different expression constructs were used for studying different parameters of *Agrobacterium* mediated transformation into eggplants.

First, we amplified the full-length coding sequence of small heat shock protein (sHSP) from eggplant (*Solanum melongena* L.) total cDNA pool by using a pair of nested PCR primers (sHSP_F1/sHSP_R1 and sHSP _F2/sHSP _R2) (Supplementary Table 3) and cloned into pCR-4-TOPO vector (Invitrogen, USA) by following the standard TA cloning procedure. After confirmation by sequencing, the *sHSP* gene was sub-cloned into a modified Gateway compatible entry vector 1 (pL12R34-Ap, modified in crop improvement lab, ICGEB) between 3X CaMV35S promoter and Nos terminator. The 3X CaMV35S promoter was cloned into *Kpn*I and *Nde*I restriction sites and *sHSP* gene into *Nde*I and *Not*I sites. The Nos terminator was cloned between *Not*I and *Sac*I restriction sites. The final construct in entry clone 1 was sequentially confirmed, first by colony PCR followed by restriction digestion (Supplementary Fig. 1A). The complete gene cassette was then transferred into the binary expression vector pMDC100 (Invitrogen, USA) by using the LR recombinase mediated gateway cloning process. The final expression cassette of *sHSP* gene was transformed into *Agrobacterium tumefaciens* strain EHA105 by electroporation (Supplementary Fig. 1B). In order to study the *Agrobacterium* mediated transformation efficiency into different eggplant explants, we generated two expression constructs with two different reporter genes *i.e.;* green fluorescent protein (mGFP) and β-glucuronidase (GUS) genes. Green fluorescent fusion protein (mGFP) based expression vector pCAMBIA1302 used for the study has hygromycin (*hptII*) as the plant selection marker gene. To fuse the full-length cDNA of *sHSP* with the open reading frame (ORF) of mGFP protein, the stop codon of *sHSP* gene was removed from the reverse primer of the PCR primer set (sHSP-GFP_F1/sHSP-GFP_R1) (Supplementary Table 3). The PCR amplified coding region of *sHSP* gene without the stop codon was subsequently inserted into pCAMBIA1302 binary vector between the CaMV35S promoter and mGFP with NOS terminator (NosT) by cloning within the *Nco*I and *Spe*I restriction sites (Supplementary Fig. 1C, D). Similarly, for the construction of GUS expression vector under kanamycin (*nptII)* selection, we amplified the coding region of GUS along with Nos terminator from pCAMBIA1301 plasmid with primer pair, GUS_F/NosT_R (Supplementary Table 3) using KOD plus high-fidelity DNA polymerase (Toyobo, Japan). This PCR amplicon was then cloned into the modified entry clone 1 (pL12R34-Ap) under CaMV35S promoter between *Nco*I and *Xho*I restriction sites and later transferred into pMDC100 binary vector contain *nptII* selection marker gene using LR recombinase mediated Gateway™ (Invitrogen, USA) cloning. *Agrobacterium tumefaciens* EHA105 strain was used to transfer of this GUS expression vector into eggplant. A separate plant expression cassette was also prepared by cloning plant codon-optimized *Streptococcus pyogenes Cas9* (*spCas9*) gene under the regulation of CaMV35S promoter and Nos terminator to generate a constitutively expressing *spCas9* platform, which would be useful for gene targeting in eggplant. The *spCas9* gene was codon optimized with 3X SV40 nuclear localization signal (NLS) to retain the expressed protein inside the nucleus. The CaMV35S promoter was cloned into *Kpn*I and *Bam*HI restriction sites, plant codon-optimized *spCas9* gene into *Bam*HI and *Not*I sites and Nos terminator into *Not*I and *Sac*I sites in the Gateway compatible modified entry vector 1 (pL12R34-Ap). The complete gene cassette (35SPromoter:SpCas9:NosT) was transferred into the expression vector pMDC100 (Invitrogen, USA) by the gateway cloning method (Supplementary Fig. 1E).

2.4. Preparation of Agrobacterium culture for transformation into eggplant

The following factors related to *Agrobacterium* transformation were evaluated in this study to obtain a maximum frequency for *Agrobacterium* mediated transformation in eggplant:-(i) density of *Agrobacterium* cell suspension used for infection, (OD_{600}= 0.2, 0.4, 0.6, 0.8 and 1.0), (ii) eggplant compatible antibiotic selection pressure (kanamycin 50 mg/l, 75 mg/l, 100 mg/l or 150 mg/l and hygromycin 5 mg/l, 10 mg/l, 12 mg/l or 15 mg/l), (iii) infection time (5, 8, 10, 15 or 20 min) and (iv) co-cultivation duration (1, 2, 3 or 4 days). Briefly, a single colony of *Agrobacterium* containing our expression cassette was inoculated in 10 ml yeast extract-mannitol (YEM) broth with appropriate antibiotics *i.e.*, rifampicin 25 mg/l and kanamycin 50 mg/l and growth at 28 °C with gentle shaking. After 24 h, 1% of this primary culture was inoculated into a 250 ml conical flask containing 50 ml YEM broth. Then this secondary culture was grown at 28 °C until the OD_{600} reached 0.6. The cells were pelleted down by centrifugation at 3500 rpm for 25 min at 4 °C. The pellet was resuspended in an appropriate volume of liquid MS medium to reach the target cell density. Acetosyringone at 100 mM concentration was added to the suspension and co-cultivation medium to induce expression of *Agrobacterium vir* genes which facilitate the transfer of T-DNA from *Agrobacterium* into plant cells. At the same time, 12 to 14 days old newly germinated sterile eggplant seedlings were used for the collection of different explants such as cotyledonary leaves, hypocotyls and roots which were then cut into two pieces. These freshly cut explants were dipped in the acetosyringone treated *Agrobacterium* suspension for 10 min. The *Agrobacterium* infected explants were dried for 5 min on sterile Whatman No. 3 filter paper. Explants were then transferred to the co-cultivation medium and incubated at 27 ± 1 °C in the dark for around 48 h. Next, the explants were gently washed with double distilled water containing 250 mg/l cefotaxime for 8–10 min and allowed to dry on sterile Whatman No. 3 filter paper. After complete drying, the explants were transferred into the first selection medium and incubated for 12 days in a growth room under fluorescent illumination on a 16 h photoperiod at 25 ± 2 °C. After three rounds of selection (each selection period is 10 days of incubation) in the regeneration medium, the transformed cells were found to develop green shoots. We carefully excised the 3,4 cm long *in vitro* shootlets and dipped them into filter-sterilized IBA (1.0 mg/l) solution for 4–5 min. The shootlets were then transferred into two different rooting medium, *i.e.*, half of the newly regenerated shootlets were transferred into the MS rooting medium and rest were directly planted into the soil:compost (2:1) mixture. Putative transgenic plants were allowed to acclimatize to the greenhouse condition for the first 12 days. Subsequently, these hardened plants were transplanted in the earthen pots for further growth and development observation inside the green house.

2.5. GUS staining and GFP fluorescence assay

Transformation efficiency was determined in terms of the number of explants showing GUS staining and expression of the green fluorescent fusion gene (mGFP) according to the following formula:

Transformation efficiency (%) = Number of GUS positive explants/ Number of explants infected with Agrobacterium × 100%

GUS assay was conducted using control and transformed cotyledons, hypocotyls and root explants according to the description of (Jefferson et al., 1987). pMDC100:GUS construct was used for transformation and explants were selected in 100 mg/l kanamycin for three consecutive

rounds. After staining, 70% (v/v) ethanol was used for removing the chlorophyll and then viewed under a microscope (Zeiss SteREO Discovery.V12, Germany). For GFP assay, eggplant cell suspension culture was prepared by inoculating 2,3 pieces of friable calli (approximately 50 mg) derived from both transfected and control explants with pCAMBIA1302:sHSP::mGFP fusion construct selected with 10 mg/l hygromycin. The calli were incubated with 50 ml autoclaved liquid MS media having the same concentration of hygromycin as above for one week. After two or three weeks of sub-culture, 1 ml of the culture was used for centrifugation at 3000 rpm for 5 min and washed three times with fresh sterile water. The pellet was resuspended in 20 ul of water and used for preparing slides which were then photographed in the dark field of laser scanning confocal microscopy (Zeiss LSM510, Germany).

2.6. Molecular confirmation of putative transgenic plants

Genomic DNA was extracted from all the putative T_0 transgenic lines developed with three independent expression vectors, *i.e.*, pMDC100:sHSP, pMDC100:spCas9 (both with *nptII* gene as selection marker) and pCAMBIA1302:sHSP::mGFP (*hptII* gene as selection marker) along with control eggplant leaves using PureLink™ genomic plant DNA purification kit (Invitrogen, USA) according to the manufacturer's instructions. To confirm transgene insertion for both the expression cassettes, PCR screening was carried out to trace the presence of *nptII* gene sequence in the respective T-DNAs with the primer pair (NptII_F/NptII_R) which produced a 480 bp amplicon. Further confirmation was carried out using junction primer pair of sHSP_F2/NosT_R1 and spCas9 primer pair (Cas9_Screen_F/Cas9-Screen_R) for the respective constructs (Supplementary Table 3). For the putative T_0 transgenic lines developed from the pCAMBIA1302:sHSP::mGFP vector, primary screening was carried out with primer pair (Hygro_F/Hygro_R). After the intial selection of the putative T_0 transgenic lines, the first generation of stable transgenic plants (T1 generation) were selected by germination of T_0 seeds in 150 mg/l kanamycin for plants generated from pMDC100:sHSP and pMDC100:spCas9 constructs and 10 mg/l hygromycin for pCAMBIA1302:sHSP::mGFP. These T1 transgenic plants were further used in the analysis for transgene integration and determination of copy number by Southern blot analysis. We used 20 μg of genomic DNA from each of the independent lines and control plants. Restriction enzymes, *Nde*I and *Eco*RV were used for overnight digestion of genomic DNA of the control plants and transgenic lines. Fragments of the digested DNA were separated by electrophoresis in 0.8% agarose gel. Hybond™ nylon positive membrane (GE Healthcare Limited, UK) was used for capillary transfer of DNA fragments. Construct specific digoxigeninlabelled probes (DIG probe) were used for subsequent hybridization. PCR DIG probe synthesis kit (Roche, Germany) was used for the synthesis of each DIG probe from sHSP (sHSP-Probe_F1/sHSP-Probe_R1) and spCas9 (Cas9-Probe_F/Cas9-Probe_R) (Supplementary Table 3) cDNA coding regions. The blots were detected according to the manufacturer's instructions (DIG High Prime DNA Labeling and Detection Starter Kit II, Roche, Germany).

2.7. Statistical analysis

All data were acquired from three batches consisting of 12 individual plants of each batch. All experiments were repeated at three times. All data are presented as the mean ± standard error. Data were statistically analyzed by one-way ANOVA and Newman-Keuls test ($P < 0.05$) to identify significant differences. Regeneration and transformation results from each trial were analyzed following completely randomized design (CRD) with three replications.

3. Results

3.1. In vitro regeneration protocol of eggplant

Initiation of multiple shoots in most of the explants was observed within four to five weeks of culture duration. For the three types of explants used from the three varieties, the highest number of regenerated plants was obtained for the media composition of MS+2.5 mg/l BAP (Supplementary Table 1). The highest frequency of regeneration was observed from cotyledonary explants for all the three varieties. Among the three varieties, maximum regeneration frequency was recorded to be 75.0 ± 0.13% in BARI Begun 4 along with the highest number of shoots per explants (5.08 ± 0.08). Similarly, the number of shoot induction from hypocotyl explant was observed to be 4.08 ± 0.14 and almost 83.33 ± 0.13% of the culture was found to be regenerated, whereas, root explants showed a reduced number of shoot development, with only 2.08 ± 0.08 per explant and about 41.67 ± 0.14% of the explant was found to be regenerated in the same media (Supplementary Table 1) (Fig. 2). Similarly, shoot regeneration frequency was observed more than 70% for BARI Begun 2 and 6 (Supplementary Figs. 2, 3). Results indicate that the number of shoots increased with a gradual increase (up to 2.5 mg/l) in the concentration of BAP. However, the regeneration efficiency decreased significantly when the concentration of BAP was increased.

With MS+2.5 mg/l BAP, hyperhydricity was found to be a major and frequently observed problem for most of the shoots in all the three varieties (Fig. 1A–D). In order to minimize the formation of hyperhydrated shoots, we examined the effect of casein hydrolysate and sorbitol on the regeneration media. A significant reduction in hyperhydrated shoot formation (16.67 ± 0.11%) was observed when casein hydrolysate and sorbitol were used in the same media composition (*i.e.*, MS+2.5 mg/l BAP) at a concentration of 0.2 mg/l and 0.1 mg/l respectively (Fig. 1E-H). Casein hydrolysate had previously been reported to be effective in decreasing the rate of tissue hyperhydricity (Mayor et al., 2003). However, we for the first time observed that the addition of small amount of sorbitol can greatly reduce tissue hyperhydricity *in vitro* culture of eggplants. Based on these observations, we were able to generate good shootlets from all the three explants with the composition of 0.2 mg/l casein hydrolysate, 0.1 mg/l sorbitol and 2.5 mg/l BAP. Next, the regenerated shootlets were separated and the small shootlets were transferred to the medium for further growth. For standardizing the shoot elongation media, we reduced the concentration of BAP in MS medium so that the regenerated shoot could sufficiently elongate and become suitable for root induction. In this case, the best elongation was obtained using MS + BAP (0.2 mg/l) + casein hydrolysate (0.2 mg/l) + sorbitol (0.1 mg/l) in the media for all three explant types after 12–14 days of culture (Fig. 2D). However, the highest percentage of elongation was observed in cotyledonary explants and also in BARI begun 4.

For root induction of the elongated shoots, full or half-strength MS media were supplemented with different types and concentrations of auxins. We observed that 80% of shoots rooted well within 9 days of culture. Each micro-cutting section produced 6.66 ± 0.14 mean number of roots in full strength MS medium supplemented with 1.0 mg/l IBA (Supplementary Table 2). The freshly regenerated shoots were transferred to our optimized rooting media (Fig. 2E). After adequate root development, plantlets were transplanted to small plastic pots containing soil and compost (2:1) (Fig. 2F). Rate of survival of the transplanted plantlets was found 80% in potted soil condition. Acclimatized plantlets were transferred to larger pots. The plants were allowed to mature, set flowers and normal fruits with viable seeds for average seven to eight weeks (Fig. 2G, H).

3.2. Optimization of antibiotic concentration for transgenic plant selection

To determine the concentration of selectable marker for the screening of putative transformed lines, eggplant shoots and cotyledonary leaves were inoculated into optimal shoot regeneration medium supplemented with different concentrations of kanamycin (*i.e.* 50, 75, 100, and 150 mg/l) and hygromycin (*i.e.* 5, 10, 12 and 15 mg/l) (Fig. 5A). Shoot regeneration from the control explants were initially (10–12 days of incubation) inhibited by 100 mg/l of kanamycin and 10

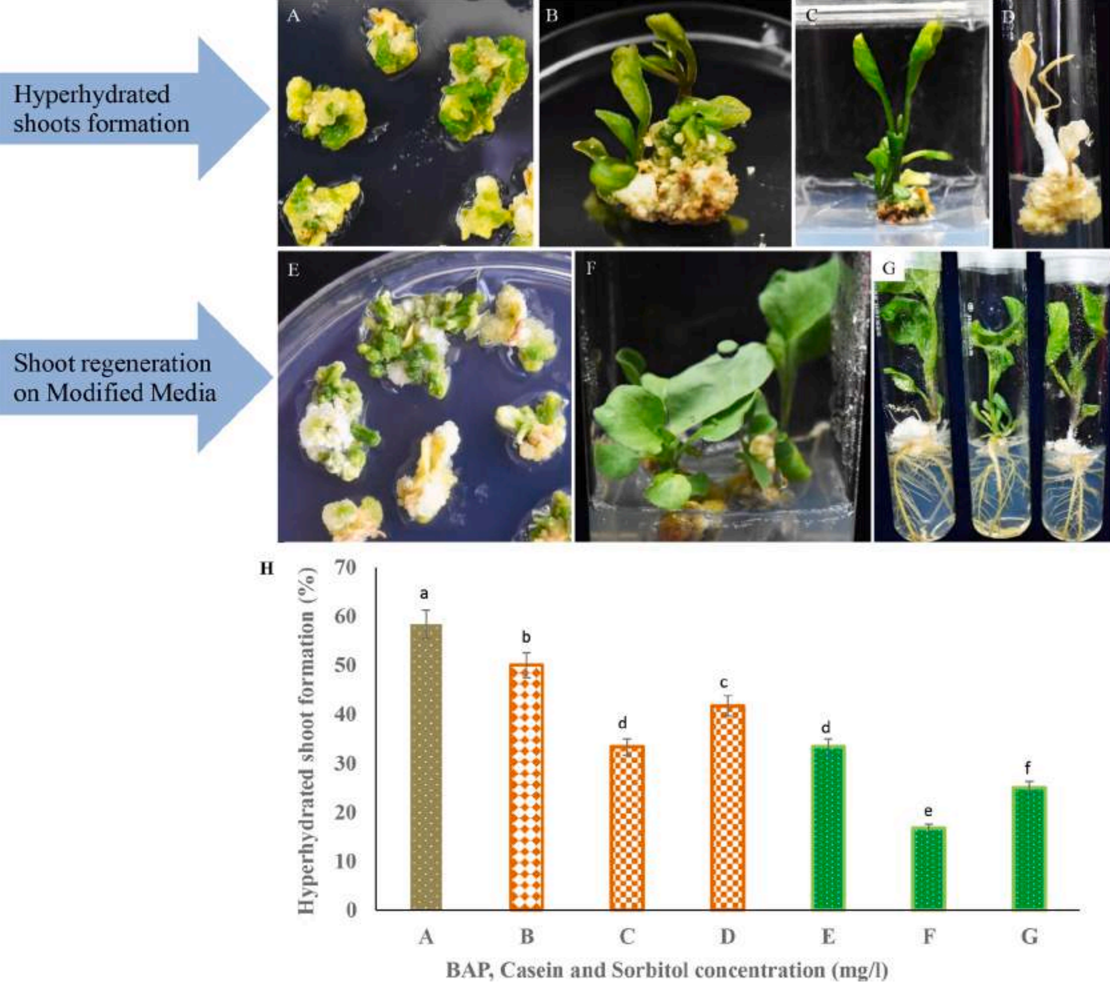

Fig. 1. Different media composition to control hyperhydricity and its effects on shoot and root formation on *in vitro* eggplant (variety BARI begun 4 transformation. (A) Initiation of hyperhydricity from cotyledon explants. (B, C) Elongation of hyperhydrated shoots. (D) No root formation occurs from hyperhydrated shoots and died eventually (E) Modified media showing normal and healthy *in vitro* shoots with no effect of hyperhydricity. (F, G) *In vitro* multiplication and well rooted regenerated shoots in modified media composition. (H) Percentage (%) of hyperhydrated shoot formation caused by the effect of casein hydrolysate and sorbitol on regeneration media. A: 2.5 mg/l BAP, B: 2.5 mg/l BAP+ 0.1 mg/l casein hydrolysate, C: 2.5 mg/l BAP+ 0.2 mg/l casein hydrolysate, D: 2.5 mg/l BAP+ 0.3 mg/l casein hydrolysate, E: 2.5 mg/l BAP+ 0.2 mg/l casein hydrolysate+ 0.05 mg/l sorbitol, F: 2.5 mg/l BAP+ 0.2 mg/l casein hydrolysate+ 0.1 mg/l sorbitol, G: 2.5 mg/l BAP+ 0.2 mg/l, casein hydrolysate+ 0.2 mg/l sorbitol.

mg/l of hygromycin and became brown within 3 weeks of culture. Hence, 100 mg/l of kanamycin and 10 mg/l of hygromycin were found to be suitable for transgenic shoot selection (Fig. 3A–I). Further, to determine the right antibiotic concentration for germination selection of T1 transgenic lines from T0 seeds, control eggplant seeds were first germinated on media supplemented with two different concentrations of kanamycin (*i.e.* 100 mg/l and 150 mg/l) and hygromycin (*i.e.* 10 mg/l, 15 mg/l) to check the antibiotic sensitivity of control plants. MS medium supplemented with 150 mg/l of kanamycin prevented root formation of germinated seedlings whereas, at 10 mg/l of hygromycin control seeds failed to germinate. Hence, 150 mg/l of kanamycin and 10 mg/l of hygromycin were determined to be suitable for selecting T1 transgenic eggplant lines (Fig. 3J–M). Moreover, *Agrobacterium* overgrowth was controlled in the MS medium supplemented with different concentrations of cefotaxime (*i.e.* 100, 200, 250, and 350 mg/l) (Supplementary Fig. 4A). *Agrobacterium* overgrowth was observed at a concentration of 100 mg/l cefotaxime selection, whereas; severe cell damage was occurred at 350 mg/l of cefotaxime exposure. Nonetheless with 250 mg/l of cefotaxime the growth of *Agrobacterium* was effectively inhibited and minimum cell damage was observed after the co-cultivation (Supplementary Fig. 4B-D). Henceforth, 250 mg/l of cefotaxime was used for controlling the growth of Agrobacterium.

3.3. Overview of eggplant genetic transformation for the generation of independent events

The optimized *in vitro* regeneration protocol was used to check for *Agrobacterium* mediated stable transformation and regeneration efficacy of eggplants. First we used two different binary expression vectors (pMDC100:GUS and pCAMBIA1302:sHSP::mGFP) (Fig. 7B, C) contain two different selection markers, kanamycin and hygromycin respectively, for plant selection. We first checked the transformation efficiency of different explants, *i.e.*, cotyledonary leaves, stem and roots with the both pMDC100:GUS and pCAMBIA1302:sHSP::mGFP vector. GUS staining revealed significant expression of GUS in explants transformed with pMDC100:GUS (with *npt*II selection) construct but not in the control explants (*i.e.*, non-transformed explants) (Fig. 4A–C). We have also observed that compared to root explants the cotyledonary leaves and hypocotyls were found to have higher transformation efficiency (Fig. 4D–G). Prominent expression of GFP was observed in cell cultures derived from cotyledons transformed with pCAMBIA1302:sHSP::mGFP containing hygromycin selection marker (Fig. 4H–K). Apart from the antibiotic selection pressure, the transformation efficiency of cotyledon, hypocotyl and root explants were also studied under different density of *Agrobacterium* culture during co-cultivation. We found the highest

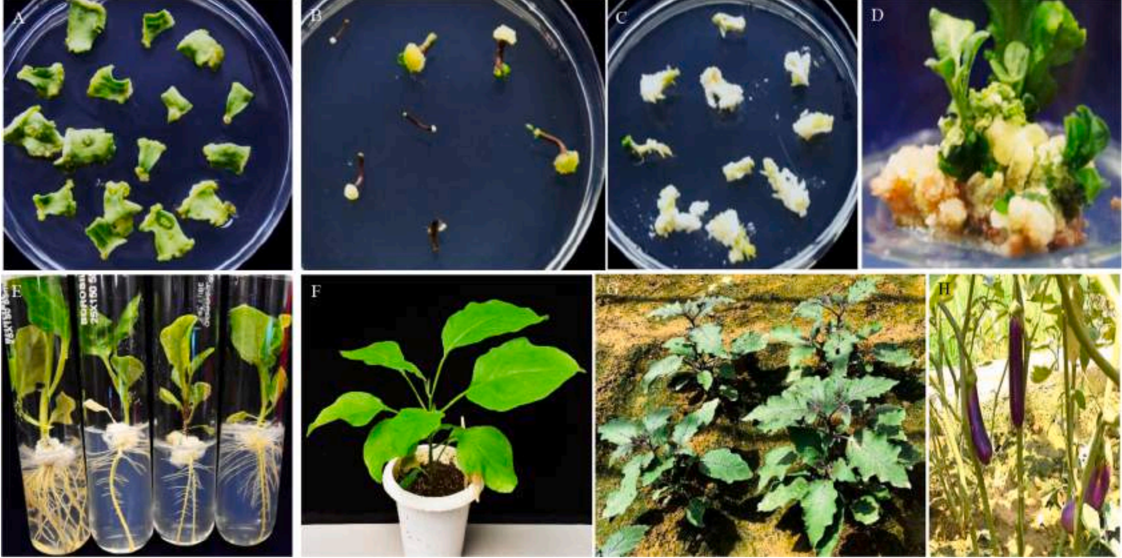

Fig. 2. *In vitro* regeneration of eggplant from cotyledon, hypocotyl and root explants from BARI begun 4. (A, B, C) Initiation of shoot formation from cotyledon, hypocotyl and root explants respectively. (D) Multiple shoot development from all three types of explants. (E) *In vitro* root formation of the regenerated shoots. (F) Acclimatization of *in vitro* plantlets. (G, H) Field establishment and setting of subsequent flowers and fruits in the *in vitro* regenerated eggplants.

transformation efficiency when the optical density of *Agrobacterium* was 0.6 at OD 600 nm, together with 10 min of infection and two days of incubation in co-cultivation medium (Fig. 5B–D). Moreover, we found that the addition of a lower concentration of acetosyringone (*i.e.*, 50 μmol) in the *Agrobacterium* suspension and co-cultivation medium significantly improved the transformation efficiency.

Based on these observations, we used cotyledons and hypocotyls of 10 day-old eggplant seedlings as a source of explants for the stable transformation with *Agrobacterium* containing the pCAMBIA1302:sHSP::mGFP, pMDC100:sHSP and pMDC100:spCas9 expression vectors. The transformed cells were selected on MS medium supplemented with appropriate antibiotics. After three rounds of selection, the explants began to develop green shoots. Explants which did not show any sign of regeneration or callus initiation, even after 2,3 successive transfers on selection medium were discarded.

After sufficient elongation, the regenerated shootlets were transferred to rooting media. However, we noticed that the initiation of *in vitro* rooting had stalled after *Agrobacterium* mediated transformation. Using our previously optimized rooting medium (MS+ 1.0 mg/l IBA), only 40% of transformed shoots were found to develop proper roots after transformation. This step was subsequently optimized to accelerate rooting frequency in the putative transformed plants. We tried both *in vitro* and *ex vitro* rooting induction strategies. When the lower part of the micro shoots was dipped into filter sterilized IBA solution (1.0 mg/l) for 4,5 min before transfer to *in vitro* MS rooting medium or directly planted into pot, root induction frequency was found to increase approximately to 85% (Fig. 6A–F). Once the micro shoots showed good rooting frequency, they were transferred to plastic pots containing soil and compost (2:1). The putative transgenic plants were acclimatized and transferred to greenhouse. Media composition and the critical stages of eggplant transformation together with troubleshooting the same have been summarized in Table 1.

3.4. Molecular confirmation of the transgenic plants

We have developed three different stable transgenic lines with a set of three different expression vectors such as pCAMBIA1302:sHSP::mGFP (with *hptII* as selection marker), pMDC100:sHSP and pMDC100:spCas9 (both with *nptII* as selection marker). An average of 40 different putative T_0 transgenic eggplant lines were generated from pMDC100:sHSP and pMDC100:spCas9 constructs using our optimized transformation protocol. A slightly lower regeneration efficiency was observed in case of pCAMBIA1302:sHSP::mGFP (25 putative transgenic plants). Primary PCR confirmation of transgene integration was carried out using specific primer pairs for all the three different expression constructs. Putative T_0 transgenic plants developed from pCAMBIA1302:sHSP::mGFP were PCR screened with a set of primer for *hptII* gene (Hygro_F/Hygro_R) and junction primers of *sHSP* and *mGFP* (HSP Fused_F1/GFP_R1) (Supplementary Table 3). In case of putative T_0 transgenic plants developed from pMDC100:sHSP construct, we used primer pairs of *nptII* (NptII_F1/NptII_R1) gene and *sHSP* and Nos terminator primer (HSP_F2/NosT_R1). Similarly, *nptII* and *spCas9* gene specific primer pairs were used for PCR confirmation of putative transgenic lines developed from pMDC100:spCas9 expression construct. On PCR screening for the presence of kanamycin and hygromycin genes, average than 32 and 22% of transformation efficiency was observed in T_0 transgenic plants respectively (Supplementary Table 4). Kanamycin selected transgenic lines showed the expected 480 bp DNA fragment for the *nptII* gene. Approximately a 900 bp DNA fragment was obtained for *sHSP* and Nos terminator primer set and a 563 bp DNA fragment with *spCas9* primer set, whereas no such DNA fragment was amplified from the wild type (wt) eggplant genomic DNA (Fig. 7E). Southern analysis was performed to confirm the copy number of transfer deoxyribonucleic acid (T-DNA) insertions using *sHSP* and *spCas9* cDNA specific probes (Fig. 7F, G). We obtained 2 single-copy, and 2 double-copy transgenic events for *sHSP* transgenic lines and all single-copy events for *spCas9* lines.

4. Discussion

Transformation of recombinant DNA and regeneration of transformed cells into plantlets depend on the availability of an efficient protocol. Inefficient transformation and regeneration protocol is the major problem which limits the successful delivery of genes of interest for overexpression or gene editing components such as Cas9, guide RNA for precisely targeting agronomically important traits. Since the first report (Guri and Sink, 1988) of *Agrobacterium* mediated transformation and regeneration in eggplant, the same protocol has been followed with modifications in later on (Pratap et al., 2011; Chen et al., 2016; Jadhav et al., 2015 and Foo et al., 2018). But this low success rate of transformation is still not enough to proper analysis of transformation and genetic engineering of eggplant. Therefore, in this study, we tried to comprehensively addressed various key factors particularly type of

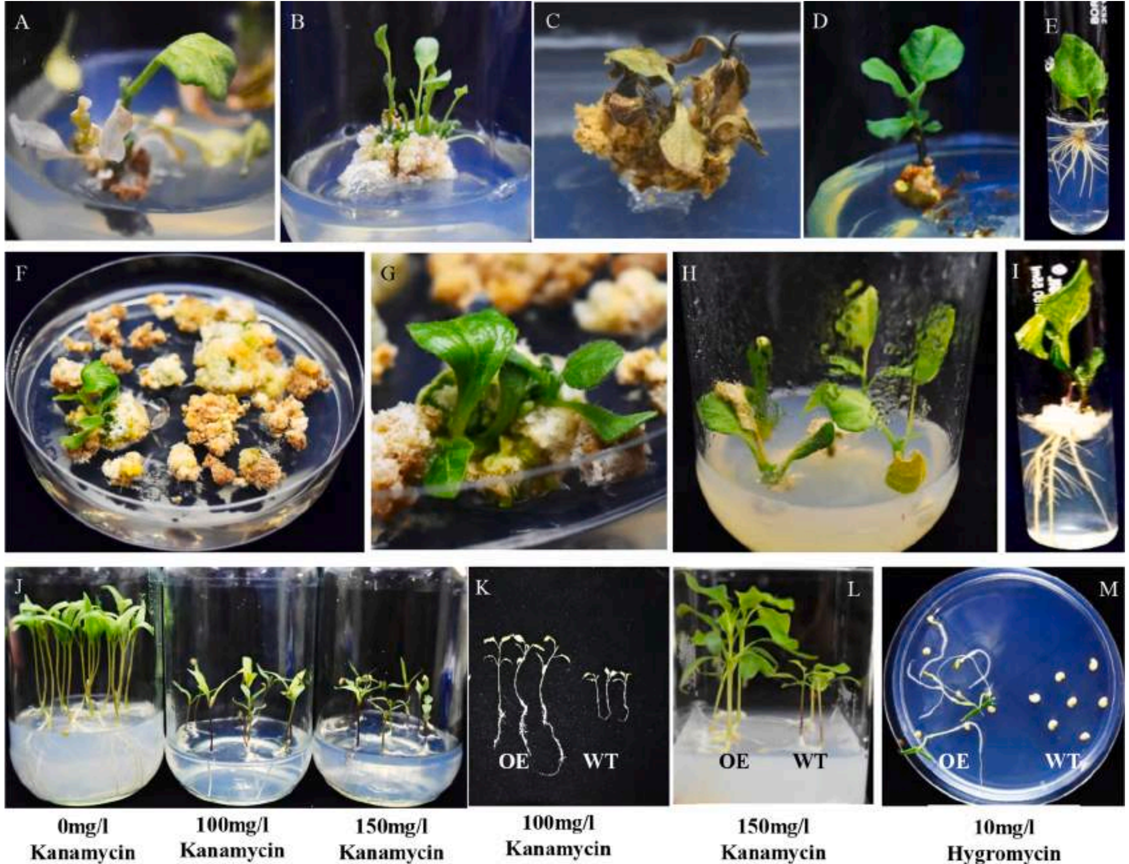

Fig. 3. Optimization of different antibiotic concentrations for selection of transgenic plants. (A) Browning of non-transformed shoot from BARI begun 4 under 50 mg/l kanamycin selection. (B) Transformed shoot from BARI begun 4 remain green under 50 mg/l kanamycin selection. (C) Non-transformed shoots from the same variety died under 100 mg/l kanamycin selection. (D) Transformed shoots remain green and grow well at 100 mg/l kanamycin selection. (E) Well rooted transformed shoots at 100 mg/l kanamycin. (F, G) Non-transformed cotyledonary leaves from BARI begun 4 became brown and shoot formation inhibited under 5 mg/l hygromycin whereas transformed cotyledon starts to form shoots and grow well in 10 mg/l of hygromycin. (H) Multiple transformed shoots growing well in 10 mg/l hygromycin. (I) Transformed shoots show good rooting in 10 mg/l hygromycin. (J) Germination assay with control eggplant seeds under different selection pressures in the media (concentration ranging from 0 mg/l, 100 mg/l and 150 mg/l kanamycin) to find the suitable concentration for selecting T1 transgenic lines. (K, L) MS medium supplemented with 100 and 150 mg/l of kanamycin prevented root formation of control seedlings (WT) whereas transgenics lines (OE) show well rooting. (M) Control seed (WT) could not germinate at 10 mg/l of hygromycin but transgenic lines (OE) germinated well at the same selection pressure (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

explant, efficacy of transformation and regeneration, selection of balanced plant growth regulators, reduction of tissue hyperhydricity, acceleration in rooting frequency and identification of compatible selection pressure to overcome the low frequency of regeneration and transformation in eggplant cultivars from Bangladesh.

Several previous studies reported different combination of auxin and cytokinein for eggplant morphogenic responses such as benzylaminopurine with naphthalene acetic acid (BAP+NAA) (Rahman et al., 2006; Scoccianti et al., 2000) or indole aceteic acid (BAP+IAA) (Sabina et al., 2018; Yarra and Kirti, 2019) as well as kinetin (BAP+Kinetine) (Kaur et al., 2011; Shivaraj and Srinath, 2011). Recently Garcia-Fortea et al. (2020) have demonstrated zeatin riboside to be the best shoot inducing hormone working independently of eggplant genotype. In our study, we first examined the shoot regeneration response of the three high yielding eggplant varieties (BARI Begun 2, 4 and 6) of Bangladesh under different auxin and cytokinin concentration. Among all the different combinations, we found that 2.5 mg/l BAP alone is the best supplement for a maximum shoot regeneration of cotyledon, hypocotyl and root explants. Similarly, maximum shoot elongation was achieved by only reducing the concentration of BAP (0.2 mg/l) in the same MS medium and this hormone concentration was successfully applied for all the subsequent transformation events. BAP is a synthetic cytokinin that alone can promote cell division, differentiation and growth of various tissues (Ruzic and Vujovic, 2008). Also, prolonged exposure of eggplant embryogenic callus into auxin containing media progressively lose regeneration capacity (Reynolds, 1986). Although, in previous studies reported the variation of tissue differentiation and regeneration in eggplants in genetic background such as *S. melongena, S. melongena* var. insanum, and their F1 hybrids, cultivars, and inbred lines (Mir et al., 2008; Zayova et al., 2008; Kaur et al., 2013).

After the initial studies on shoot regeneration response of our three varities, we further extensively studied several key factors that significantly affect eggplant transformation frequency. One such important factor is the application of antibiotic concentration on selecting stably integrated transformed cells. Depending on the combination of antibiotics can inhibit the regenerative response of different explants. In previous studies, it has been reported that cefotaxime treatment alone can reduce 52% of embryogenesis (Magioli et al., 2001). Cefotaxime was found to enhance callus fresh weight, but also causing a decrease on the rate of regeneration (Picoli et al., 2002). We found that 250 mg/l of cefatoxime is lethal for *Agrobacterium* but doesn't have significant adverse effect on callus development and regeneration in BARI begun 2, 4 and 6. Also, we found superior number of shoot induction with optimized kanamycin concentration of 100 mg/l (4.16 shoots/explant) compared to hygromycin concentration of 10 mg/l (2.16 shoots/explant) although chances of getting non-transgenic shoots could not be

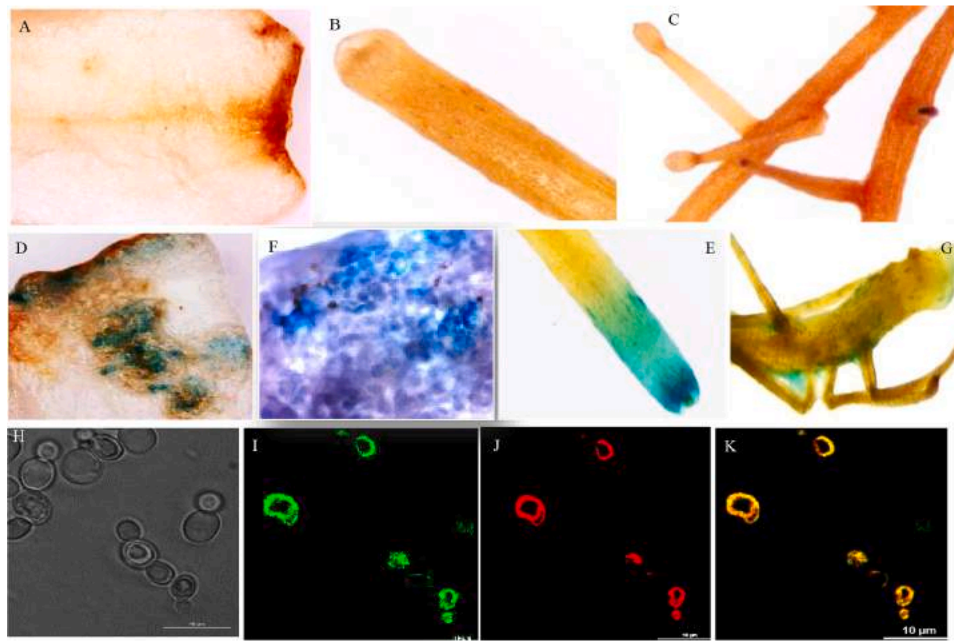

Fig. 4. *Agrobacterium* mediated transformation efficiency in different explants through GUS histochemical assay and green fluorescent fusion protein (mGFP) based expression analysis. (A, B, C) Untransformed (Control) explants *i.e.*, - cotyledon, hypocotyl and root showing no GUS expression. (D, E) Transformed explants showing strong GUS expression in cotyledon. (F, G) GUS expression in hypocotyl and root explants. (h-k) Stably transformed eggplant suspension cells directly observed under confocal microscopy. H: GFP fluorescent signal in transformed cells, I: Co-localization of mitochondria specific dye MitoTracker Red, J: Bright field merged, K: Dark field merged. The bar represents 10 μm (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

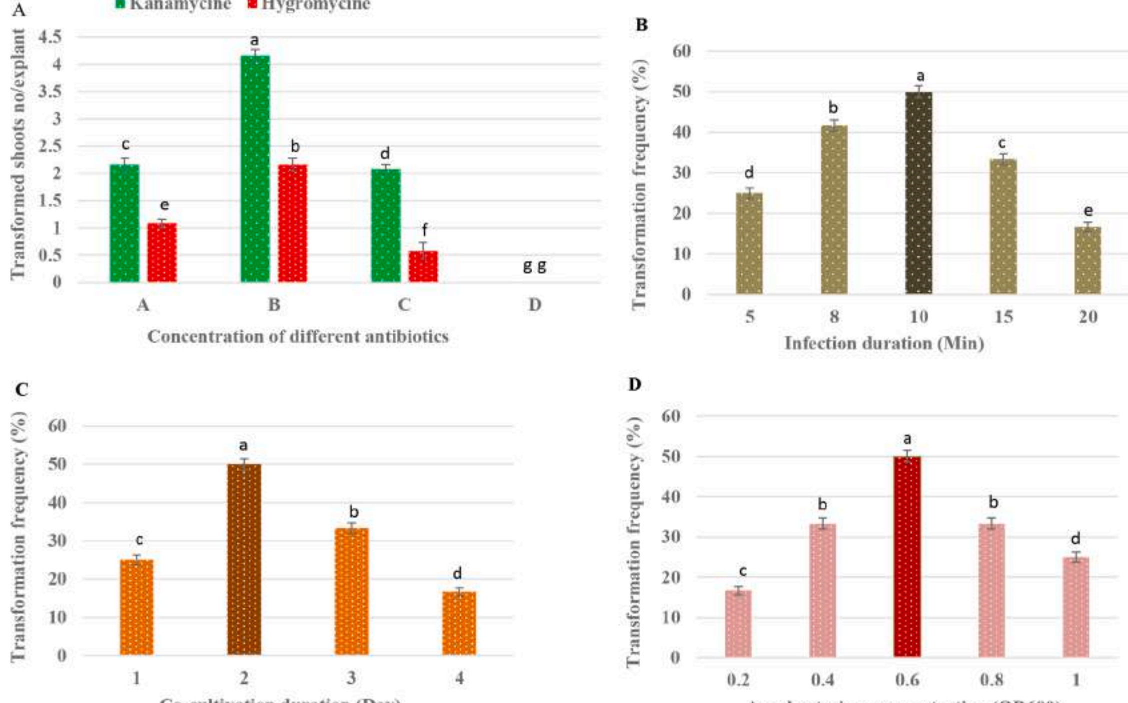

Fig. 5. Factors affecting the frequency of *Agrobacterium* mediated transformation in eggplant. (A) Number of transformed shoots generated from different explants under different concentrations of antibiotics. (Kanamycin, A: 50 mg/l, B: 75 mg/l: C: 100 mg/l, D: 150 mg/l. Hygromycin, A: 5 mg/l, B: 10 mg/l: C: 12 mg/l, D: 15 mg/l). (B) Transformation frequency (%) for different infection times (5, 8, 10, 15 or 20 min). (C) Transformation frequency (%) against different co-cultivation durations (1, 2, 3 and 4 days). (D) Transformation frequency (%) against different *Agrobacterium* cell densities (OD600= 0.2, 0.4, 0.6, 0.8 and 1.0). The results are presented as the means and standard errors from three independent experiments. Different letters indicate statistical significance at $P < 0.05$ level.

excluded at this concentration. This is not unusual as similar observation has also been reported for different plant species and it is considered that non transgenic cells can survive because of incomplete selection of adjacent transformed cells (Yevtushenko and Misra, 2010). Previous studies also support our finding that kanamycin is better for selecting eggplant transformants than other selection agents (Franklin et al., 2004; Sagare and Mohanty, 2012).

Another important factor that affects eggplant transformation frequency is the density of *Agrobacterium* suspensions used for infection of explants. We observed the highest transformation frequency when *Agrobacterium* suspension had an optical density (OD) of about 0.6 at 600 nm. Our findings are in agreement with previous studies which have found that a moderate range (0.2–0.6 at OD 600 nm) of *Agrobacterium* density is suitable for maximum transformation frequency for different

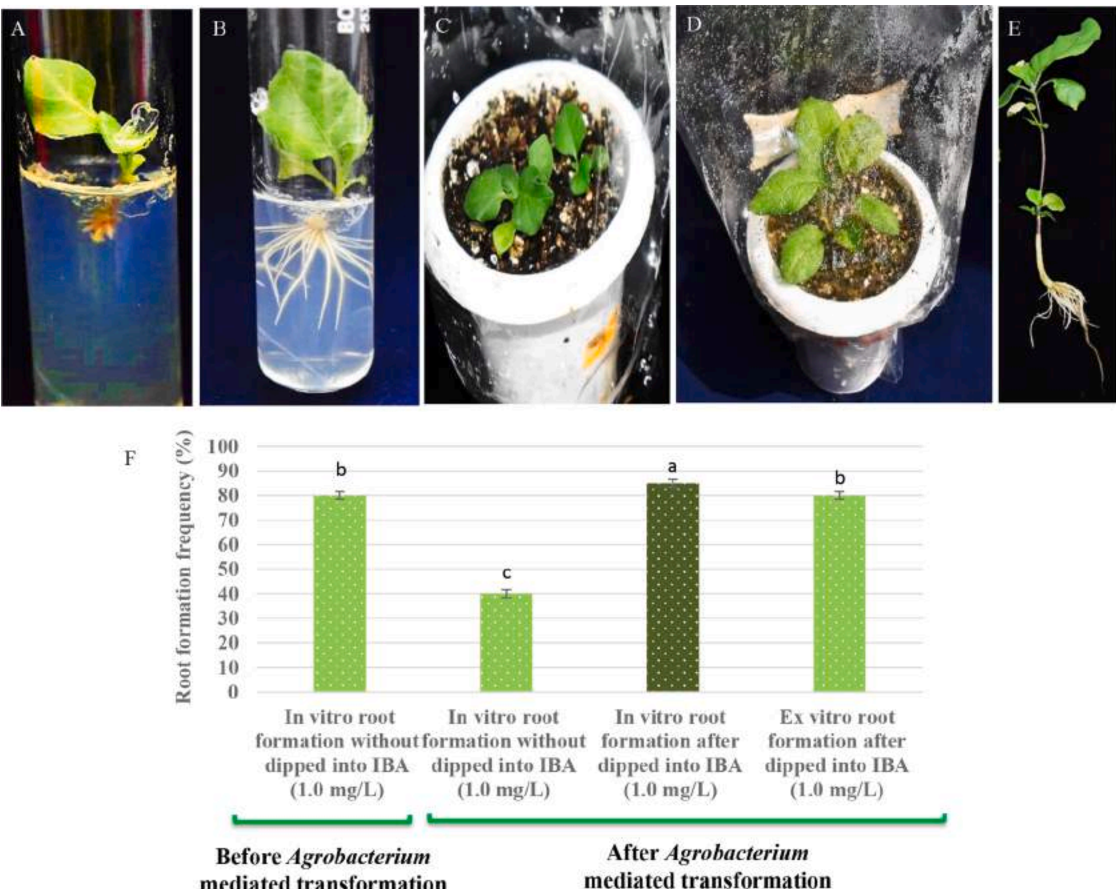

Fig. 6. *In vitro* and *ex vitro* rooting induction in transformed micro-shoots of eggplant. (A, B) Root initiation after micro-shoot dipping in filter sterilized 1.0 mg/l IBA solution. (C, D, E) Ex vitro root induction (bottom portion of micro-shoot dipped in filter sterilized 1.0 mg/l IBA solution then transferred directly to soil and compost mixture (2:1).

genetic backgrounds of eggplant (Kumar and Rajam, 2005; Pratap et al., 2011). They observed the regeneration ability to be critically inhibited when bacterial OD reached more than 0.6. Along with bacterial OD, infection and co-cultivation times with *Agrobacterium* are equally important to achieve the highest transformation efficiency. We observed that 10 min of infection and two days of co-cultivation is optimal for maximum transformation. Extended infection and co-cultivation times than our observed time *i.e.*, 10 min of infection time and two days of co-cultivation duration were found to cause severe damage such that the explants were beyond regeneration. Similar observation was also made for a different eggplant variety (Yarra and Kirti, 2019). Yesmin et al. (2014) also reported that a co-cultivation period of three days was most suitable for obtaining high transformation efficiency. However, this was also found to impede the growth and survival of explants due to an overgrowth of Agrobacterium. Earlier studies found pre-culturing of explants prior to transformation to be vital as it increased explant competence for inducing *vir* gene expression of *Agrobacterium* (Prabhavathi and Rajam, 2007; Yarra and Kirti, 2019; Yesmin et al., 2014), Conversely, our study suggest fresh-cut explants to be more competent in inducing *Agrobacterium vir* gene expression when only 50 μmol of acetosyringone is added to the re-suspension media.

While studying different key factors important for inducing a high rate of regeneration frequency and transformation events in BARI begun 2, 4 and 6, we faced two serious problems *i.e.*, hyperhydricity and root induction frequency. These were either neglected or had little focus in previous studies. Several studies also mentioned hyperhydricity as serious physiological deformities of *in vitro* regenerated shoots (Gleddie et al., 1986; Saito and Nishimura, 1994; Magioli et al., 2001). However, no noticeable efforts are known to have been made to lessen or escape from the same (Kevers et al., 2004). It is reported that hyperhydricity condition can be overcome by better ventilation of the culture vessel, manipulation of solidifying media, lower cytokinin and ammonium nitrate concentrations, frequent subculture and adding anti-hyperhydricity agents (Bairu et al., 2007; Peârez-Tornero et al., 2001). In our study, we tried to explore the role of casein hydrolysate and sorbitol in reducing hyperhydricity and as a growth promoter of *in vitro* tissues. We used various concentrations of these two agents for eggplant. Casein hydrolysate at a concentration of 0.2 mg/l and sorbitol at 0.1 mg/l were found to significantly reduce the rate of hyperhydric shoot formation. Previous studies also agreed to our findings that application of casein hydrolysate successfully decrease the incidence of hyperhydricity and accelerate the growth of *in vitro* tissue (Chandrika et al., 2015; Mayor et al., 2003). Chandrika et al. (2015) also reported a gradual decrease in the levels of vitrification as the concentration of casein hydrolysate was incremented from 5–15 mg/l in *Eryngium foetidum* L. Similar observations of usefulness of casein hydrolysate in increasing shoot multiplication and accelerating the growth rate and formation of embryogenic calli and somatic embryos in Chinese Plum and Date palm respectiveley (Zou, 2010; Al-Khayri et al., 2011). Although, the actual mechanism of casein hydrolysate in reducing tissue hyperhydricity is not clearly undrstood, however; it is well known that casein hydrolysates can be a source of calcium, phosphate, several microelements, vitamins and most importantly, a mixture of up to 18 amino acids (Salehi et al., 2017; Amera et al., 2017). It also provides easy accessibility to reduced nitrogen essential for growing callus and the shootlets (Baskaran et al., 2012; Amera et al., 2017; Samiei et al., 2021) and might contain some unknown plant growth promoting factors which promote callus growth (Inoue and Maeda, 1982; Salehi et al.,

Table 1
Outline of overall *in vitro* regeneration and transformation proceduressss.

Stage	Media composition	Duration	Photograph	Trouble shooting
1. **Seed germination**: Sterile eggplant seedsgerminatedon half strength MS medium without any hormone and kept under light fluorescent illumination on 16 h photoperiod at 25±2 °C	Half strength MS (1L: MS 2.2 *g* + Sucrose (15 g) + pH (5.7) + Gelrite (4 g)	12–14 days		• Explants (cotyledon, hypocotyl and root) should be collected from 12–14 days old *in vitro* germinated seedlings to increase transformation rate
2. ***Agrobacterium* culture preparation**: *Agrobacterium* cell grown in YEM medium with continuous shaking of 250 rpm at 28 °C upto OD	YEM (100 ml): Yeast extract (0.04 g) + Mannitol (1 g) + K_2HPO_4 (0.05 g) + $MgSO_4$. $7H_2O$ (0.020 g) + NaCl (0.01 g)	24h: OD 600 nm 0.6		• *Agrobacterium* cell density should be at OD 600 nm of 0.6 to overcome poor transformation efficiency
3. **Infection with explants:** *Agrobacterium* cell re-suspended in liquid MS medium under aseptic condition and infected with cotyledone, hypocotyl and root explants	Resuspension (500 ml): MS (1.1 g) + Sucrose (7.5 g) + Glucose (5 g) + pH (5.2) + Acetosyringone (50 µM)	10 min		• Infection time should not exceed more than 10 min • Discarded damage or defective explants
4. **Co-Cultivation with Agrobacterium:** After infection cotyledon, hypocotyl and root explants transferred to co-cultivation medium and kept under dark at 28 °C	Co-cultivation media (1 L): MS (4.4 g) + Sucrose (30 g) + Glucose (10 g) + BAP (2.5 mg/l) + pH (5.2) + Gelrite (4 g); After autoclave, add 50 µM acetosyringone when temperature below 60 °C.	48 h		• Incubation of explants should not be more than 48 h under dark to reduce *Agrobacterium* overgrowth and explants cholorosis
5. **Washing:** After 48 h of co-cultivation all explants washed with proper antibioticcontaining water solution under aseptic condition	100 ml of sterile water + carbenicillin (250 mg/l) + cefatoxime (250 mg/l)	8–10 min		• Washing time should not exceed more than 8–10 min to reduce tissue damage and hyperhydricity in explants • Avoid excessive blotting to dry explants
6. **Selection 3 to 4 times (Shoot regeneration):** Transformed shoots selected on appropriate antibiotic containing selection plate for 3 to 4 times and selection plates kept under light fluorescent illumination on 16 h photoperiod at 25±2 °C	Selection media (1 L): MS (4.4 g) + Sucrose (30 g) + Glucose (10 g) + BAP (2.5 mg/l) + Casein hydrolyste (0.02 mg/l) + Sorbitol (0.1 mg/l) + pH (5.7) + Gelrite (4 g); After autoclave, add kanamycin (100 mg/l) or hygromycin (10 mg/l) + Cefatoxime (250 mg/l) when temperature below 60 °C	10–12 days		• Use freshly prepared cefotaxime 250 mg/l on selection medium • Subculture to fresh selection plate within 10–12 days to suppress *Agrobacterium* growth on media
6. **Shoot elongation:** *In vitro* regenerated shootlets shifted to elongation medium and placed under light fluorescent illumination on 16 h photoperiod at 25±2 °C	Elongation media (1 L): MS (4.4 g) + Sucrose (30 g) + Glucose (10 g) + BAP (0.2 mg/l) + Casein hydrolysate (0.02 mg/l) + Sorbitol (0.1 mg/l) +pH (5.7) + Gelrite (4 g); After autoclave, add kanamycin (100 mg/l) or hygromycin (10 mg/l) + Cefatoxime (250 mg/l) when temperature below 60 °C	12–15 days		• Use low concentration of BAP to allow proper elongation of *in vitro* transformed eggplant shoots
7. ***In vitro* Rooting:** Sufficiently elongated shoots transferred to root induction medium and kept under fluorescent illumination on 16 h photoperiod at 25±2 °C	Rooting media (1 L): MS (4.4 g) + Sucrose (30 g) + IBA (1.0 mg/l) + pH (5.7) + Gelrite (4 g) After autoclave, add kanamycin (100 mg/l) or hygromycin (10 mg/l) + Cefatoxime (250 mg/l) temperature below 60 °C	15–18 days		• Bottom portion of micro shoot dipped in filter sterilized 1.0 mg/l IBA solution for 4–5 min before transfer to rooting medium or directly to soil

2017). On the other hand, few reports have found sorbitol as a water stress inducer for various plant species which can therefore be expected to act as an anti-hyperhydricity agent (Bidabadi et al., 2012; Vanhove et al., 2012). Similarly, others have reported that the addition of sorbitol significantly promotes *in vitro* callus induction and regeneration (Geng et al., 2008; Li et al., 2004; Ye et al., 2017).

Successful rooting of transformed micro-shoots is another crucial step to facilitate their establishment in soil. Most studies have shown that different concentrations of IBA exhibit maximum root formation in eggplant (Padma Mallaya and Ravishankar, 2013; Sabina et al., 2018). We witnessed severely decreased root induction frequency in our studied variety in the same medium described in previous studies. Therefore,

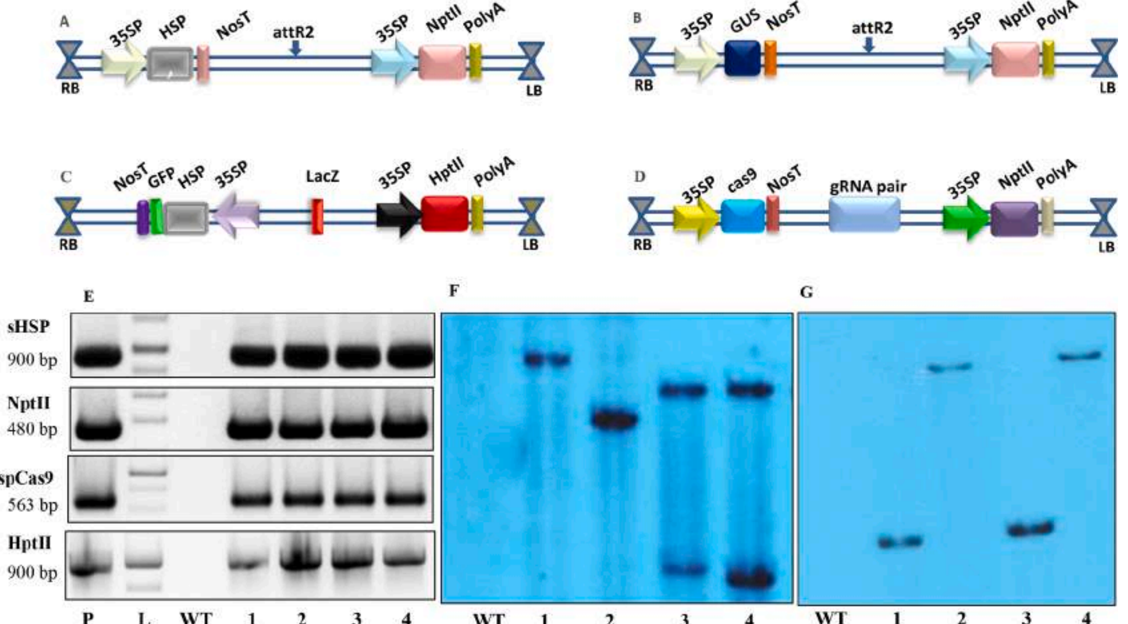

Fig. 7. Molecular analysis and schematic diagrams of different expression cassettes used in eggplant (BARI begun 4) transformation. (A) Schematic diagram of small heat shock protein (sHSP) expression cassette pMDC100:sHSP. Full length sHSP cDNA cloned into expression vector pMDC100 under cauliflower mosaic virus (CaMV) promoter (35SP) and nopaline synthase (NOS) gene terminator (NosT) with kanamycin (*nptII*) as the selection marker. (B) Schematic diagram of GUS expression cassette pMDC100:GUS. PCR amplified GUS along with CaMV promoter (35SP) and NOS gene terminator (NosT) cloned into expression vector pMDC100 with kanamycin (*nptII*) as the selection marker. (C) Schematic diagram of green fluorescent protein (mGFP) fused with sHSP expression cassette, pCAMBIA1302: sHSP::mGFP. Coding sequence of *sHSP* gene lacking the stop codon was fused in-frame to green fluorescent protein (mGFP) under CaMV promoter (35SP) and NOS gene terminator (NosT), and hygromycin (*hptII*) genes as the selection marker. (D) Schematic diagram of spCas9 expression cassette pMDC100:spCas9. Plant codon optimized spCas9 cDNA was cloned under CaMV promoter (35SP) and NOS gene terminator (NosT) with kanamycin (*nptII*) as the selection marker. (E) PCR amplification of sHSP+Nos terminator, and kanamycin (*nptII*) gene from putative transgenic lines developed from pMDC100:sHSP construct. Hygromycin (*hptII*) gene amplified from putative transgenic lines generated from pCAMBIA1302:sHSP::mGFP construct and spCas9 genes amplified from pMDC100:spCas9 transformed eggplant lines using specific primers in wild type (WT) and four positive T2 transgenic lines (1–4). L: 1 Kb DNA ladder, P: positive PCR control (plasmid DNA). (F, G) Southern blot analysis of WT and four positive T2 transgenic lines developed from pMDC100:sHSP and pMDC100:spCas9 constructs using sHSP and spCas9 cDNA specific probes respectively (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

we have optimized rooting efficiency in our variety. We applied both *in vitro* and *ex vitro* root induction strategies. For the *in vitro* strategy, we found that the best results are obtained when the basal portion of a well-elongated micro-shoot is dipped into filter sterilized IBA solution (1.0 mg/l**)** before their transfer (IBA shock) to the rooting medium. Similarly, in the *ex vitro* strategy, the micro-shoots were directly transferred to pots containing either soil or vermiculite after dipping in the same IBA solution. A similar *ex vitro* root induction strategy was applied in eggplant which also showed a good response rate (Shekhawat et al., 2015). This process can be considered as a cost-effective strategy and the problems encountered during acclimatization could be overcome easily (Baskaran and Van Staden, 2013; Ranaweera et al., 2013).

5. Conclusions

We have made significant progress in overcoming the existing bottlenecks in *Agrobacterium* mediated transformation and *in vitro* regeneration efficiency of eggplants with special concern to high yielding varieties from Bangladesh. It can be inferred from our study that hyperhydricity, rooting and shootlets regenerations are the key factors in *Agrobacterium* mediated transformation in eggplant. Tissue hyperhydricity in eggplant *in vitro* culture can be reduced by fortifying the culture medium with casein hydrolysate and sorbitol at requisite concentrations analyzed in our study. Similarly, IBA shock with 1.0 mg/l concentration of freshly regenerated shootlets significantly increases rooting frequency in both of *in vitro and ex vitro* conditions. Apart from these key factors we also studied the *Agrobacterium* culture density which found to be 50% transformation efficient at a concentration of OD600 = 0.6 with an infection time of 2 days in co-cultivation media. This study provides an optimized *Agrobacterium*-mediated transformation protocol for eggplant varieties of BARI begun 2, 4 and 6 and would be a useful reference for improving transformation efficiencies in other plant species.

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CRediT authorship contribution statement

Muslima Khatun: Investigation, Formal analysis. **Bhabesh Borphukan:** Visualization, Investigation, Formal analysis. **Iftekhar Alam:** Supervision, Writing – review & editing. **Chaman Ara Keya:** Writing – original draft, Visualization. **Haseena Khan:** Supervision, Writing – review & editing. **Malireddy K. Reddy:** Writing – original draft, Visualization. **Md. Salimullah:** Visualization, Writing – original draft, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scienta.2021.110716.

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