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Original Article

Evaluation of Factors Influencing *In Vitro* Regeneration and Transformation Protocols to Produce Salinity Tolerant Tomato (*Solanum lycopersicum* L.)

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Abstract

Introduction: Tomato (*Solanum lycopersicum* L.) is one of the essential vegetables worldwide, for consumption and mitigating malnutrition. Genetic transformation conceivably overcomes its yield challenges due to salinity, a crucial constraint for the economical use of 30% of cultivable lands in the coastal region of Bangladesh. Therefore, a robust and reproducible protocol has been established for *in vitro* regeneration and transformation to develop transgenic salt-tolerant tomato plants.

Materials and Methods: During micropropagation, cotyledonary leaf explants were excised and cultured on MS media containing different combinations and concentrations of plant growth regulators. In transformation, the pre-cultured explants were inoculated and co-cultivated with *Agrobacterium*. Then they were transferred to the antibiotics-supplemented media to achieve salt-tolerant putative transformed plants. The transformation was confirmed by β-glucuronidase (GUS) assay and PCR for the antiporter gene.

Results: Maximum regeneration response was achieved from the explants abaxially positioned at a 1.5 cm distance apart. BARI Tomato 14 and BINA Tomato 3 showed the highest shoot regeneration response (%) on MS media supplemented with 2 mg/L BAP and with 0.1 mg/L IAA for BARI Tomato 2 and BARI Tomato 15. Bacterial culture of OD_{600} 0.68 for 30 min and a Co-cultivation period of 48 h resulted in the highest transformation frequency (47%) in *Agrobacterium*-mediated transformation with pBI121 in BARI Tomato 3. The highest regeneration frequency (20.5%) was obtained in transformation with pH7WG2_OsNHX1_1.6.

Conclusions: The optimized procedure is simple, efficient, and can be used for micro-propagation and the production of tolerant varieties to increase yield in saline areas.

Keywords: Tomato, *In Vitro* Regeneration, *Agrobacterium*-mediated Transformation, Salt Tolerance, Phytohormones, Explants Spacing, Orientation
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Introduction

The world is facing an imminent global food emergency due to a pandemic, which is a greater threat to food and nutritional security.[1] It is a huge challenge for a highly populated country, like Bangladesh. Increasing the productivity of food and vegetables can be the best alternative to avoid such a situation.[2] Tomato (*Solanum lycopersicum* L.), which is a major vegetable crop in Bangladesh can play a vital role in providing health benefits because of the presence of vitamins, minerals, bioactive phenolic compounds and lycopene, which exhibit many pharmacological activities including anticancer, antioxidant, antidiabetic and anti-allergic.[3,4] The increasing demand for tomato cultivation can also generate higher income for the farmers and laborers, which is an essential approach for developing countries.[5] However, the production is being hampered by a variety of environmental stress such as salinity.[6] In Bangladesh, the climatic challenges are posing additional difficulty in crop production with the adverse effect of salt water intrusion into the coastlines; which mainly affects the production system, biodiversity and human health.[7] Tomato yield and morphological properties have been affected by salinity due to ion deficiencies initiated from the excessive amount of sodium uptake.[8] The reduction of leaf area index, total chlorophyll content and 12-32% yield has been reported when the electrical conductivity was higher than 3-5.5 dS/m.[9] Fifty percent yield loss has also been revealed as a consequence of salinity enhancing physiological disorders such as blossom end rot.[10] Salinity stress has also been reported to be growth-stage specific and yield reduction arises due to the lesser amount of fruit production.[11] Conventional breeding can be employed

to improve the situation, but the absence of required tolerant traits within the compatible germplasm makes it unsuitable.[12] Therefore, genetic transformation can be the only approach for developing salinity-tolerant varieties. However, the establishment of *in vitro* regeneration protocol is a prerequisite for genetic engineering.

Tomato regeneration protocols have been reported by several authors in the last few decades, but these reports vary considering the variation in the genotypes.[13] Cotyledonary leaves were selected as explants which have been reported to be the most responsive towards high-frequency shoot regeneration.[14] The protocols for regeneration[15] and transformation[16] have been established for Bangladeshi tomato BINA Tomato 3, BINA Tomato 5 and Bahar varieties. Apart from genotype, i*n vitro* regeneration is also dependent on culture methods, growth regulators and many other factors.[17] This study aimed to develop salinity tolerant tomatoes through obtaining the efficient tissue culture and transformation protocols. In tomato, several genes have been identified for the development of plants by enhancing salt tolerance with transgenic approaches.[18] The well-characterized Na^+/H^+ antiporter genes show a positive influence in salt homeostasis, which helps plants to grow better in higher salinity conditions.[19] For instance, a significant improvement has been observed in transgenic tomato plants through the over-expression of *AtNHX1*, a single-gene controlling vacuolar Na^+/H^+ antiporter gene from *Arabidopsis thaliana*.[20] The transgenic plants grew, set flowers, and produced fruits in the presence of 200 mM NaCl in greenhouse hydroponics. The transgenic plants acquired a halophytic response to salt stress, accumulating salts in the cell and sequestering them in the vacuole. This *NHX1* gene can efficiently be utilized for various plants in crop science.[18]

Thus, the present investigation was designed in order to establish various parameters that are necessary for developing successful regeneration and transformation protocols using Bangladeshi local varieties to produce transgenic salt-tolerant tomato plants, which could be applicable for other varieties as well.

Materials and Methods

During this study, the optimum concentration of growth hormones along with the position and their orientation of the explants on the culture medium were assessed for successful regeneration and the reproducibility of the protocol was tested. Factors affecting transformation protocol were also obtained using pBI121 and then pH7WG2_OsNHX1_1.6 was used in the regeneration of salt-tolerant putative transformed plants. Finally, molecular analysis was done for confirmation.

Plant Materials

Five farmer popular tomato varieties, namely, BARI Tomato 2, BARI Tomato 3, BARI Tomato 14, BARI Tomato 15, and BINA Tomato 3 were selected for this study. Seeds were collected from the Bangladesh Agricultural Research Institute (BARI) and Bangladesh Institute of Nuclear Agriculture (BINA) respectively.

Explant Preparation

The tomato seeds were surface sterilized as mentioned by Islam et al.[16] with a slight modification as 30% sodium hypochlorite was used. Seeds were then transferred on to germination media with 30 g/L sucrose and incubated at 25 °C ± 2 with 16 h photoperiod. Cotyledonary leaves were collected from 7-day-old seedlings and cut into small pieces. Twenty-five to thirty explants were inoculated as explants on hormone supplemented MS media for each variety, and the experiment was done in triplicate.

Optimization of Explants Position and Orientation

Explants were positioned at three different distances, *i.e.*, 1.0, 1.5, and 2.0 cm apart and the regeneration responses were determined by the fresh and dry weight of the explants, measurement of chlorophyll content, and the number of regenerated shoots.[21] Total chlorophyll content was calculated according to Arnon's equation[22] and expressed as "µg chlorophyll/g fresh tissue." Furthermore, to determine whether regeneration ability is affected by its orientation on the media or not, the explants were placed in adaxial (upper side) and abaxial (lower side) orientations.

Shoot and Root Regeneration

Explants were cultured on MS[23] medium supplemented with BAP (6-Benzylaminopurine) with/without IAA (Indole-3-Acetic-Acid) for shoot regeneration. Successfully regenerated shoots were sub-cultured every three to four weeks to fresh media for achieving healthy shoots. Visually well-developed shoots were transferred to IAA supplemented rooting media. The regeneration percentages of all the varieties were analyzed by ANOVA (Microsoft Office Excel 2010).

Transplantation

The well-developed plantlets were carefully transferred from the rooting media to a pot containing autoclaved soil. After acclimatization, the plantlets were placed in the net house for flowering and fruit formation.

Analysis of the Reproductive Response of the Regenerated Plantlets

Following acclimatization of regenerated plantlets survivability, flowering and fruit setting response in the natural environment were assessed by fruit weight, fruits number per plant, seed number, etc. Finally, the viability of seeds from mature fruits of *in vitro* regenerated plants was also tested.

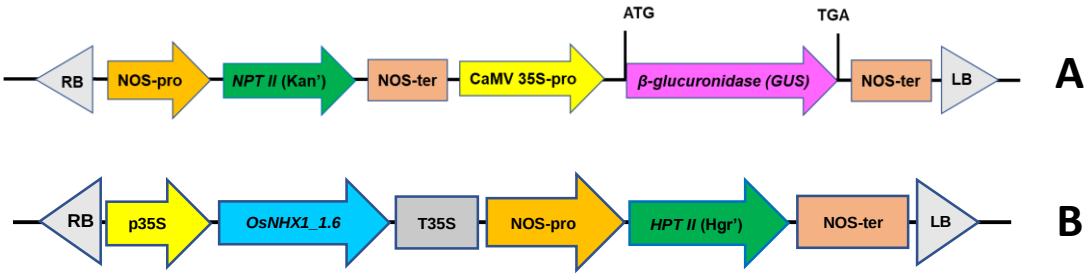

Figure 1. Schematic Diagram of **A.** the T-DNA region of the binary *pBI121*, **B.** Constructed vector pH7WG2_OsNHX1_1.6.

Data Analysis

The experiments of shoot regeneration and rooting were replicated thrice. In all the experiments, 25-30 explants were inoculated. The results of all experiments were analyzed by ANOVA at 5% significance (p<0.05) level in Microsoft Excel 2010.

Determination of Baseline Saline Tolerance Level of Tomato Seedlings

Initiating to determine the basal salt tolerance level, the effect of salinity on the germination of tomato seeds was investigated for the five mentioned varieties. Salinity conditions were represented by different concentrations of NaCl ranging from 5-100 mM (0.5-10 dS/m). The MS media was prepared with different amounts (5, 10, 20, 50, and 100 mM) of NaCl in every 100 ml of media.

Gene Construct and Plasmid Vectors

Agrobacterium tumefaciens strain LBA4404 containing two individual plasmid constructs was used for the establishment of the transformation (Figure 1). The plasmid pBI121 contained a chimeric gene for β-glucuronidase (GUS) gene and kanamycin-resistance (*nptII*), each driven by CaMV35S promoter. *A. tumefaciens* strain LBA4404 harboring pH7WG2_OsNHX1_1.6 was used in transformation with the established condition. The plasmid pH7WG2_OsNHX1_1.6 carried antiporter *OsNHX1* gene and hygromycin-resistance (*hptII*) gene, each expressed under the CaMV35S promoter. It contains hygromycin resistance for selection in plants and spectinomycin and streptomycin resistance for selection in bacteria.

Effect of Hygromycin and Kanamycin on Regeneration

Hygromycin and kanamycin have been used here as selectable markers for incorporation of the plasmid, while cefotaxime has been used as a bacteriostatic agent to control *Agrobacterium* overgrowth during co-cultivation. Optimal levels of these antibiotics have been tested to avoid growth adversity due to antibiotic in media. Regeneration media containing different concentrations of kanamycin (50, 100, 150, and 200 mg/L) and hygromycin (1, 3, 4, 5, 10, 20, and 30 mg/L) was prepared. A control for each set was also maintained which contained no antibiotics.

Transformation Protocol

Cotyledonary leaves from 7-10 days seedlings were used as the explants for transformation. *A. tumefaciens* strain LBA4404 (pBI121) was cultured in YMB liquid medium supplemented with 200 mg/L kanamycin and 50 mg/L streptomycin while *A. tumefaciens* strain LBA4404 containing pH7WG2_OsNHX1_1.6 was grown in a YMB medium supplemented with 200 mg/L spectinomycin and 100 mg/L streptomycin. Bacterial cultures were grown in a shaker at 28 ºC for 24 to 48 h in order to obtain the OD_{600} of both 0.45 and 0.68. The explants were soaked in *A. tumefaciens* suspension for both 30 and 60 min and co-cultivated on regeneration medium for 48 h. Explants were then transferred to the medium supplemented with 100 mg/L cefotaxime to control the overgrowth of *A. tumefaciens* and selected antibiotics (100 mg/L kanamycin and 10 mg/L hygromycin for pBI121 and pH7WG2_OsNHX1_1.6, respectively) to obtain regeneration from transformed explants. After 45-60 days of culture, regenerated shoots were placed on rooting media to develop roots.

Preparation of Histochemical Reagent (X-gluc) Solution and GUS Assay

The preparation of reagent (X-gluc) and GUS assay was performed according to a previous study.[20] Slides of transformed explants were prepared for observation under the microscope.

Data Collection

The number of survived explants with regenerated shoots was recorded after 45-60 days of inoculation following the start date. Transformation efficiency was also calculated by the number of regenerated putative transgenic shoots on selection by using the following Eq. 1:

$$\text{Efficiency (\%)} = \frac{\text{the number of shoots germinated}}{\text{total number of explants inoculated}} \times 100$$

DNA Isolation and PCR Analysis

Total genomic DNA was extracted from the leaves of

transformed plants and non-transformed control plants by the plant genomic DNA isolation procedure.[24]

Results and Discussion

Effect of Explants Orientation and Spacing on the Shoot Regeneration

Explants placed in abaxial orientation showed 20% more shoot regeneration compared to the adaxial orientation. In agreement with this, the abaxial regeneration showed 70% to 90% shoot regeneration in 4 to 7-day old seedlings, whereas only 45% shoots regeneration was achieved in adaxial orientation after 5 weeks in a study with MicroTom tomatoes.[25]

To get an insight into the regeneration response in relation to nutrient availability, the explants were placed on regeneration media at 1 cm, 1.5 cm and 2 cm apart. The highest fresh and dry weights of the explants were found when they were 2 cm apart and the lowest value was found at 1 cm distance (Table 1). The result was found similar for all varieties, but data are shown only for BARI Tomato 15. The highest chlorophyll content and shoot number were obtained at 1.5 cm distance apart from each other (Figure 2A-2C). Similar results were found for *Linum usitatissimum* explants indicating better photosynthetic activity and proper health of the tissue.[21] They did not require competing for light and produced less chlorophyll at a distance of 2 cm. At a distance of 1 cm, they produced the minimum amount of chlorophyll among the three tested groups due to the inadequate amount of nutrients.[21] Similarly, the explants density of nine explants in a Petri dish was observed to have the highest shoot regeneration.[25]

Table 1. Fresh and Dry Weight, Shoot Number and Chlorophyll Content of Explants Cultured at Three Different Placing Distances

Space between Explants (cm)	Average Fresh Weight (g) ±SE[1]	Average Dry Weight (g) ±SE[1]	Mean No. of Shoot ± SE*	Chlorophyll *a* (mean±SE)[1]	Chlorophyll *b* (mean±SE)[1]	Total Chlorophyll content (mean±SE)[1]
1	0.0803 ± 0.005	0.0068 ± 0.006	9.6 ± 0.4	0.078 ± 0.003	0.144 ± 0.006	0.330 ± 0.009
1.5	0.0883 ± 0.011	0.0091 ± 0.001	10 ± 1.4	0.152 ± 0.012	0.279 ± 0.02	0.588 ± 0.022
2	0.1092 ± 0.012	0.0092 ± 0.001	7.3 ± 1.0	0.1 ± 0.006	0.184 ± 0.011	0.388 ± 0.014

[1]Average values are from 3 replications (data are presented for BARI Tomato 15)

Figure 2. Factors Affecting Regeneration, Root Formation and the Reproductive Response **A.** BARI Tomato 15 at A. 1 cm distance, **B.** 1.5 cm distance, and **C.** 2 cm distance (Photos were taken after 3 days and 60 days after inoculation), **D.** Regenerated BARI Tomato 2 on regeneration media containing 2 mg/L BAP after 60 days of inoculation. **E.** BARI Tomato 2 on media supplemented with 7 mg/L after 30 days of their inoculation, **F.** Abnormal morphology of BARI Tomato 15 explants in BAP 7 mg/L after 30 days of inoculation, **G.** Vitrification was observed in BARI Tomato 2 while sub-cultured on media containing higher BAP concentration (7 mg/L), **H.** BARI Tomato 2 on regeneration media containing 2 mg/L BAP + 0.1 mg/L IAA after 60 days of inoculation, **I.** Root formation of BARI Tomato 14 in MS+0.1 mg/L IAA, **J.** BARI Tomato 2 in MS+0.5 mg/L IAA, **K.** BARI Tomato 15 in MS +1 mg/L IAA **L.** BINA Tomato 3 in MS+0.2mg/L IAA concentration **M.** Taproots are formed in 2 mg/L BAP and no IAA concentration in BARI Tomato 15, **N.** Regenerated plants of BARI Tomato 15 growing in a pot, **O.** Flowering of BARI Tomato 3, **P.** Fruits of BARI Tomato 2, **Q.** Fruits maturation, **R.** Seed germination test from mature fruits.

Table 2. Effect of Hormonal Concentrations on Shoot Regeneration in all Five Tomato Varieties

Hormonal Concentration (mg/L)		BARI Tomato 2		BARI Tomato 3		BARI Tomato 14		BARI Tomato 15		BINA Tomato 3	
BAP	IAA	Mean shoot no. ± SE[1]	Days required for shoot formation	Mean shoot no. ± SE[1]	Days required for shoot formation	Mean shoot no. ± SE[1]	Days required for shoot formation	Mean shoot no. ± SE[1]	Days required for shoot formation	Mean shoot no. ± SE[1]	Days required for shoot formation
1	-	4.3±0.4	14	6.6±1.0	17	8.3±0.4	12	7.6±0.4	16	8.6±0.4	15
2	-	7.6±0.4	15	7.0±0.7	17	8.6±1.0	14	8.0±1.4	17	9.0±0.7	15
5	-	6.0±1.8	16	5.7±0.4	18	5.0±0.7	14	7.0±1.8	16	4.3±0.8	16
7	-	8.0±0.7	16	2.6±0.4	18	5.3±0.8	14	0	N/A	3.3±0.4	16
1	0.1	6.3±0.3	14	5.0±0.5	16	4.6± 0.3	14	4.6±0.6	14	3.0±0.5	15
1	0.2	5.0±0.7	15	7.0±0.5	15	5.6± 0.6	15	5.6± 0.3	14	4.3±0.3	15
1	0.5	5.3±0.6	16	6.3±0.3	16	6.3± 0.6	16	4.00±0.3	15	4.0±0.0	16
1	0.7	4.6±0.3	16	4.0 ±0.3	16	4.0± 0.3	16	3.6± 0.3	15	4.3±0.3	16
1	1	5.0±0.0	17	3.6 ±0.3	15	3.6± 0.3	16	5.00± 0.3	15	6.3±0.3	15
2	0.1	8.3±0.4	17	6.6± 0.8	17	6.3± 0.3	17	8.3± 0.4	17	4.6±0.3	15
2	0.2	7.0±0.5	16	5.3±0.6	18	4.0± 0.3	17	8.0±0.4	16	3.6±0.3	15
2	0.5	5.6±0.3	15	5.0±0.3	18	5.6±0.6	16	3.00±0.0	15	4.3± 0.3	16
2	0.07	3.3±0.3	16	6.3±0.3	12	4.3± 0.8	16	3.6±0.3	15	3.3±0.3	16
2	1	2.3±0.3	16	4.0±0.3	14	6.3± 0.8	17	4.3±0.3	14	6.0±0.5	16
5	0.1	2.3±0.3	15	3.6±0.8	14	1.0± 0.5	14	3.00±0.0	14	2.3±0.8	16
5	0.2	5.6±0.6	16	3.0±0.0	15	4.0± 0.3	14	2.00±0.5	14	2.3±0.3	15
5	0.5	4.0±0.0	15	2.0±0.5	15	1.0± 0.0	16	1.34±0.3	16	2.0±0.0	16
5	0.7	4.3±0.3	15	2.3± 0.3	16	2.3± 0.3	17	3.67±0.3	17	1.0±0.5	17
5	1	3.6±0.6	15	1.0 ±0.5	17	1.6± 0.6	16	3.0± 0.5	16	0.6±0.3	16
7	0.1	4.3±0.3	17	2.0±0.0	18	1.0± 0.5	16	1.0±0.5	15	1.0±0.0	15
7	0.2	5.6±0.6	17	3.0±0.5	18	3.6± 0.8	17	1.0± 0.0	15	2.0±0.5	15
7	0.5	4.0±0.0	16	2.3±0.3	16	1.0± 0.0	17	3.3± 0.3	14	1.3±0.3	15
7	0.7	3.3±0.7	16	0.6±0.3	15	1.0± 0.5	15	3.3± 0.3	14	2.6±0.6	16
7	1	4.6±0.3	16	1.3±0.6	16	2.3± 0.3	15	2.0±0.5	14	1.3±0.3	16
BAP		F Value		3.288628	*p*-Value		0.040058				
BAP+IAA		F Value		2.114509	*p*-Value		0.011082				

[1]Average values are from 3 replicates

Effect of BAP on Shoot Regeneration

The maximum number of shoots was obtained at 2 mg/L BAP concentration for all tested varieties (Table 2), which agrees with Billah et al.,[14] who found it as the most effective for shoot regeneration of cotyledonary leaf, cotyledonary node and hypocotyl explants. Maximum (95%) shoot regeneration was achieved using cotyledons explants.[26] Eight shoots per explants have been achieved from nodal explants of Cassel rock tomatoes.[27] Both 2 mg/L BAP and 7 mg/L BAP gave a similar response in BARI Tomato 2 (Figure 2D-2E). High concentrations like 7 mg/L BAP brought abnormal morphology in BARI Tomato 15 (Figure 2F) and vitrification has been found in both BARI tomato 2 and BARI Tomato 3 (Figure 2G). BAP was reported to induce vitrification more frequently compared to other cytokinins in *Dianthus caryophyllus* culture[28] and the rate increases with the higher concentration of BAP on media for Sebri Pear Cultivar.[29] Genotype-specific regeneration response of tomato to plant growth hormones has been reported earlier.[27]

Effect of BAP and IAA Combinations on Shoot Regeneration

The highest number of shoot formation (8.33 ± 0.4) was found in MS media supplemented with 2 mg/L BAP+0.1 mg/L IAA in BARI Tomato 2 (Figure 2H) and BARI Tomato 15 (Figure 2I). This hormonal combination has been reported to be the best for direct shoot regeneration from hypocotyls explants.[30] BARI tomato15 showed 86% regeneration response with 14.12 average numbers of shoots per explants on MS with 2 mg/L BAP and 0.5 mg/L IAA from cotyledonary leaf explants.[14] On the contrary, 4 mg/L BAP and 1 mg/L IAA showed the best response.[30] In the case of cotyledonary node explants, the same variety showed 100% regeneration with six average numbers of shoots per explant while on MS media with 2 mg/L BAP and 1 mg/L IAA.[14] However, BARI Tomato 3 and BINA Tomato 3 obtained the highest shoots in the combination of 1 mg/L BAP + 0.2 mg/L IAA and1 mg/L BAP + 1 mg/L IAA, respectively. MS media containing 1 mg/L BAP and 0.5 mg/L NAA was reported the best for BARI Hybrid Tomato 4 and apple tomato variety.[31]

The regeneration percentages of all the varieties were analyzed by ANOVA. The result was statistically significant in the case of shoot regeneration in media supplemented with BAP as found from one-way ANOVA analysis where F (3.3) was greater than F crit (3.0) and the *p*-value was statistically significant (<0.05). For the different concentration groups, the result significantly differed from each other as one-way ANOVA analysis showed that the results obtained for the F (2.11) was greater than F crit (1.71) and the *p*-value was statistically significant (<0.05) (Table 2).

Effect of IAA on Root Formation in Different Tomato Varieties

IAA has been reported to be more effective in producing healthy roots compared to NAA in tomato.[15] However, rhizogenic response has been found to vary depending on genotype. BARI varieties produced fibrous roots, while the BINA variety formed long and slender roots in response to IAA supplemented rooting media (Figure 2I-2L). Higher concentrations of IAA (½ MS with 0.7 mg/L and 1 mg/L both) were found optimum in BARI Tomato 15 and BINA Tomato 3, while lower (0.2 and 0.5 mg/L) concentrations of IAA were found optimum for the remaining varieties (Table 3). Most commonly, root formation occurs on media supplemented with IAA ranging between 0.1 mg/L IAA and 1 mg/L IAA for various tomato varieties.[32] Interestingly, though auxin was needed for root formation, some shoots started forming tap-roots in media containing only BAP in shooting media (Figure 2M). Rhizogenesis on auxin-free media has also been observed.[33]

Reproductive Response of the Regenerated Plants

The regenerated plants were transplanted into the pots for hardening. Acclimatization success (Figure 2N) was recorded maximum in BARI Tomato 3 (100%) and the least (70%) in BARI Tomato 14 (Table 4). All rooted plants flowered and set fruits like the non-regenerated control plants (Figure 2O-2Q). Plantlets that were transferred to nature in the month of April-May took three to four months to flower. However, plantlets transferred in September-October flowered in three to four weeks as all the varieties used in this study were winter varieties. Fruit setting (15-20 days) and maturation (4-5 weeks) followed successively. The maximum number of fruits (9.0), fruit weight (80 g/fruit), and seed number per fruit (50) were recorded in the variety BARI Tomato 14. In the present study, the fruit number and weight varied. These differences might be due to variation in transplantation season. It was reported to have a comparatively higher fruit number and more fruit weight in winter and a reduced number in summer,[34] but the seeds produced by the regenerated plants gave cent percent viability irrespective of transplantation season (Figure 2R).

Salinity Stress Tolerance Test of Tomato Varieties

Seeds were subjected to germinate on media containing different NaCl concentrations ranging from 5-100 mM (0.5-10 dS/m) to determine a baseline salinity tolerance level of untransformed tomato plants. In the present study, the germination rate of seedlings dropped to 46.4% in media containing 20 mM (2.0 dS/m) NaCl (Figure 3A-3E) and severe reduction (1.6%) was observed at 100 mM (10 dS/m) NaCl (Figure 3F). The time requirement for germination at 50 mM (5 dS/m) NaCl and above was also influenced (Table 5). Tomato seeds needed 50% and 100% additional days to germinate at 2.5 dS/m and 4.5 dS/m NaCl, respectively, than in a medium without salt.[35] This indicated that salinity severely influences plant physiology.[36] This is while some

Table 3. Effect of IAA on Root Production in Different Tomato Varieties

Tomato Varieties	Concentration of IAA (mg/L)	Percentage of Shoot Producing root	Days Required for Root initiation	Type of Root	Roots/ Shoot
BARI Tomato 2	0.1	88	9	Tap root	8
	0.2	90	9	Tap root	9
	0.5	80	8	Fibrous	9
	0.7	70	9	Fibrous	10
	1.0	80	8	Fibrous	10
BARI Tomato 3	0.1	60	8	Tap root	5
	0.2	56	8	Tap root	6
	0.5	75	8	Fibrous	6
	0.7	60	9	Fibrous	7
	1.0	70	8	Fibrous	6
BARI Tomato 14	0.1	80	9	Tap root	14
	0.2	**100**	9	Tap root	13
	0.5	90	10	Fibrous	15
	0.7	88	9	Fibrous	16
	1.0	80	8	Fibrous	12
BARI Tomato 15	0.1	90	10	Tap root	8
	0.2	80	9	Tap root	10
	0.5	80	9	Fibrous	11
	0.7	**100**	10	Fibrous	12
	1.0	**100**	9	Fibrous	14
BINA Tomato 3	0.1	75	7	Slender, Long	12
	0.2	100	7	Slender, Long	12
	0.5	90	7	Slender, Long	14
	0.7	**100**	8	Slender, Long	15
	1.0	**100**	8	Slender, Long	20

Table 4. Analysis of the Reproductive Response of the Regenerated Plants

Tomato Varieties	Percentage of Transplanted Plants	Percentage of Survived Plants in Nature	Fruits /Plants	Average Fruit Weight (g)	Average Seed No. /Fruits	Fruit Shape
BARI Tomato 2	87.5	100	8	65	44	Round
BARI Tomato 3	100	100	6	75	46	Semi-globe
BARI Tomato 14	70	100	9	80	50	Semi globe
BARI Tomato 15	91.6	100	5	65	25	Ovoid
BINA Tomato 3	90	100	7	70	35	Oval

Table 5. Effect of Salinity on Seed Germination of Tomato Varieties

NaCl Concentration into Germination Media	NaCl Concentration in dS/m	No. of Seeds Inoculated	Percentage of Germinated Seeds	Mean No. of Germinated Seeds ± SE[1]
0 mM	0	20	81.5	16.3 ± 1.4
5 mM	0.5	20	73	14.6 ± 2.2
10 mM	1	20	56.5	11.3 ± 2.4
20 mM	2	20	46.5	9.3 ± 0.8
50 mM	5	20	36.5	7.3 ± 0.8
100 mM	10	20	1.5	0.3 ± 0.4

[1]Average values are from 3 replicates

Bangladeshi BARI tomato varieties, namely, BARI Tomato 2, BARI 14 and BARI Hybrid Tomato 5 showed greater adaptation ability under saline conditions as they consistently produce more root dry matter at moderate saline conditions (4.1-8.0 dS/m).[37]

Antibiotics Sensitivity

Cotyledonary leaf explants were subjected to different concentrations of both antibiotics (Table 6). Kanamycin is the most preferable to obtain transgenic plants,[38] while hygromycin is the second most preferred used antibiotic.[39] The higher concentration was used to avoid the high frequency of non-transformed 'escapes' or chimeric plant production.[40] In the present study, explants did not regenerate at 50 mg/L of kanamycin and became albino at 100 mg/L of kanamycin (Figure 3G-3K). The kanamycin was maintained at 100 mg/L concentration for transgenic tomato shoot screening. A higher concentration (200 mg/L) of kanamycin was used for Bahar, BINA Tomato 3, BINA Tomato 5 and Pusa Ruby.[16] Lower concentrations (50 mg/L) were used for Dhanshree.[41] The explants became albino and then eventually started necrotizing from 5 mg/L hygromycin (Figure 3L-3T). The amount gradually increased to 10 mg/L hygromycin for the selection. About 25 mg/L hygromycin was standardized for the selection of transformed tomato explants cvs. Riogrande, Roma[42] and Summer set.[43] However, further increases of 40 mg/L and 50 mg/L hygromycin were effective for drought-tolerant tomato cv. Pusa Ruby and tomato cv. Riogrande selection respectively.[44,45] In the present study, 100 mg/L cefotaxime has been used in the media as it was found to be the most favorable for morphogenesis.[46]

Determination of Factors Affecting Transformation Efficiency

Agrobacterium strain LBA4404 harboring pBI121 was used to check its compatibility with five different varieties of tomato. In this study, GUS histochemical assay was done to

Table 6. Effect of Various Kanamycin and Hygromycin Concentrations on the Regeneration of Tomato Cotyledonary Leaf Explants

Antibiotic Concentration (mg/L)		Percentage of Shoot Formation[1]	Percentage of Survival (%)[1]	Visual Appearance
Kanamycin	0	60	100	Normal, green
	50	0	0	Albino
	100	0	0	Albino
	150	0	0	Brown
	200	0	0	Brown
Hygromycin	0	50	100	Green
	1	20	41	Green
	2	18	37	Green
	3	10	28	Green
	4	0	0	Albino
	5	0	0	Albino
	10	0	0	Brown
	20	0	0	Brown
	30	0	0	Brown

[1]Out of ten cotyledons; Data was collected after 45 days of inoculation

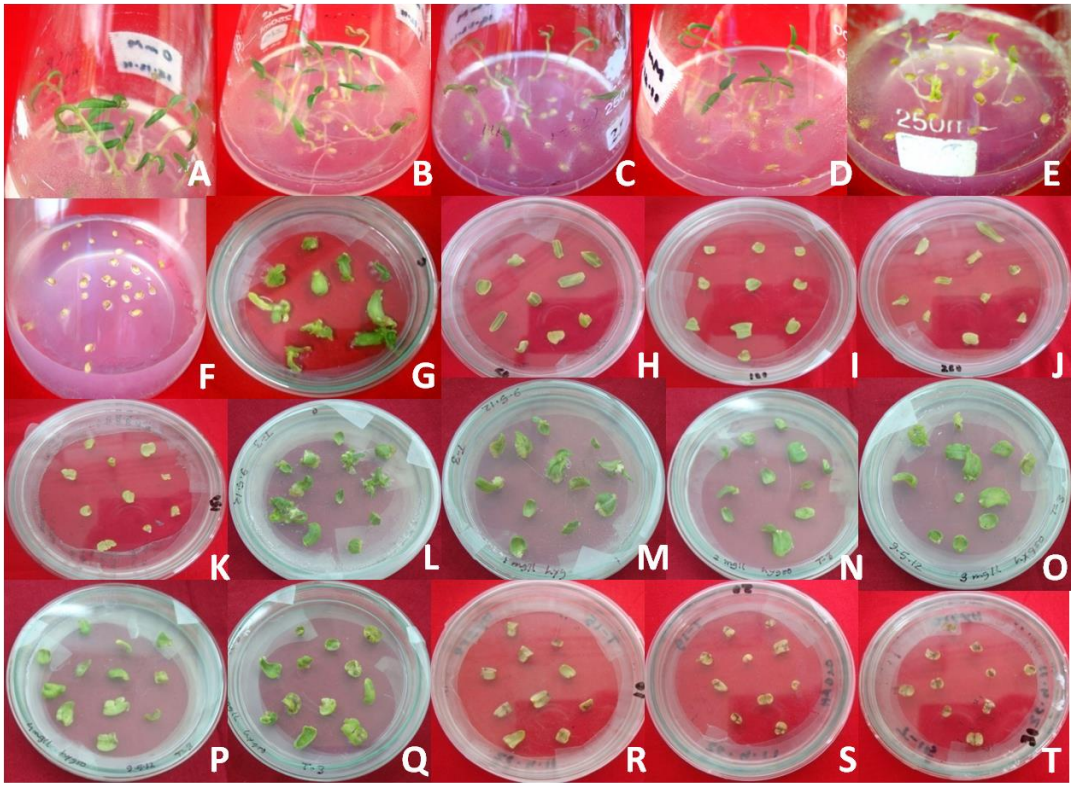

Figure 3 Seed Germination in the Presence of Different Amounts of NaCl. **A.** 0 mM, **B.** 5 mM, **C.** 10 mM, **D.** 20 mM, **E.** 50 mM and **F.** 100 mM; Effect of various concentrations of kanamycin on tomato cotyledonary explants of BARI Tomato 2, **G.** Control (0 mg/L kanamycin), **H.** 50 mg/L, **I.** 100 mg/L, **J.** 150 mg/L, and **K.** 200 mg/L kanamycin respectively; Effect of various concentration of hygromycin on tomato cotyledonary explants of BARI Tomato15, **L.** 1 mg/L, **M.** 2 mg/L, **N.** 3 mg/L, **O.** 4 mg/L, **P.** 5 mg/L **Q.** 8 mg/L, **R.** 10 mg/L, **S.** 20 mg/L, and **T.** 30 mg/L hygromycin respectively [photos were taken after 45 days of inoculation].

observe the transfer of marker gene *uidA* (β-glucuronidase) to determine factors influencing transformation. The transformation rate was found to be proportional to the relationship between infected (transformed) explants and inoculation time, co-cultivation period, bacterial suspension concentration, and selection antibiotic concentration.[47]

Effect of Pre-Culture On Transformation Efficiency

Interestingly this factor did not influence transformation efficiency but had a positive effect on regeneration initiation. It helped in the regeneration of putative transgenic shoots (Figure 4A-4B). The highest shoot number was found in pre-cultured BARI Tomato 14 explants among the five varieties tested in the present study (Table 7). Pre-culture enhances the regeneration percentage as explants are considerably swelled during this treatment which helps cells or tissue to overcome the stress, followed by co-cultivation with *Agrobacterium*[48] thus improving the transformation frequency in tomato. One day pre-culture was also used in the transformation of tomato hybrids, namely Felina, Siena and Dan Jose.[49]

Effect of Bacterial Culture Density on Transformation Efficiency

Maximum GUS positive explants (100% positive transient

Table 7. Effect of Pre-culture on Transformation Efficiency in Five Tomato Varieties

Tomato Varieties	Infected Explants	% of GUS Positive Explants	Days Required for Regeneration	Mean No. of Shoot ± SE[1]
BARI Tomato 2	Pre-cultured	80	16	4.33 ± 0.40
	Non-pre-cultured	82	20	3.33 ± 0.40
BARI Tomato 3	Pre-cultured	90	15	4.00 ± 0.70
	Non-pre-cultured	100	23	2.6 ± 0.40
BARI Tomato 14	Pre-cultured	94	14	5.5 ± 0.35
	Non-pre-cultured	100	19	4.0 ± 0.70
BARI Tomato 15	Pre-cultured	86	12	4.33 ± 0.80
	Non-pre-cultured	90	20	2.0 ± 0.70
BINA Tomato 3	Pre-cultured	82	18	5.3 ± 0.40
	Non pre-cultured	86	25	4.3 ± 1.08

[1]Average values are from 3 replicates

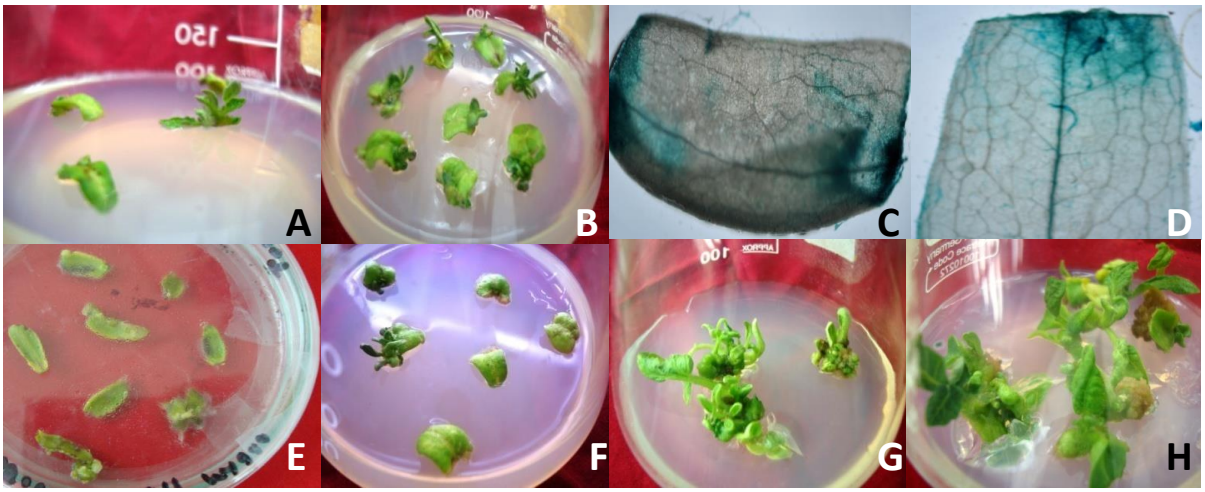

Figure 4. Factors Affecting Transformation **A.** non-pre-cultured BARI Tomato 2 explants in regeneration media containing 150 mg/L kanamycin and **B.** pre-cultured BARI Tomato 2 explants in regeneration media containing 150 mg/L kanamycin after 60 days of inoculation followed by transformation assay **C-D.** Stereomicroscopic view of GUS activity within the tissue underneath the epidermis **E.** Agrobacterial over growth after 3 days of co-cultivation in BARI Tomato 15, **F.** BARI Tomato-3 explants on selection media containing 100 mg/L kanamycin in transformation with pBI121; **G.** Shoot formation of BARI Tomato 14 on selection media containing 5 mg/L hygromycin after 30 days of inoculation, and **H.** Putative plantlets on selection media containing 10 mg/L hygromycin after 60 days of inoculation.

GUS expression) were found at OD_{600} 0.68 for BARI Tomato 3, BARI Tomato 14, and BINA Tomato 3 (Table 8). A similar result was reported to obtain maximum transformation (100%) at OD_{600} 0.79 for Bahar and BINA Tomato 3[16] and (95%) at OD_{600} 0.8 for BARI tomato 8.[50] In contrast these, bacterial suspension concentration (OD_{600} 0.5) showed the best result in tomato transformation with *Agrobacterium* strain EHA105.[47] The super-virulence expression was probably due to the extra copy of *vir* gene in the cell compared to moderately virulent LBA strains.[16]

Effect of Incubation Period on Transformation Efficiency

In most cases, higher culture density (OD_{600} 0.68) gave better transformation in 30 min incubation period (Table 8). Cent percent transformation was observed in BARI Tomato 3, BARI Tomato 14 and BINA Tomato 3. The efficiency of the transformation system mediated by *Agrobacterium* was reported to be influenced by the inoculation period which differs among plant species.[47] Incubation period of 30 min was reported to be optimum for tomato varieties Pusa Ruby, Arka Vikas and Sioux when transformed with *Agrobacterium tumefaciens* strain, AGL1, carrying either pCTBE2L or pRINASE2L construct[51] and for tomato variety Summer set in transformation with LBA4404 containing pITB-AFP.[43] In contrast to this, using the same strain LBA4404, 30 to 40 min of incubation was reported to be optimum.[52] Apart from these, transformation efficiency was found to decline above 15 min of inoculation period using LBA4404 in Bahar, BINA tomato 3, BINA tomato 5 and Pusa Ruby transformation.[16]

Effect of the Co-Cultivation Period on Transformation Efficiency

The co-cultivation period was one of the main factors affecting transformation as 'too long period' resulted in bacterial overgrowth and 'too short period' preceded in declination of transformation frequency indicating explants death on selection media.[47] Co-cultivation periods of 48 h were found best for all five tomato varieties. The highest response of transient GUS assay was obtained by both BARI Tomato 3 and BARI Tomato 14 (Figure 4C-4D). The percentage of positive GUS expression was decreased with the decrease of the co-cultivation period (Table 9). Explants having a co-cultivation period of three or more days showed overgrowth of bacteria (Figure 4E). Thus, they failed to regenerate and

Table 8. Effect of Optical Density (OD_{600}) and Incubation Period in *Agrobacterium* Suspension

Tomato Varieties	OD_{600}	Incubation Period (min)	No. of Explants Used in GUS	% of GUS Positive Explants
BARI Tomato 2	0.45	30 min	10	80
		60 min	12	87
	0.68	30 min	10	80
		60 min	11	65
BARI Tomato 3	0.45	30 min	10	40
		60 min	10	60
	0.68	30 min	10	100
		60 min	10	80
BARI Tomato 14	0.45	30 min	9	75
		60 min	10	80
	0.68	30 min	13	100
		60 min	11	93
BARI Tomato 15	0.45	30 min	10	51
		60 min	10	65
	0.68	30 min	10	65
		60 min	10	60
BINA Tomato 3	0.45	30 min	10	80
		60 min	10	90
	0.68	30 min	10	100
		60 min	13	77

Table 9. Effect of Co-Cultivation Periods on Transformation Efficiency of Different Tomato Varieties

Tomato Varieties	Co-cultivation Period*	No. of Explants Assayed in GUS Assay	Percentage of GUS Positive Explants
BARI Tomato 2	24 hours	7	86
	48 hours	8	88
BARI Tomato 3	24 hours	8	88
	48 hours	8	100
BARI Tomato 14	24 hours	9	89
	48 hours	8	100
BARI Tomato 15	24 hours	7	72
	48 hours	10	90
BINA Tomato 3	24 hours	9	67
	48 hours	7	86

finally, necrosis was found. It has been stated that two days of the co-cultivation period was ideal in tomato cvs. Megha L-15,[53] Pusa Ruby, Arka vikas,[54] and hybrid tomatoes[49] transformed with *Agrobacterium* in various studies. The one-day co-cultivation period was appropriate for Micro-Tom tomatoes.[47] In the present study, transformation efficiency was adversely affected by the *Agrobacterium* growth in the medium after two days of co-cultivation period. The same result was found in tomato cv. Riogrande transformation.[45] However, three days of co-cultivation was recommended for Bahar, BINA tomato 3, BINA tomato 5, Pusa Ruby[16] and BARI Tomato 8.[50] The reason behind this may be related to the tomato genotype and the use of different plant tissue as explants, different *Agrobacterium* strain and genes that have been transformed.[47]

Transformation Frequency

In this present study, the transformation of five tomato varieties with *Agrobacterium* strain containing pBI121, gave higher transformation efficiencies by transient GUS expression than the regeneration frequencies of transformed shoots. It was reported earlier that a big difference was observed between transformation efficiency obtained through transient GUS expression and regeneration on selection media during the transformation of tomato cv. Moneymaker with various combinations of binary vectors and *Agrobacterium* helper strains.[55]

The highest average percentage of regeneration (47%) was found in BARI Tomato 3 in kanamycin supplemented media and the incubation period was 30 min for all of the varieties (Figure 4F). In BARI Tomato 2, the regeneration response was the lowest (34%) at OD_{600} 0.68. In transformation with pH7WG2_OsNHX1_1.6, the regeneration media containing hygromycin (4 mg/L and 10 mg/L) was used in order to observe its effect on regeneration following the transformation of explants of five varieties (Figure 4G-4H). BARI Tomato 3 attained the highest regeneration response (20.5%) at OD_{600} 0.68 within 30 min of the incubation period (Table 10). The lowest regeneration response (13.5%) was found to be in BARI Tomato 15.

A range of tomato cultivars was used in transformation according to the findings of previous studies[43] and transformation frequencies showed variation among cultivars *i.e*, 5.1%[47] and 19.1%[56] and 6% to 49.5%[57] for Micro-Tom, 8% for Dotaerang,[58] 6%, 20.83%, and up to 35.3-44.3% for Pusa Ruby.[59-61] In the present study, the highest transformation frequency (47%) was obtained in transformation with pBI121 and 20.5% in the case of pH7WG2_OsNHX1_1.6 based on the regeneration percentage of transgenic shoots of BARI Tomato 3 on selection media. A similar result was found that 49% of the tomato cv. Riogrande shoots were transformed with *Agrobacterium tumefaciens* strain EHA101 harboring pBI333.[45] These results differed from each other due to the differences in bacterial strain,[62] plasmid construct,[45] plant genotype,[63] and transformation procedure.[64]

Table 10. Transformation Frequency based on Regeneration on Media Containing Kanamycin and Hygromycin

Varieties	Incubation Period (min)	OD_{600}	Average Transformation Frequency (% ± SE)[3]	
			In Media Containing Kanamycin[1]	In Media Containing Hygromycin[2]
BARI Tomato 2	30 min	0.68	34 ± 1.4	14.9 ± 3.0
BARI Tomato 3	30 min	0.68	47 ± 1.7	20.5 ± 2.9
BARI Tomato 14	30 min	0.68	43 ± 1.6	17.3 ± 1.71
BARI Tomato 15	30 min	0.68	42 ± 1.0	13.5 ± 2.9
BINA Tomato 3	30 min	0.68	37 ± 1.0	18.2 ± 3.5

[1] *Agrobacterium* strain LBA4404 containing pBI121
[2] *Agrobacterium* strain LBA4404 containing pH7WG2_OsNHX1_1.6
[3] Values were presented from three independent experiments

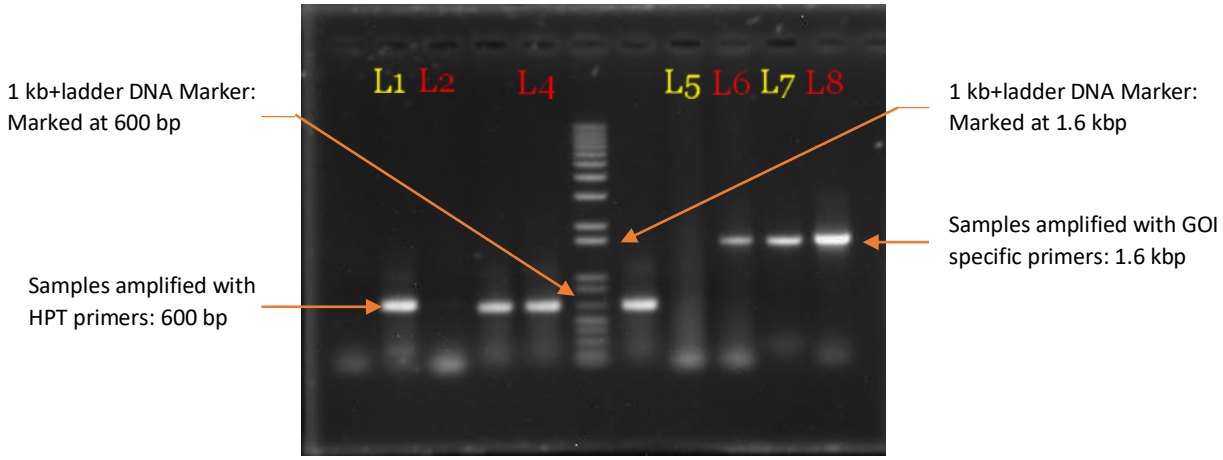

Figure 5. PCR of Putative Transgenic Shoot (60 days after infection) of BARI Tomato 3 Using HPT Primer: Primers for Hygromycin gene, GOI Specific Primer: Primers for coding sequence for OsNHX1 (L1: Positive control; L2: Negative control; L3-L4: Samples amplified with HPT Primers. L5: Negative control; L6: Positive control; L7-L8: Samples amplified with GOI Primers).

Molecular Analysis of Regeneration of Putative Transformed Shoots

Putative shoots (60 days old) regenerated on 4 mg/L hygromycin containing selection media were subjected to molecular analysis through PCR (Figure 5). Molecular analysis of putatively transformed explants was done by PCR for confirmation of pH7WG2_OsNHX1_1.6 (*OsNHX1*, Na^+/H^+ antiporter gene) incorporation in BARI Tomato 3, which was found to have the highest transformation efficiency.

Conclusion

Bangladesh has a very positive attitude in adopting Genetically Engineered (GE) food crops. Since 2013, Bt brinjal is commercially cultivated while many more food crops are undergoing the biosafety approval process to combat malnutrition in the country. The *in vitro* regeneration system established in this study for five farmer popular tomato varieties is efficient, reproducible and suitable to be used for transgenic research. The reported protocols can easily be used for future improvement of other cultivars to incorporate tolerant genes for the improvement of resistance plants in the molecular breeding of this crop. In the future, evaluations will be carried out for transgenic F_1 plants.

Authors' Contributions

API designed the study and guided the work. MMF and AD carried out the laboratory work. MMF analyzed the data and wrote the manuscript. All authors read and approved the final version of the manuscript.

Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

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