

INFLUENCE OF PH OF CO-CULTIVATION MEDIUM, CLAFORAN® CONCENTRATION, CO-CULTIVATION PERIOD, PRE-SELECTION DURATION AND SELECTION REGIME ON TOMATO TRANSFORMATION

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Abstract

Tomato fruit is enriched with lycopene and β -carotene which is an initiator of vitamin A, whereas lycopene has a shielding effect against prostate cancer. The tomato crop is very sensitive to cold stress which is a great threat to the yield of cultivated tomato. Genetic transformation offers the basis of foreign genes integration in the plant for the improvement of crop. Therefore, the present study was carried out to optimize different parameters of gene transformation in three tomato genotypes. These three tomato genotypes were transformed for cold tolerance via *DREBIA* gene with inducible promoter (Lip9). Optimal conditions to achieve 14.16-16.45% transformation efficiency (TE) was found as pH ranged between 5.6-5.7 for co-cultivation media, 500 mg/l claforan®, 6-d pre-selection period, 48-h co-cultivation duration and 35 mg/l hygromycin. Specific gene primers were used for screening and real time semi quantitative PCR assay that proved the insertion of *DREBIA* gene and its expression in T₀ and T₁ transformed lines. During the cold stress period, the increase in expression pattern was confirmed by the results of semi-quantitative PCR. Under normal growth settings, there were no considerable differences among transformants and non transformants based on morphological characteristics. Convincingly, *DREBIA* gene over expression in transgenic tomato plants increased the protection and delivered cold tolerance.

Key words: *Agrobacterium tumefaciens*, *DREBIA* gene, Lip9 promoter, Semi-quantitative RT PCR, Transgenic tomato.

Introduction

One of the fresh low cost vegetable crops and easy to handle in the food industry is known as tomato (*Solanum lycopersicum* Mill.). This tomato fruit is enriched with lycopene and β -carotene which is an initiator of vitamin A, whereas lycopene has a shielding effect against prostate cancer. The tomato crop is very sensitive to cold; an abiotic stress which is a great threat to the yield of cultivated tomato (Shah *et al.*, 2015a). In order to combat this challenge, transgenic tomato varieties need to be produced (Shah *et al.*, 2015d). Genetic transformation offers the basis of foreign genes integration in the plant for the improvement of crop. The introduction of new traits such as cold tolerance is needed in tomato varieties. In order to improve existing tomato varieties, various gene integration techniques for different crops have been developed (Fahad *et al.*, 2015). The integrated genes may express the functional proteins that perform a vital role in the biosynthesis pathways of chemicals involved in osmosis (Cho *et al.*, 2014). Procedures standardization for *In vitro* regeneration, co-cultivation time, pH of co-cultivation media, cefotaxime salt concentration, pre-selection time and selection procedure can offer improvements for the development of tolerant varieties against stress (Shah *et al.*, 2013; 2014a, b; 2015b; 2015c; Jan *et al.*, 2015).

Studies showed that the competence to the cold acclimatization in order to produce tolerance in plants is linked with the cascade of signaling pathways that may

trigger cold-regulated (COR) genes (Castonguay *et al.*, 2013). Rae *et al.* (2011) reported that a group of genes (*CBF/DREB*) is linked to increase the stress tolerance which is called as C-repeat binding factors. A motif (CCGAC) is found in the labeled CRT/DRE DNA regulatory elements which bind with the *DREBIA/CBF3* cold responsive binding factor 3 in tomato to regulate the cold responsive gene function and maximize the tolerance under chilled condition (Kasirajan *et al.*, 2014).

Previously, steady growth and yield of transgenic tomato plants having increased molecular expression of *DREBIA* gene in tomato by incubation and piercing technique of direct integration of gene have already been reported (Shah *et al.*, 2015a). In this study, numerous aspects that affect the transformation facilitated by *agrobacterium* in tomato are studied. We have successfully established an effective and positive AMTT (*Agrobacterium* mediated tomato transformation) by integrating *DREBIA* gene in three tomato varieties under the lip9 promoter. In order to confirm the inducible nature of promoter (lip9), the agronomic characteristics such as yield under natural environment were also tested.

Materials and Methods

***DREBIA* gene characterization by tomato transformation:** The pBIH cloning vector used in the current research was acquired from National Institute for Genomics & Advanced Biotechnology (NIGAB), NARC,

Pakistan. For tomato transformation, the *Agrobacterium* strain (EHA105) was used that contained the binary vector (pBIH) comprising of *DREB1A* gene (649 bp) and inducible by promoter *lip9* and hygromycin phosphotransferase (*hpt*) gene. To increase the transformation rate, the following parameters were optimized via *Agrobacterium*-mediated genetic transformation in three tomato lines namely Rio Grande, Moneymaker and Roma. Optimum cell O.D. for *Agrobacterium* was set up to 0.2 at 600 nm during this study. The explant was prepared by excising the leaf discs and hypocotyls of about 0.5 cm from 15 days old seedling grown in aseptic conditions. The explants excised were then co-cultivated after 3 min of infection with *Agrobacterium*, and shifted to co-cultivation media having varying range of pH (5.3-5.8) for multiple time periods (24 h to 96 h) in command to estimate the response of pH of media and co-cultivation time on the transformation efficiency (TE). After completion of co-culturing time, explants were rigorously washed away four to six times with ddH₂O. Afterward, the explants were washed with various concentrations of claforan® (200 to 700 mg/l) for 40 min, to minimize/inhibit the bacterial division in co-infected plants through regeneration. For optimization of pre-selection time, the explants were shifted for different time durations (0-8 days) to pre-selection medium.

Three varieties of tomato were studied to check the effect of hygromycin B (0 to 60 mg/l) (Phyto Technology) concentration on *In vitro* shoots. The two explants were subjected to elongation medium having various levels of hygromycin to conclude optimal concentration of hygromycin for maximum persistence of transformed explants. The incubation was done at 24-25°C under 16 h/8 h light/dark photoperiod, 50-51 micromoles m⁻²s⁻¹ fluorescence light and 64-72 percent relative moisture were maintained. Both explants were then moved to a selection medium added with hygromycin and antibiotic cefotaxime and kept at 24 -25 °C in low to no light to handpick the transformed plants.

Molecular assays: The characterization of T₀ – T₂ generation was done by molecular approach as follows:

Extraction of DNA and PCR analysis: CTAB protocol was used to extract the plant genomic DNA (Murray and Thomson, 1980). Afterward, the PCR analysis investigated the presence of gene of interest. The target 649 bp section of *DREB1A* was amplified by specific gene marker pair (*DREB1A* forward primer 5'-TGAACCTATTTCTGCTTT-3' and *DREB1A* reverse primer 5'-TAATAACTCCATAACGATA-3'). Similarly, 399 bp section of *hpt* was amplified by specific gene marker pair (*hpt* forward primer 5'-TCGTGCTTT CAGCTTCGATG-3' and *hpt* reverse primer 5'- TCCATCACAGTTT GCCAGTG-3'). The PCR reagents optimization was done to identify the both genes (*DREB1A* and *hpt*) which are given as 2 µl of 10 X Taq buffer, 2.4 µl of 50 mM MgCl₂, 0.4 µl of 10 mM dNTPs, 10 mmol of forward and reverse primer in final reaction volume, 0.3 unit of Taq DNA polymerase, 10.9 µl of DEPC-treated water and 2 µl of template DNA in order to make reaction volume up to 20 µl. PCR profile was optimized and used for PCR analyses is given as one cycle of initial denaturation at

94°C for 4 min followed by 35 cycles of denaturation, annealing and initial extension at 94°C, 50°C, 72°C, respectively for 1 min and final extension of one cycle at 72°C for 10 min in PCR thermocycler (Applied Biosystem).

Extraction of RNA and semi-quantitative RT-PCR:

The total RNA was extracted from transgenic as well as non-transgenic (NT) tomato plants via NucleoSpin RNA kit, GmbH, Germany using company's instructions. In first step of RT-PCR, cDNA was synthesized and then the 2 µl cDNA worked as template DNA to perform PCR with the same conventional PCR optimized conditions.

Phenotypic analyses: Phenotypic comparison of T₂ transgenic and NT lines were performed on thirty days old plants. CTAB method was used to extract the DNA, and *DREB1A* and *hpt* genes were confirmed through PCR analysis. Selection was based on the Mendelian Segregation Ratio for morphological characterization under typical growth settings for the plants.

Statistical analysis

CRD in a factorial system was used for all the experiments. ANOVA at $p \leq 0.05$ and LSD test to compare the mean differences via The Statistic 8.1 (Steel *et al.*, 1997).

Results and Discussion

Assessment of pH of co-cultivation medium: To determine the effect of pH of the co-cultivation medium on the TE, two explants i.e. hypocotyls and leaf discs were infected and cultured simultaneously in different media with different range of pH (5.3, 5.4, 5.5, 5.6, 5.7 and 5.8). The pH 5.6 was recorded to be the best for producing the highest transformation efficiency (16.18%) documented in Rio Grande culturing hypocotyls, trailed by Moneymaker (15.94%) and Roma (15.89%) culturing hypocotyls as explant sources. While the highest efficiency of gene transformation (11.92%) found in Roma, 10.97% in Moneymaker and 9.86% in Rio Grande at pH 5.7 co-cultivation medium (Fig. 1). The significant ($p \leq 0.05$) reduction in transformation efficiency in all genotypes was observed above or below the pH 5.6-5.7 (Fig. 1). Whereas, the optimal pH for the co-cultivation medium was observed for explants hypocotyls and leaf discs to be pH 5.6 and 5.7, respectively to achieve the highest TE. Similar type of findings was observed in an earlier research study in which it was concluded that co-cultivation media pH (5.6) greatly influenced the TE (Mondal *et al.*, 2001). The optimal pH 5.0 enhanced the TE in three genotypes of tomato by culturing cotyledon explants (Rai *et al.*, 2012). Formerly, the maize plant inbred lines culturing immature embryos as explant have been investigated for pH levels of co-cultivation medium to check the efficiency of *Agrobacterium* strains. EHA105 strain has been found to be better as compared to LBA4404 and GV310. Already this strain has shown high TE at pH 5.4 (Huang & Wei, 2005).

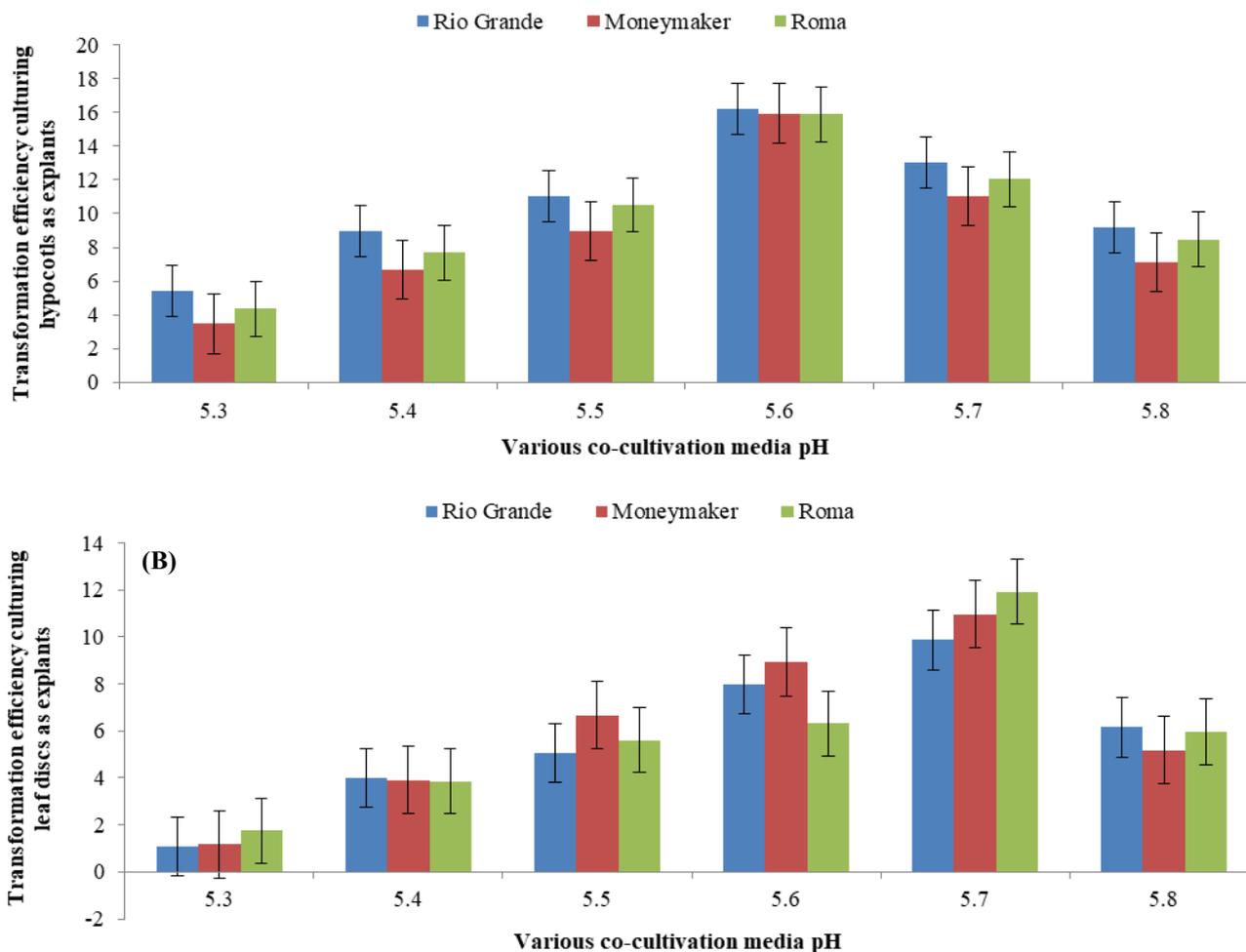


Fig. 1. (A) Evaluation of co-cultivation media pH on transformation efficiency (TE) via agrobacterium using hypocotyls as explant base in 3 tomato genotypes. (B) Evaluation of co-cultivation media pH on TE via agrobacterium using leaf discs as explant base in 3 tomato genotypes.

Assessment of claforan® concentration: The data was observed after one month which clearly indicate the direct relationship of claforan® (cefotaxime sodium) with the transformation and ultimately TE in all the cultivars (Fig. 2). However claforan® at concentration of 500 mg/l enhanced the TE but if the dose increased further the TE in all the genotypes suppressed (Fig. 2). Claforan® at the concentration of 500 mg/l, the highest transformation efficiency was observed in Roma (16.45%) trailed by 16.22% in Rio Grande and 14.32% in Moneymaker culturing hypocotyls. Likewise, the Roma (14.16%) showed the highest transformation efficiency when leaf discs explants were used to check TE at (500 mg/l) claforan® concentration. This concentration was found to be optimal as no bacterial growth was observed without any negative effect on plant growth for tomato transformation. Six concentrations of claforan® were estimated to overcome the unwanted growth of *Agrobacterium*. Below this level, the bacterial overgrowth in the medium was observed in all varieties. Above this dose, bacterial progression was not observed but affected the regeneration of two explants used. The low regeneration response was observed due to high pressure on the plant tissues. Similar type of findings were observed with that of Roy *et al.*, (2006), according to his studies claforan® at 500 mg/l was found to be the best for inhibition of the bacterial

growth in tomato. Our finding was not identical to the Bihao *et al.*, (2012) who reported the concentration of 400 mg/l claforan® for the inhibition of *Agrobacterium* on co-cultured explants. Another researchers' group controlled the bacterial growth using cefotaxime sodium at 300 mg/l (Cortina and Culianez-Macia, 2004). El-Siddig *et al.*, (2009) however reported 25 mg/l cefotaxime sodium to be sufficient for bacterial growth control.

Assessment of co-cultivation duration: In this study, we found that co-cultivation duration of 48 hours produced the best results regarding the transformation efficiency. In case of hypocotyls, the cultivar Roma produced the highest transformation efficiency (14.83%), whereas, for using leaf discs, the transformation efficiency was recorded to be the highest in Roma (11.45%) trailed by Rio Grande (9.89%) and Moneymaker (8.26%) at 48 h co-cultivation time (Fig. 3). The extended duration of co-cultivation might result in poor plant health because of overgrowth of bacteria, browning at the wound part and no growth response by explants. Co-cultivation duration is an important step for uniform transformation of a gene in plants therefore; duration of co-cultivation needs to be optimized (Ren *et al.*, 2012). Duration of co-cultivation has already been a matter of discussion and reported to impact on the regeneration

response in different plant species (Seo *et al.*, 2011). In this study, the co-cultivation times (24, 48, 72 and 96 h) along with persistent O.D. and infection duration was examined to obtain the maximum number of transformants. 48 h duration was found to be optimal for the optimal TE in three tomato cultivars. However, decreasing the time period decreased the TE dramatically because of poor growth of bacteria. Inversely, increasing this time period, TE and regeneration response decreased due to overgrowth of bacteria. Our results were in line with Singh *et al.*, (2011) who stated the optimum co-cultivation duration of 48 h for tomato transformation. Whereas our findings were contrary to other research study stated by Gao *et al.*, (2009) where high TE was reported at 72 h for co-cultivation.

Assessment of pre-selection period: Six days for pre-selection gave the maximum TE in Rio Grande, Moneymaker and Roma i.e. 14.44, 10.58 and 10%, respectively for hypocotyls explant (Fig. 4). The explant leaf discs of Rio Grande, Roma and Moneymaker gave maximum TE i.e., 9.15, 7.49 and 6.86% (Fig. 4). During this investigation, the six days of pre-selection against hygromycin gave a high survival percentage. The gradual decline was observed with the elevation of this period. In contrast, the explant shifted directly to the

medium containing hygromycin arrested the growth. These findings were in line with the earlier report in which pre-selection duration of 7 days created the maximum TE in tomato genotypes (Afroz *et al.*, 2011). Pre-selection stage was found to be the most important factor influencing the TE in our study.

Antibiotic sensitivity tests: In our study the results showed the inverse relation of plant regeneration to the hygromycin concentration (Fig. 5; Fig. 6a-c). Regeneration of *In vitro* shoot formation stopped within seven days for both the explants kept in 35 mg/l hygromycin containing medium. Beyond 35 mg/l concentration of hygromycin, all the cultured explants showed browning initially and ultimately died. An ideal selection of transformants was done on 35 mg/l hygromycin (Fig. 6d-l). The lethal dose optimization for hygromycin is the mandatory step as this concentration is tissue and genotype specific in tissue culture based transformation (Datta *et al.*, 2004). In understudied genotypes, both explants were dead on 35 mg/l hygromycin. In another research, Roy *et al.*, (2006) produced transgenic tomato plants against *bspA* gene at hygromycin concentration of 40 mg/l. Shah *et al.*, (2010) proclaimed 25 mg/l of hygromycin against the tomato transformed plants with *ech42* gene.

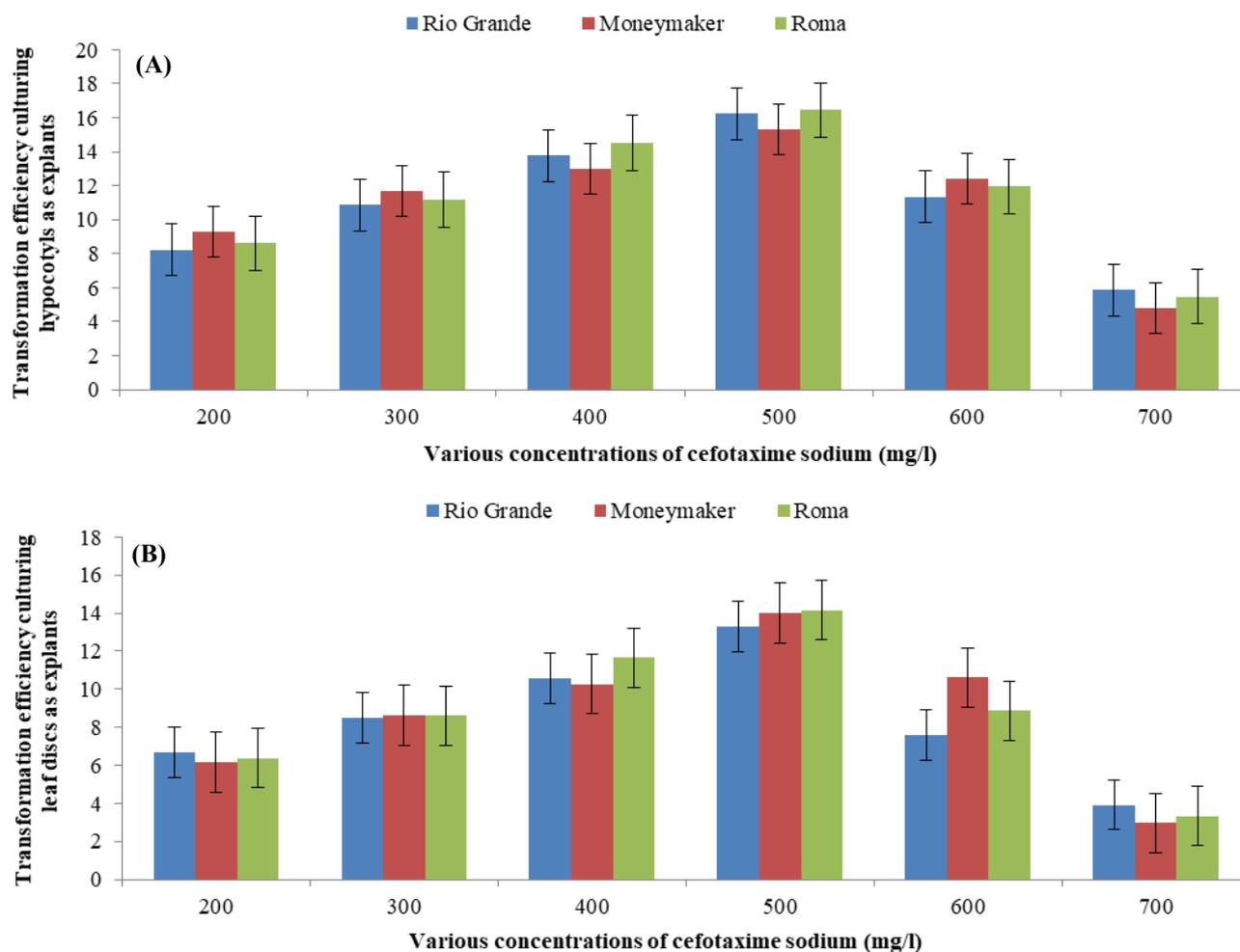


Fig. 2. (A) Valuation of claforan[®] (cefotaxime sodium) concentration on transformation efficiency via agrobacterium using hypocotyls as explant base in 3 tomato genotypes. (B) Valuation of cefotaxime on transformation efficiency via agrobacterium using leaf discs as explant base in 3 tomato genotypes.

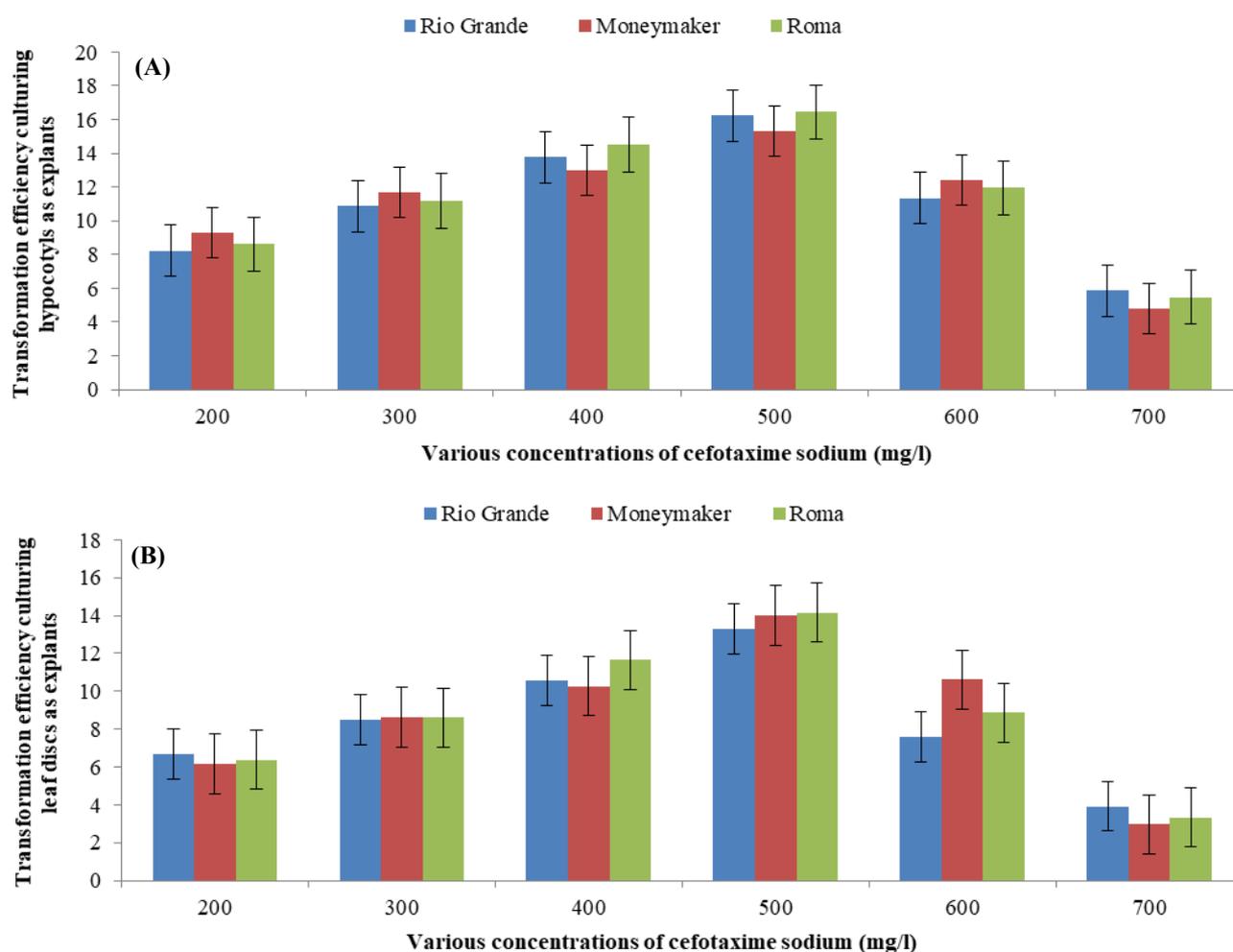


Fig. 3. (A) Valuation of co-cultivation duration on transformation efficiency (TE) via agrobacterium using hypocotyls as explant base in 3 tomato genotypes. (B) Valuation of co-cultivation duration on TE via agrobacterium using leaf discs as explant base in 3 tomato genotypes.

Molecular assays

Validation of desired gene: PCR was performed to check the presence of desired genes i.e., *DREB1A* and *hpt*. DNA of non-transformed plants was kept as negative control. Plasmid extraction was done from *A. tumefaciens* integrated with gene of interest and run as +ve control. The gene specific markers i.e. *DREB1A* and *hpt* genes were used to identify the transgenic plants by PCR analysis. In transgenic plants, the desired band size of 649 bp and 399 bp of *DREB1A* and *hpt* genes, respectively were recorded, while no band was observed in non transformants (Fig. 7a & b). In command to ratify the segregation pattern, the T₁ generation of transformed lines with their counterpart NT was checked by gel electrophoresis. All the transformed lines showed (3:1) Mendelian ratio using simple PCR (Fig. 7c-e).

Semi-quantitative PCR for expression analysis: To check the *DREB1A* expression in T₁ generation of transformants, semi-quantitative RT-PCR was performed. First of all the total RNA was extracted from liquid nitrogen treated transformed lines and non-transformed lines, and then cDNA was prepared that worked as a template for normal PCR. Differential expression was

observed in transformed lines whereas no expression was noticed in non-transformed plants (Fig. 7f). In conclusion to this experiment, only transformed lines exhibited the expression of gene of interest under cold stress.

Phenotypic characterization of transformants and NT plants:

The following traits were compared between transgenic and non-transgenic plants: mean weight of fruit, first fruit set and seeds' number per fruit under standard development practices. No significant difference was observed in the plants having single gene integration and their isogenic NT plants in all the phenotypic characteristics studied (Tables 1-4). Our findings were similar to the Singh *et al.*, (2011) who stated that no significant difference was observed in Shalimar variety showing increased expression *AtCBF1* gene, which did not display noticeable alteration in the phenol-agronomical features under normal growth conditions. The expression of *DREB1A* gene did not link with any of the yield factors in tomato without any cold stress. The lip-9 cold inducible promoter might be the reason of this factor because it induced the expression of *DREB1A* gene in cold stress but not in normal conditions resulted in no measureable difference among the transformed and non-transformed lines.

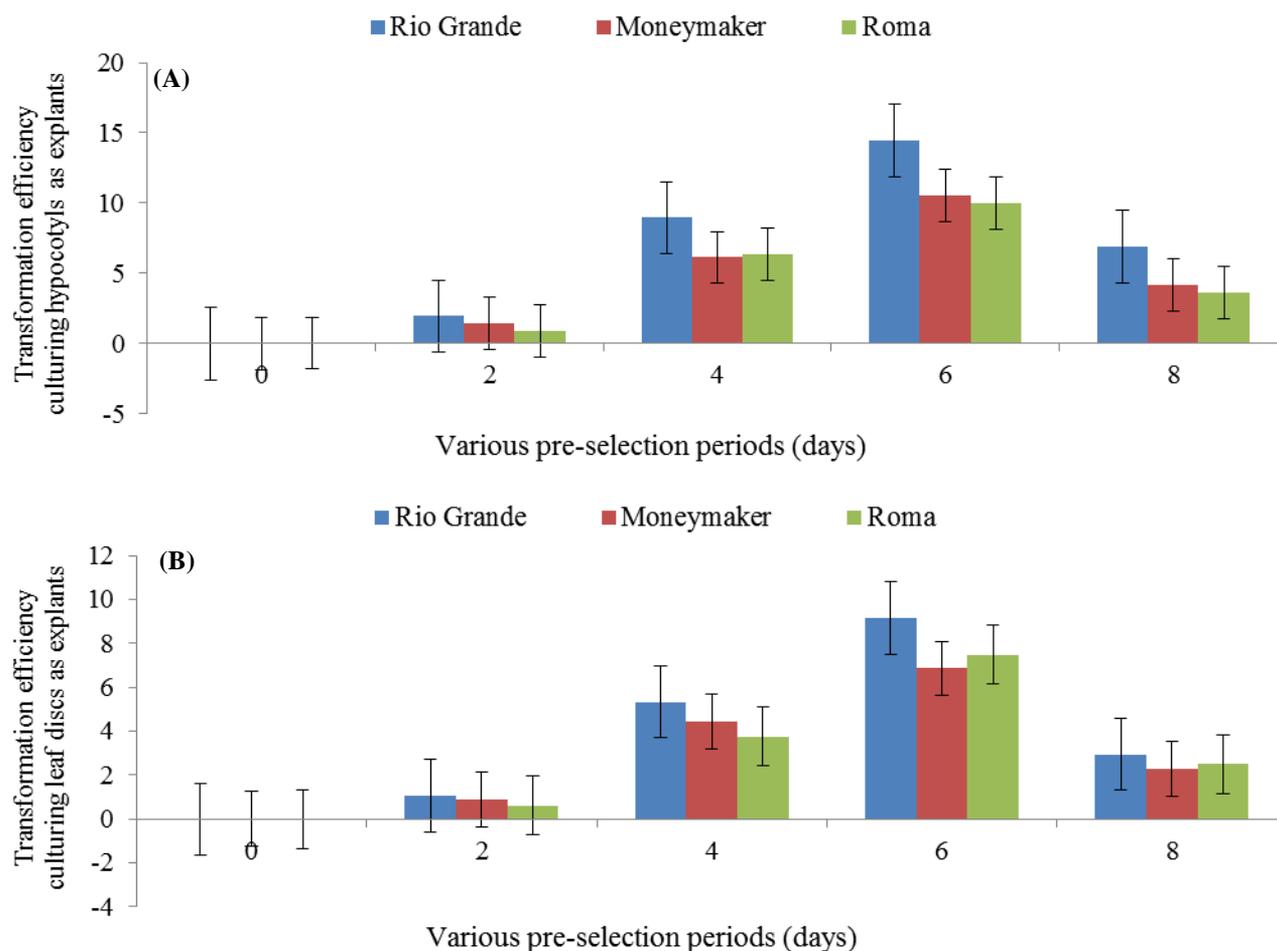


Fig. 4. (A) Valuation of pre-selection duration on transformation efficiency via agrobacterium using hypocotyls as explant sources in 3 tomato genotypes. (B) Valuation of pre-selection duration on transformation efficiency via agrobacterium using leaf discs as explant sources in 3 tomato genotypes

Table 1. Comparison of thirty days old T₂ transgenic plants carrying *AtDREB1A* gene, relative to their isogenic NT plants of the same age based on the days to flowering.

Plants	Rio Grande	Moneymaker	Roma	Mean
Transgenic plants	55 ± 5.57	67 ± 7.94	62 ± 5.29	61.33
NT plants	52 ± 5.29	65 ± 4.58	61 ± 4.58	59.33
Mean	53.5	66	61.5	

Data was recorded under normal growth conditions without any chilling stress. Three replicates' average of each data was taken. Standard deviation have been shown after ± sign (n=3). LSD = 10.02 ($p \leq 0.05$)

Table 2. Comparison of thirty days old T₂ transgenic plants carrying *AtDREB1A* gene, relative to their isogenic NT plants of the same age based on days to first fruit set.

Plants	Rio Grande	Moneymaker	Roma	Mean
Transgenic plants	65 ± 5.2	76 ± 6.08	70 ± 5	70.33
NT plants	62 ± 4.36	74 ± 6.24	69 ± 5.29	68.33
Mean	63.5	75	69.5	

Data was recorded under normal growth conditions without any chilling stress. Three replicates' average of each data was taken. Standard deviation have been shown after ± sign (n=3). LSD = 10.44 ($p \leq 0.05$)

Table 3. Comparison of thirty days old T₂ transgenic plants carrying *AtDREB1A* gene, relative to their isogenic NT plants of the same age based on the mean weight of fruit.

Plants	Rio Grande	Moneymaker	Roma	Mean
Transgenic plants	39.59 ± 4.17	30.84 ± 3.71	35.65 ± 4.82	35.36
NT plants	42.72 ± 4.91	32.45 ± 4.35	38.31 ± 5.96	37.82
Mean	41.15	31.64	36.98	

Data was recorded under normal growth conditions without any chilling stress. Three replicates' average of each data was taken. Standard deviation have been shown after ± sign (n=3). LSD = 8.19 ($p \leq 0.05$)

Table 4. Comparison of thirty days old T₂ transgenic plants carrying *AtDREB1A* gene, relative to their isogenic NT plants of the same age based on no. of seeds/fruit.

Plants	Rio grande	Moneymaker	Roma	Mean
Transgenic plants	120.78 ± 9.91	110.37 ± 6.91	116.67 ± 7.76	115.94
NT plants	123.87 ± 5.18	115.23 ± 5.87	121.59 ± 4.26	120.23
Mean	122.32	112.8	119.13	

Data was recorded under normal growth conditions without any chilling stress. Three replicates' average of each data was taken. Standard deviation have been shown after ± sign (n=3). LSD = 12.79 ($p \leq 0.05$)

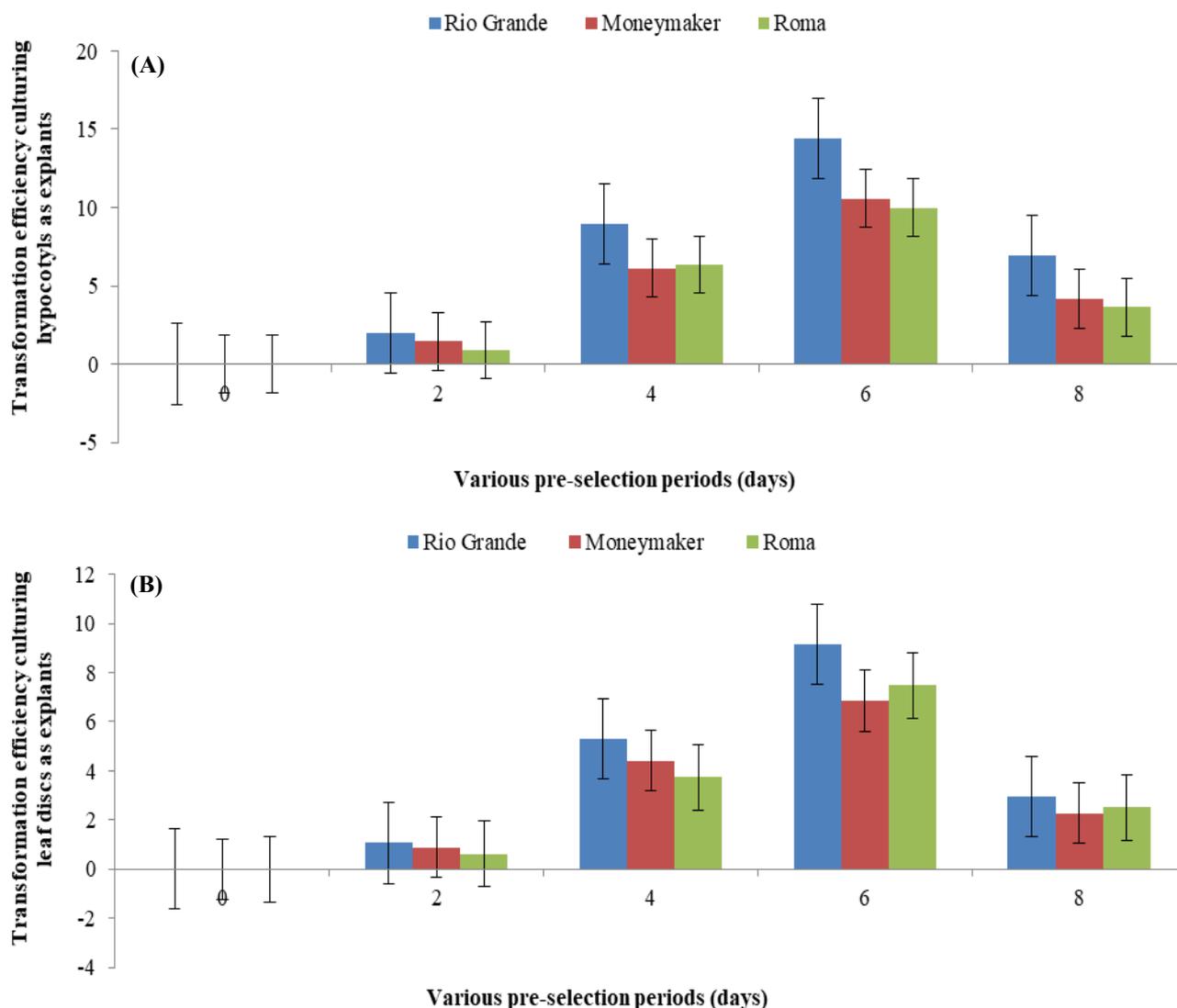


Fig. 5. Optimization of hygromycin level for transformation via *Agrobacterium* in 3 tomato genotypes (A) Evaluation of hygromycin levels on *In vitro* shoot regeneration using hypocotyls as explant base in three tomato genotypes. (B) Evaluation of hygromycin levels on *In vitro* shoot regeneration culturing leaf discs as explant base in three tomato genotypes.

Conclusion

A novel method AMTT was developed which is more efficient. We also unveiled many prime factors that lead to stable integration of foreign gene in three indigenous tomato lines by integration of *DREB1A* gene. We recommend this method as realistic approach in identification of other transcription factor related studies in tomato cultivars. This technique may be adopted for other species of plants to produce transformants without the need of advanced skills. Under normal conditions, the

plants were screened out and no significant difference was observed on the basis of yield and agronomic features because of cold inducible nature of promoter used.

Acknowledgments

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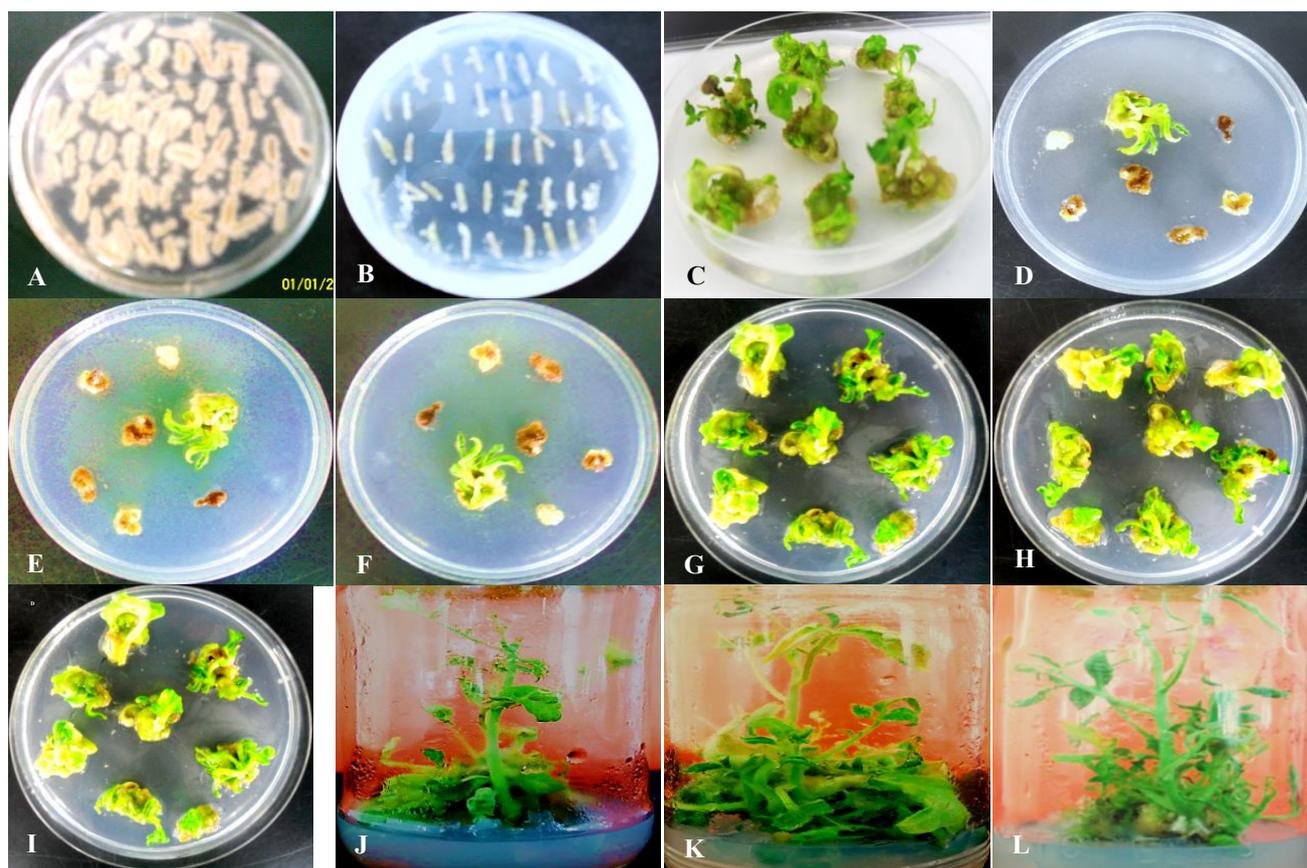


Fig. 6. Hygromycin resistance plant in 3 varieties of (*Solanum lycopersicum* Mill.). (A) Degree of overgrowth of *Agrobacterium* at 300 mg L⁻¹ claforan®, O.D_{600nm} = 0.2, acetosyringone 60 µM. (B) Minimal overgrowth of *Agrobacterium* at 400 mg L⁻¹ claforan®, O.D_{600nm} = 0.2, acetosyringone 60 µM. (C) Competent regeneration at 500 mg L⁻¹ claforan®, O.D_{600nm} = 0.2, acetosyringone 60 µM after 48-h of co culture. (D, E, F): Selection against hygromycin leaf discs at 35 mg L⁻¹ (G, H, I): Morphogenesis in resistant calli under aseptic conditions taken from hypocotyls 35 mg L⁻¹ hygromycin after 3-4 weeks (J, K, L): Transformed plants obtained after culturing hypocotyls earlier acclimatization in 3 varieties of tomato.

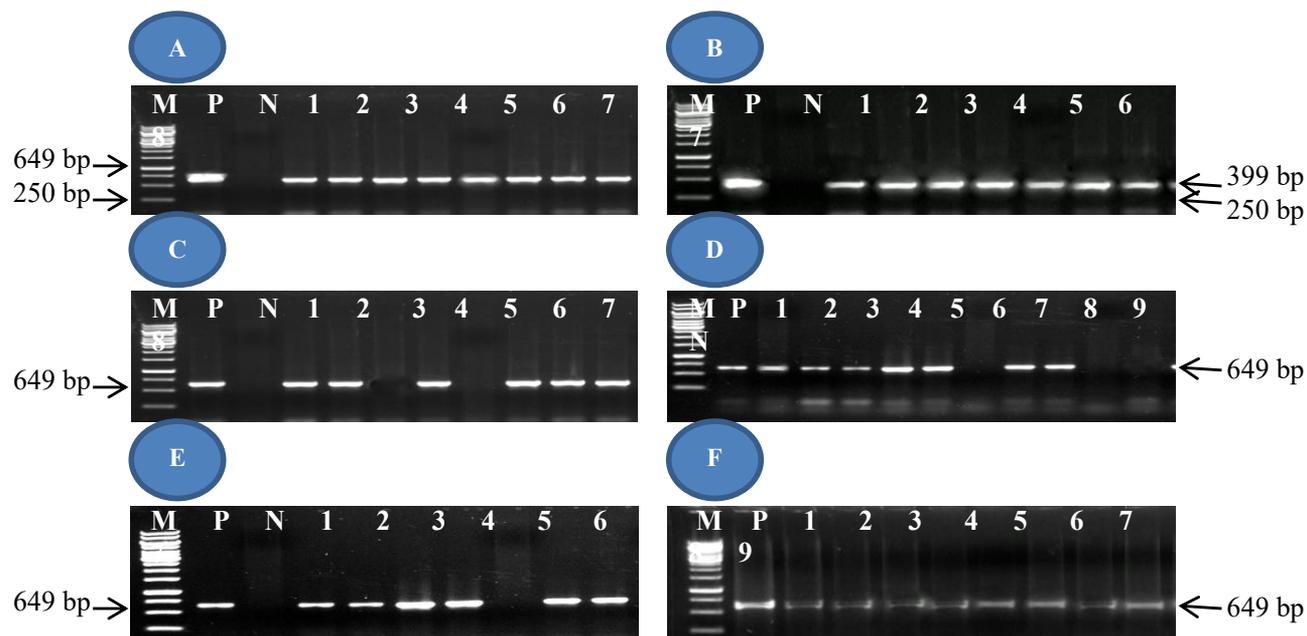


Fig. 7. Characterization of transformed lines (T₀ and T₁ generation) for incorporation and expression of *DREB1A* and *hpt* genes via PCR, segregation assay and semi-quantitative RT-PCR. (A) Verification of *DREB1A* gene fragment (649 bp) Lane M = DNA ladder (1 kb). Lane P = +ve control; Lane N = -ve control. Lanes 1–8 = transgenic plants of three varieties (B) Identification of *hpt* gene fragment (399 bp) via PCR. Lanes M, P and N = DNA Ladder, +ve control, -ve control, respectively. Lanes 1–7 = Transformed plants. (C, D, E) Segregation scrutiny of Moneymaker, Roma and Grande T₁ transgenic line. (F) *DREB1A* gene's expression assay by semi-quantitative RT-PCR investigation in T₁ generation of three tomato varieties.

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