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Original article

Highly efficient *Agrobacterium*-mediated transformation and plant regeneration system for genome engineering in tomato

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ABSTRACT

Tomato (*Solanum lycopersicum* L.) is an important vegetable and nutritious crop plant worldwide. They are rich sources of several indispensable compounds such as lycopene, minerals, vitamins, carotenoids, essential amino acids, and bioactive polyphenols. Plant regeneration and *Agrobacterium*-mediated genetic transformation system from different explants in various genotypes of tomato are necessary for genetic improvement. Among diverse plant growth regulator (PGR) combinations and concentrations tested, Zeatin (ZEA) at 2.0 mg l^{-1} in combination with 0.1 mg l^{-1} indole-3-acetic acid (IAA) generated the most shoots/explant from the cotyledon of Arka Vikas (36.48 shoots/explant) and PED (24.68 shoots/explant), respectively. The hypocotyl explant produced 28.76 shoots/explant in Arka Vikas and 19.44 shoots/explant in PED. In contrast, leaf explant induced 23.54 shoots/explant in Arka Vikas and 17.64 shoots/explant in PED. The obtained multiple shoot buds from three explant types were elongated on a medium fortified with Gibberellic acid (GA_3) (1.0 mg l^{-1}), IAA (0.5 mg l^{-1}), and ZEA (0.5 mg l^{-1}) in both the cultivars. The rooting was observed on a medium amended with 0.5 mg l^{-1} indole 3-butyric acid (IBA). The transformation efficiency was significantly improved by optimizing the pre-culture of explants, co-cultivation duration, bacterial density and infection time, and acetosyringone concentration. The presence of transgenes in the plant genome was validated using different methods like histochemical GUS assay, Polymerase Chain Reaction (PCR), and Southern blotting. The transformation efficiency was 42.8% in PED and 64.6% in Arka Vikas. A highly repeatable plant regeneration protocol was established by manipulating various plant growth regulators (PGRs) in two tomato cultivars (Arka Vikas and PED). The *Agrobacterium*-mediated transformation method was optimized using different explants like cotyledon, hypocotyl, and leaf of two tomato genotypes. The present study could be favourable to transferring desirable traits and precise genome editing techniques to develop superior tomato genotypes.

1. Introduction

Tomato (*Solanum lycopersicum* L.) belongs to the Solanaceae family, a significant vegetable and nutritionally important crop worldwide. It is cultivated as an annual crop, growing under temperate/tropical conditions (Atherton and Rudich, 2012). The plant crops have grown the maximum number of times in a year because of their easy cultivation, short duration, and seed number. Hence, it is considered as a model crop plant to understand several biological processes, such as functional transcriptomics, proteomics, metabolomics, and genomics (Gerszberg et al., 2015; Shikata et al., 2016; Chaudhary et al., 2019). Tomato is one of the most common vegetable crops containing many valuable nutrients grown worldwide for the human diet (Yusufe et al., 2017). Tomatoes are rich in several nutritional compounds such as lycopene, vitamins (A, C, and E), minerals, carotenoids, essential amino acids and bioactive polyphenols (Raiola et al., 2015; Martí et al., 2016; Gorecka et al., 2020). The polyphenols of tomato have several

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physiological benefits: anti-inflammation, antioxidant, blood vessel relaxation, and capillary wall stabilizing activity (Pek et al., 2010; 2011; Hassellund et al., 2013). The lycopene (carotenoid) acts as a potent antioxidant to neutralize free radicals to fight against cancers cells (Martí et al., 2016; Gorecka et al., 2020). The lycopene also defends the cells from oxidative damage caused to DNA, lipids, and proteins (Cheng et al., 2019; Han et al., 2019; Gorecka et al., 2020) and reduces the risk of heart diseases (Perveen et al., 2019; Tierney et al., 2020). Tomatoes play a valuable role in human health as a good blood purifier, boosting immunity, improving vision, a natural antiseptic, controlling cell cycle progression, cell signaling, and transcriptional modulation, and also helping to prevent gallstones and control sugar levels (Palozza et al., 2012; Tierney et al., 2020). Tomatoes are used in different preserved foodstuffs like tomato soup, juice, ketchup, salads, salsa, spaghetti sauce, chutney, paste, and pizza sauce, and these are primary dietary sources of lycopene (Storniolo et al., 2019; Gorecka et al., 2020).

The demand for tomatoes has increased recently because of the significant nutritional value of its fruit and to feed the increasing global population (Chaudhary et al., 2019; Cheng et al., 2019; Han et al., 2019; Kabir et al., 2020). Several abiotic and biotic stresses might drastically reduce tomato yield (Krishna et al., 2017; Singh et al., 2017). Many consistent factors include variety, cultivation, climatic conditions, degree of ripeness during harvest, and long-term storage conditions of tomatoes (Tilahun et al., 2017; Raheem et al., 2019). It is necessary to develop a proficient plant regeneration and genetic transformation system in different tomato genotypes resilient to biotic and abiotic stresses and yield high through genome editing tools (Gerszberg et al., 2015; Gupta and Van Eck, 2016; Reem and Van Eck, 2019; Van Eck, 2020). Plant regeneration methods have been reported from various genotypes using cotyledon, cotyledonary node, hypocotyl, leaf, node, shoot tip, stem, pistil, and inflorescence as explants (Koul et al., 2014; Senapati, 2016; Alatar et al., 2017; Lee et al., 2020). The different types of experiments with varying concentrations of hormones are designed based on previous studies (Senapati, 2016). An efficient plant regeneration is utilized for gene transfer methods to develop transgenic plants to get the desired character. The PGR concentrations and combinations play a crucial role during the in vitro morphogenesis of tomato culture. Other parameters also affect the tomato plant regeneration frequency, such as the age of explants, type of medium, and components (Senapati, 2016; Alatar et al., 2017; Lee et al., 2020).

Efficient and repeatable plant regeneration and transformation system are required to improve tomato cultivars through genetic engineering and genome editing techniques (Gupta and Van Eck, 2016; Reem and Van Eck, 2019; Van Eck, 2020). Many of these reports do not present critical information on the transformation because the *Agrobacterium*-mediated genetic transformation efficiency strongly depends on several factors that significantly influence the transformation efficiency (Gerszberg et al., 2015; Senapati, 2016). The transformation efficiency was increased considerably by evaluating the various factors such as genotype/cultivars (Stavridou et al., 2019), explant type (Kumar et al., 2017), different PGRs regimes (Koul et al., 2014), pre-culture of explants (Koul et al., 2014; Stavridou et al., 2019), acetosyringone (Gupta and Van Eck, 2016). Other factors like pH in the medium (Rai et al., 2012), *Agrobacterium* cell density (Bamishaiye et al., 2017), *Agrobacterium* strains (Koul et al., 2014; Rajesh et al., 2016), plasmid constructs (Chetty et al., 2013), vacuum infiltration and sonication (Koul et al., 2014; Rajesh et al., 2016) have been reported.

Agrobacterium-mediated genetic transformation protocols have successfully generated transgenetic tomato plants with agronomically important traits. The human lactoferrin (Lf) gene expression significantly increased resistance to fungal pathogens in transgenic tomato plants (Buziashvili et al., 2020). The transgenic tomato plants with overexpression of AtDREB1A and BcZAT12 genes showed tolerance to drought and increased fruit production (Krishna et al., 2021). The watermelon *ClERF069* gene overexpression resulted in delayed fruit ripening in transgenic tomato plants (Zhou et al., 2020). The mouse *ODC* gene overexpressed with the fruit-specific promoter (*2A11*) increases tomato fruit quality (Pandey et al., 2015). The carotenoid gene expression in tomato increased beta carotene content (Romer et al., 2000).

Recently a new genome-editing tool CRISPR-Cas system derived from the prokaryotic immune system of bacteria, and it is popular due to its fast and simplicity (Alok et al., 2018). The CRISPR-Cas tool was based on recognizing target sequences in the host genome, and Cas nuclease creates the breaks in the targeted DNA (Sandhya et al., 2020). This technique was applied successfully in many plants like tomato, wheat, and rice (Sandhya et al., 2020; Jogam et al., 2021). The CRISPR-Cas-based genome editing tool was attempted in tomato and achieved good results (Chandrasekaran et al., 2021). The *SlHyPRP1* gene of tomato was edited using the CRISPR-Cas multiplexing tool and achieved salt stress tolerance in tomato plants (Tran et al., 2021). The editing of tomato transcription factor *SlLBD40* using the CRISPR-Cas tool in tomato increased drought tolerance (Liu et al., 2020). The editing of the *SlMlo1* gene in tomato plants resulted in powdery mildew resistance (Nekrasov et al., 2017).

The present study was conducted to determine the relative importance of explants, cultivars (Arka Vikas and PED), and different concentrations of PGRs for multiple shoot induction, proliferation, shoot elongation, and successful recovery of complete plantlets after rooting. Furthermore, we have undertaken a study to establish an efficient *Agrobacterium*-mediated genetic transformation protocol by optimizing various factors. Different factors like explant type, genotype, hygromycin concentration for selection transformants, pre-culture duration, bacterial cell density and infection time, co-cultivation duration, and acetosyringone were evaluated to improve the transformation efficiency in both genotypes of tomato.

2. Methods

2.1. Seed material and preparation of explants

Seeds of tomato cultivars viz; Arka Vikas kindly provided by ICAR-Indian Institute of Horticultural Research, Bengaluru, Karnataka, India and PED (Pusa Early Dwarf) procured from Division of Vegetable Science, ICAR-Indian Agriculture Research Institute, Pusa, New Delhi were used as starting material. Sodium hypochlorite (4%) solution was used to sterilize seeds for 10 min, then washed thoroughly with sterile distilled water five to six times and placed on half-strength Murashige and Skoog (Murashige and Skoog, 1962) medium for germination. Cotyledon and hypocotyl explants were prepared from 10 to 12 d old seedlings. Leaf explants prepared from 4 weeks-old seedlings were used for further experiments.

2.2. Culture media and conditions

All experiments were conducted using MS basal medium augmented with diverse concentrations of cytokinins and auxins. The medium supplemented with sucrose (3%), then solidified with agar (0.8%). The pH was adjusted to 5.8 with either 0.1 N NaOH or 0.1 N HCl, then autoclaved for 20 min at 103.4 pKa (121 °C). All cultures were kept in the culture room and maintained the photoperiod (16/8h) with cool fluorescent light (50 $\mu Em^2\ S^{-1}$) and 24 ± 2 °C.

2.3. Effect of PGRs on multiple shoot induction

Cotyledon, hypocotyl, and leaf explants of both genotypes were inoculated onto a shoot induction medium. The shoot induction medium was amended with PGRs such as BAP (1.0 to 5.0 mg l^{-1}), TDZ (0.5 to 2.5 mg l^{-1}), and ZEA (0.5 to 2.5 mg l^{-1}) individually and in combination with IAA/ IBA/NAA (0.1 and 0.2 mg l^{-1}) were employed (Tables 1–3) to find out their role on shoot bud initiation and multiplication.

2.4. Shoot elongation and rooting

The multiple shoot buds were isolated from a bunch of shoots and transferred on a shoot elongation medium amended with different ZEA, IAA, and Gibberellic acid (GA3) concentrations. After two weeks of culture, the elongated shoots were transferred onto a fresh medium, for further elongation of shoots produced from various explant types of both tomato genotypes.

The shoots were isolated from all three explants, viz., cotyledon, hypocotyl, and leaf explants of two tomato genotypes. The shoots were transferred to a root induction medium fortified with diverse auxins like IAA, IBA, and NAA with concentrations (0.1 to 2.0 mg l^{-1}) individually to find the appropriate auxin type and suitable auxin concentration for efficient root induction in tomato. After two weeks of culture, data on the rooting efficiency of shoots was recorded in various auxin treatments.

2.5. Hardening and acclimatization

The plantlets were carefully removed from the culture tubes and washed under tap water to remove the media traces from the roots. The plantlets were kept in 0.1% bavistin solution for 10 min, then placed in sterile liquied medium for 2 days. The plantlets were planted in paper cups filled with soilrite™ and cocopeat. The paper cups were covered with small polyethylene bags with minute holes and placed in the culture room for 2 weeks to acclimatize the plants. After two weeks of acclimatization, the plants were moved to pots. The plants were kept in the greenhouse conditions for the successful establishment of in vitro regenerated plantlets. The survival percentage of in vitro regenerated plants was recorded after six weeks for both genotypes of tomato.

2.6. The sensitivity of cotyledon explants to hygromycin

To check the influence of selection agent, a preliminary study was carried out using different concentrations of hygromycin. The cotyledon explants were cultured in a shoot induction medium containing 2.0 mg l^{-1} ZEA and 0.1 mg l^{-1} IAA. The medium was fortified with various concentrations (0 to 25 mg l^{-1}) of hygromycin. The cotyledon explants cultured on a medium without hygromycin was served as a control.

2.7. Agrobacterium culture preparation

The *Agrobacterium* strain LBA4404 with plasmid pCAMBIA1305.1 contains *hptII* and *gusA* genes employed for evaluation of various parameters. Bacterial culture was inoculated to YEM medium agumented with streptomycin (50 mg l^{-1}), kanamycin (50 mg l^{-1}), and rifampicin (30 mg l^{-1}). The culture was kept in an orbital shaker at 28 °C until the bacterial cell density reached 1.0 at OD600. The bacterial pellet was obtained after centrifugation of *Agrobacterium* culture at 5,000 rpm for 10 min. Different bacterial cell densities were prepared as per requirement by diluting the bacterial pellet using liquid MS with sucrose (3%) and acetosyringone (100 μM). The *Agrobacterium* cell suspension was used for infection of explants.

2.8. Agrobacterium-mediated transformation

The cotyledon explants prepared from the 10–12 d old seedlings of both genotypes were used to optimize the different parameters influencing *Agrobacterium*-mediated transformation. The parameters include pre-culture duration, co-cultivation duration, bacterial cell density, bacterial infection time, and acetosyringone concentration. The cotyledon explants were pre-cultured for various time durations (0 to 4 days) on a shoot induction medium supplemented with 2.0 mg l^{-1} ZEA and 0.1 mg l^{-1} IAA, then infected with *Agrobacterium*. The precultured explants were infected with different bacterial cell densities (0.2 to 1.0 at OD_{600}) and with various infection durations (5 to 30 min). The infected explants were placed onto co-cultivation medium containing (2.0 mg l^{-1} ZEA and 0.1 mg l^{-1} IAA) with various concentrations of acetosyringone (0 to 200 μM). Then infected explants were co-cultivated for diverse time durations (0 to 4 days). The cotyledon explants of both genotypes were subjected to infection using *Agrobacterium* suspension with shaking at regular intervals.

2.9. Regeneration of putatively transformed plants

After optimizing different factors that affect *Agrobacterium* mediated genetic transformation, the cotyledon explants were co-cultivated on medium containing 2.0 mg l^{-1} ZEA and 0.1 mg l^{-1} IAA for two days. The co-cultivated explants were treated with a sterile liquid medium amended with cefotaxime (400 mg l^{-1}) to remove the bacterial cells adhering to the explants. After washing, the explants were blotted on sterile paper and transferred to the MS medium containing 2.0 mg l^{-1} ZEA, 0.1 mg l^{-1} IAA, 400 mg l^{-1} cefotaxime, and hygromycin (10 mg l^{-1}). The explants with shoot buds were transferred onto fresh media amended with hygromycin (20 mg l^{-1}) and cefotaxime (400 mg l^{-1}) for three to four successive subcultures with a 10-day interval. The multiple shoots were inoculated onto shoot elongation medium containing GA_3 (1.0 mg l^{-1}), ZEA (0.5 mg l^{-1}), and IAA (0.05 mg l^{-1}) and supplemented with hygromycin (20 mg l^{-1}) and cefotaxime (250 mg l^{-1}). The cultures were incubated for two to three weeks. The shoots were isolated from the cluster of shoots and transferred onto root induction medium fortified with 0.5 mg l^{-1} IBA and amended with 10 mg l^{-1} hygromycin and 200 mg l^{-1} cefotaxime. After two weeks, the plantlets were shifted into paper cups filled with soilrite™, cocopeat and placed in a culture room for two weeks. The putatively transgenic tomato plants were moved to pots and kept in the greenhouse for acclimatization. Different factors were evaluated to improve the transformation efficiency using cotyledon explants of both genotypes. Then hypocotyl and leaf explants of two genotypes were used to assess the transformation efficiency.

2.10. GUS histochemical assay

The GUS histochemical assay was performed to verify different explants after each treatment using GlucA solution (Jefferson et al., 1987). The chlorophyll pigments were removed from explants by washing with methanol for 2 h. The transformed and non transformed (wild-type) explants were stained using GlucA solution under similar conditions. The putatively transformed plantlets showed blue staining, which was considered positive and non-staining plantlets considered negative.

2.11. Molecular analysis of putatively transgenic plants

Genomic DNA from leaflets of the putatively transformed and wild-type tomato plants of both genotypes (Arka Vikas and PED) was isolated using the CTAB method (Doyle and Doyle, 1990). The genomic DNA of non-transformed plant and plasmid pCAM-

Table 1
Effect of different plant growth regulators on multiple shoot induction from cotyledon explants in two genotypes of tomato (Solanum lycopersicum L.).

Plant growth regulators (mg l^{-1})		Arka Vikas		PED	
BAP		Percentage of response (%)	Mean no. of shoots/explant	Percentage of response (%)	Mean no. of shoots/explant
0.0		00.00	00.00	00.00	00.00
1.0		62.43 ± 0.82^{o}	05.64 ± 0.65^{o}	45.64 ± 0.38^{q}	04.62 ± 0.74 ^{lm}
2.0		88.24 ± 0.78^{ef}	18.78 ± 1.33 ^{fg}	74.64 ± 0.56^{ijk}	10.82 ± 0.65^{gh}
3.0		86.38 ± 0.47 ^{g}	14.52 ± 0.76^{hi}	77.56 ± 0.68^{gh}	10.24 ± 0.82^{gh}
4.0		84.65 ± 0.73^{ij}	10.78 ± 0.64^{jk}	73.29 ± 0.62 ^{k}	07.65 ± 0.58^{jk}
5.0		80.56 ± 0.58^{jk}	10.26 ± 0.48^{jkl}	68.56 ± 0.84 ^{lm}	06.58 ± 0.64^{kl}
TDZ					
0.5		52.63 ± 0.69^{q}	03.26 ± 0.68^{p}	41.83 ± 0.94^{r}	03.16 ± 0.58 ^{m}
1.0		56.72 ± 0.44^{p}	07.48 ± 0.77^{mn}	49.84 ± 0.76^{p}	07.58 ± 0.49^{jk}
1.5		78.36 ± 0.54 ^{l}	09.84 ± 0.74^{kl}	70.64 ± 0.58 ^{lm}	08.32 ± 0.68^{ij}
2.0		82.64 ± 0.86^{j}	14.62 ± 0.56^{hi}	78.38 ± 0.64 ^{fg}	10.36 ± 0.74^{gh}
2.5		80.68 ± 0.81^{jk}	12.26 ± 0.48^{jk}	74.62 ± 0.45^{ijk}	08.66 ± 0.84ij
ZEA					
0.5		68.54 ± 0.72^{n}	08.62 ± 0.74^{klm}	58.33 ± 0.72^{o}	04.82 ± 0.69 ^{l}
1.0		76.35 ± 0.86 ^{m}	15.73 ± 0.82^{hi}	64.48 ± 0.56^{n}	07.92 ± 0.84^{ijk}
1.5		89.66 ± 0.73^{ef}	20.48 ± 1.26^{ef}	74.84 ± 0.68^{ijk}	13.84 ± 0.86^{ef}
2.0		94.42 ± 0.88^{bc}	26.76 ± 1.68^{bc}	81.48 ± 0.71^{def}	18.64 ± 1.24^{c}
2.5		88.23 ± 0.54^{ef}	24.58 ± 1.42 ^{cd}	70.96 ± 0.66 ^{lm}	15.48 ± 0.86^{def}
BAP	**IAA**				
2.0	0.1	93.35 ± 0.68^{bcd}	23.46 ± 1.58^{de}	83.47 ± 0.68^{bc}	15.67 ± 0.84^{de}
2.0	0.2	91.82 ± 0.93 ^{cd}	21.28 ± 1.82^{ef}	81.62 ± 0.59^{def}	14.36 ± 1.02^{ef}
TDZ	**IAA**				
2.0	0.1	86.45 ± 0.81 ^{g}	17.84 ± 1.24 ^{fg}	79.85 ± 0.82^{ef}	13.68 ± 0.86^{ef}
2.0	0.2	83.62 ± 0.76^{ij}	15.66 ± 1.36^{gh}	82.63 ± 0.54^{bc}	11.42 ± 0.92^{gh}
ZEA	**IAA**				
2.0	0.1	95.54 ± 0.76^{a}	36.48 ± 1.87^{a}	86.46 ± 0.65^{a}	24.68 ± 1.58^{a}
2.0	0.2	93.67 ± 0.82^{bcd}	28.65 ± 1.56^{b}	82.84 ± 0.58^{bc}	21.72 ± 1.46^{b}

Mean values with same letter within columns are not significantly different according to Duncan's New Multiple Range Test at 5% level.

Table 2
Effect of different plant growth regulators on multiple shoot induction from hypocotyl explants in two genotypes of tomato (Solanum lycopersicum L.).

Plant growth regulators (mg l^{-1})		Arka Vikas		PED	
BAP		Percentage of response (%)	Mean no. of shoots/explant	Percentage of response (%)	Mean no. of shoots/explant
0.0		00.00	00.00	00.00	00.00
1.0		58.35 ± 0.76^{p}	04.83 ± 0.73^{lm}	48.56 ± 0.42^{m}	02.86 ± 0.74^{mn}
2.0		89.44 ± 0.56^{ef}	16.42 ± 0.68^{de}	80.62 ± 0.82^{de}	10.74 ± 0.82^{fg}
3.0		85.44 ± 0.82^{gh}	13.94 ± 0.84^{fg}	80.38 ± 0.64^{de}	08.92 ± 0.63^{gh}
4.0		82.42 ± 0.92^{ij}	12.58 ± 0.76^{fg}	76.46 ± 0.88^{ef}	06.73 ± 0.82^{ij}
5.0		80.74 ± 0.84^{kl}	11.26 ± 0.66^{gh}	72.82 ± 0.56^{hi}	05.86 ± 0.76^{jk}
TDZ					
0.5		56.46 ± 0.73^{q}	03.68 ± 0.52^{n}	44.63 ± 0.79^{n}	02.38 ± 0.46^{mn}
1.0		63.74 ± 0.82^{o}	05.74 ± 0.64^{kl}	59.72 ± 0.48^{k}	04.65 ± 0.63^{kl}
1.5		82.68 ± 0.87^{kl}	08.82 ± 0.58^{j}	74.62 ± 0.72^{g}	06.54 ± 0.72^{ij}
2.0		86.42 ± 0.92^{gh}	12.45 ± 0.47^{fg}	82.58 ± 0.82^{cd}	08.86 ± 0.56^{gh}
2.5		84.70 ± 0.76^{ij}	10.39 ± 0.68^{i}	72.68 ± 0.58^{hi}	07.45 ± 0.62^{ij}
ZEA					
0.5		66.36 ± 0.62^{n}	05.43 ± 0.68^{kl}	52.78 ± 0.48^{l}	03.68 ± 0.45^{kl}
1.0		74.46 ± 0.82^{m}	11.68 ± 0.82^{gh}	63.86 ± 0.75^{j}	05.49 ± 0.72^{jk}
1.5		86.54 ± 0.86^{gh}	16.52 ± 0.71^{de}	78.62 ± 0.64^{ef}	11.84 ± 0.66^{fg}
2.0		93.28 ± 0.94^{de}	22.67 ± 1.26^{bc}	83.66 ± 0.82^{cd}	15.64 ± 0.74^{bc}
2.5		88.78 ± 0.68^{ef}	18.48 ± 0.58^{de}	72.44 ± 0.72^{hi}	12.56 ± 0.48^{def}
BAP	**IAA**				
2.0	0.1	94.58 ± 0.74^{bc}	19.82 ± 1.08^{cd}	82.86 ± 0.54^{cd}	13.68 ± 1.34^{def}
2.0	0.2	92.42 ± 0.82^{de}	16.36 ± 1.38^{de}	80.48 ± 0.76^{ef}	11.46 ± 1.24^{fg}
TDZ	**IAA**				
2.0	0.1	87.62 ± 0.76^{fg}	13.68 ± 0.83^{f}	83.64 ± 0.86^{cd}	12.24 ± 1.48^{def}
2.0	0.2	84.86 ± 0.82^{ij}	11.24 ± 0.89^{gh}	81.44 ± 0.67^{de}	10.56 ± 1.36^{fg}
ZEA	**IAA**				
2.0	0.1	96.76 ± 0.54^{ab}	28.76 ± 1.64^{a}	85.62 ± 0.74^{ab}	19.44 ± 1.38^{a}
2.0	0.2	94.83 ± 0.78^{ab}	24.82 ± 1.56^{b}	84.53 ± 0.82^{ab}	15.62 ± 1.26^{bc}

Mean values with same letter within columns are not significantly different according to Duncan's New Multiple Range Test at 5% level.

Table 3
Effect of different plant growth regulators on multiple shoot induction from leaf explants in two genotypes of tomato (Solanum lycopersicum L.).

Plant growth regulators (mg l^{-1})		Arka Vikas		PED	
BAP		Percentage of response (%)	Mean no. of shoots/explant	Percentage of response (%)	Mean no. of shoots/explants
0.0		00.00	00.00	00.00	00.00
1.0		53.86 ± 0.64^{pq}	02.62 ± 0.56^{no}	43.64 ± 0.68^{p}	01.94 ± 0.63^{n}
2.0		86.42 ± 0.73^{fg}	13.36 ± 0.72^{gh}	80.72 ± 0.76^{bcd}	08.48 ± 0.74^{ef}
3.0		84.64 ± 0.66^{ghi}	11.58 ± 0.63^{ghi}	77.56 ± 0.52^{ef}	06.64 ± 0.48^{gh}
4.0		82.48 ± 0.84^{ijk}	09.43 ± 0.54^{ij}	72.48 ± 0.66^{ghi}	05.46 ± 0.72^{ij}
5.0		78.54 ± 0.76^{mn}	06.33 ± 0.58^{kl}	68.26 ± 0.81^{kl}	04.78 ± 0.65^{jk}
TDZ					
0.5		54.62 ± 0.83^{pq}	02.43 ± 0.65no	43.82 ± 0.74^{p}	02.08 ± 0.34^{lm}
1.0		65.72 ± 0.58^{n}	04.63 ± 0.68^{mn}	55.64 ± 0.57^{mn}	04.36 ± 0.56^{jk}
1.5		79.66 ± 0.72^{l}	07.46 ± 0.73^{kl}	71.58 ± 0.68^{ijk}	05.76 ± 0.68^{ij}
2.0		83.63 ± 0.64^{ij}	10.76 ± 0.62^{ij}	79.54 ± 0.48^{de}	08.24 ± 0.56^{ef}
2.5		81.52 ± 0.73^{jkl}	09.45 ± 0.56^{ij}	74.41 ± 0.82^{gh}	07.36 ± 0.59^{gh}
ZEA					
0.5		63.76 ± 0.52^{o}	03.68 ± 0.56^{mn}	49.74 ± 0.63^{o}	02.86 ± 0.73^{lm}
1.0		77.63 ± 0.77^{mn}	08.74 ± 0.62^{jk}	57.46 ± 0.58^{mn}	06.68 ± 0.67^{gh}
1.5		83.76 ± 0.65^{fg}	12.62 ± 0.76^{gh}	69.53 ± 0.83^{kl}	09.76 ± 0.58^{ef}
2.0		88.62 ± 0.74^{cde}	17.86 ± 1.34^{cd}	81.54 ± 0.64^{bcd}	14.28 ± 0.86^{bc}
2.5		84.53 ± 0.62^{gh}	14.58 ± 0.85^{ef}	70.78 ± 0.66^{jk}	11.63 ± 0.57^{de}
BAP	**IAA**				
2.0	0.1	90.64 ± 0.72^{cde}	18.82 ± 1.34^{bc}	82.48 ± 0.72^{abc}	13.38 ± 1.32^{cd}
2.0	0.2	88.56 ± 0.64^{ab}	15.43 ± 0.82^{ef}	81.83 ± 0.65^{bcd}	11.62 ± 0.88^{de}
TDZ	**IAA**				
2.0	0.1	84.62 ± 0.58^{gh}	14.38 ± 0.83^{ef}	78.42 ± 0.83^{def}	11.64 ± 0.68^{de}
2.0	0.2	81.48 ± 0.72^{ijk}	11.66 ± 0.76^{gh}	77.54 ± 0.65^{ef}	09.54 ± 0.76^{ef}
ZEA	**IAA**				
2.0	0.1	91.56 ± 0.82^{ab}	23.54 ± 1.48^{a}	83.76 ± 0.67^{abc}	17.64 ± 1.26^{a}
2.0	0.2	88.72 ± 0.68^{cde}	18.32 ± 1.26^{bc}	81.64 ± 0.76^{bcd}	14.86 ± 1.44^{bc}

Mean values with same letter within columns are not significantly different according to Duncan's New Multiple Range Test at 5% level.

BIA1305.1 was served as a negative and positive control, respectively, during the amplification. The polymerase chain reaction (PCR) was performed with specific primer sets of *hptII* (*hptII* FP: 5′-TAGCGAGAGCCTGACCTATT-3′; *hptII* RP: 5′-GATGTTGGCGACC TCGTATT-3′), and *gus*A (*gus* FP: 5′- CCATCGAAGTACCATCCGTTA TAG-3′; *gus* RP: 5′-GAAGAGGGCCTCGGAAAAGT-3′) to check the presense of the *hptII* and *gus*A genes into the putative transformants of tomato. The genomic DNA denaturation was performed for 5 min at 95 °C, followed by 30 cycles of denaturation at 95 °C for 30 sec, annealing at 55 °C (*hptII* and *gus*A) for 1 min, and extension at 72 °C for 2 min, and the final reaction was completed at 72 °C for 10 min. The amplified products were resolved using electrophoresis on 0.8% agarose gel and analyzed with GelDoc (Bio-Rad, USA).

2.12. Southern hybridization

Southern blotting was conducted using genomic DNAs (10 µg) obtained from PCR positive putatively transformed and non-transformed (wild type–WT) tomato plants to authenticate and differentiate the transgenic plants. Genomic DNAs were digested with *EcoRI* for 2 h (Fisher Scientific, Pittsburgh PA, USA) and separated with 1 % (w/v) agarose gel electrophoresis. The electrophoretically separated DNA fragments were blotted onto a nylon membrane (GE Healthcare, UK) as mentioned in the DIG Application Manual (Roche Diagnostics, Germany). The PCR DIG Probe Synthesis kit synthesized the DIG-labeled probe (specific to the *hptII* gene). Hybridization was carried out at 42 °C overnight. The hybridized membrane was washed as described in the manufacturer manual (GE Healthcare, UK). The CDP Star substrate was employed to develop a chemiluminescent blot (GE Healthcare, UK). After chemiluminescent development, it was finally exposed to X-ray film (Kodak, India). The genomic DNA from wild-type tomato plants was served as a negative control.

2.13. Data analysis

The data were recorded for each treatment, and all the experiments were repeated thrice. The recorded data were analyzed and presented as means ± standard error. The significance levels were determined by employing the Duncan's multiple range test (DMRT) at a 5% level using the SPSS version 20.

3. Results

3.1. Effect of cytokinins on multiple shoot induction

An efficient and highly reproducible plant regeneration protocol is essential for the genetic advancement of tomato genotypes through genetic engineering and genome editing methods. Therefore, this investigation was initiated to establish an efficient and reproducible plant regeneration system through multiple shoot bud initiation from three (cotyledon, hypocotyl, and leaf) explants of two tomato genotypes (Arka Vikas and PED) by manipulating suitable concentrations and combinations of PGRs.

The influence of PGRs (BAP, TDZ, and ZEA) was investigated on multiple shoot bud initiation from cotyledon, hypocotyl, and leaf explants of tomato. Different BAP, TDZ, and ZEA concentrations were evaluated to determine the most suitable concentration of PGR to induce shoot buds from all three explants of two tomato genotypes. ZEA (2.0 mg l^{-1}) was recorded as a highly capable concentration of cytokinin for shoot bud initiation in three explants of two genotypes of tomato (Tables 1–3). The three explants were inoculated on different media fortified with diverse PGR concentrations, leading to the shoot bud induction within 10–15 d, depend-

ing on the type of explants. Multiple shoot buds were induced within eight to twelve days of cultures in cotyledon and hypocotyl explants. Both genotypes have induced numerous shoot buds in leaf explants within 15 d of culture. Cotyledon explants cultured on ZEA (2.0 mg l^{-1}) were recorded as the most suitable PGR concentration for producing 26.76 and 18.64 shoots/explant after six weeks of culture in Arka Vikas and PED, respectively (Tables 1–3). Hypocotyl explants induced 22.67 and 15.64 shoots/explant, and leaf explants produced 17.86 and 14.28 shoots/explant on medium amended with 2.0 mg l^{-1} ZEA in Arka Vikas and PED, respectively. Different media amended with either increased or decreased concentration of ZEA declined the number of shoots for all the types of explants in both genotypes tested (Tables 1–3). BAP showed a better response in shoot bud induction for the three explants than the TDZ. BAP (2.0 mg l^{-1}) induced 18.78 shoots/cotyledon explant and 10.82 shoots/hypocotyl explant in Arka Vikas genotype. 16.42 shoots/cotyledon explant and 9.74 shoots/hypocotyl explant PED genotype (Tables 1–3). The leaf explants produced 13.36 and 08.48 shoots/explant in Arka Vikas and PED, respectively, on medium amended with 2.0 mg l^{-1} BAP. Among different concentrations tested, TDZ fortified medium at 2.0 mg l^{-1} was observed as the most favorable concentration to produce the maximum number of shoots within a range of 2.08 to 14.62 shoots/explant in all three explants of both genotypes of tomato. When the concentrations of three cytokinins (ZEA, BAP, and TDZ) were increased, they decreased the shoot number in three explants in two tomato genotypes (Tables 1–3).

3.2. Effect of cytokinins with auxins on multiple shoot induction

Cytokinins and auxins play a significant role in several aspects by influencing growth and developmental progressions in plants. Shoot bud initiation and proliferation were affected by several factors such as genotype, explants type, and PGR concentrations and combinations. The different concentrations of BAP, TDZ, and ZEA, were amended with diverse auxins such as NAA, IBA, and IAA (0.1 and 0.2 mg l^{-1}) to examine their interactive role in the shoot initiation and proliferation of three explants of two tomato genotypes. A low concentration of auxin (0.1 mg l^{-1}) and ZEA (2.0 mg l^{-1}) was highly efficient to induce shoot buds and proliferation of the optimum number of shoots induced from all explants types of both tomato cultivars (Tables 1–3). Among the three explants of both genotypes tested, cotyledon explants were the most efficient explant type than hypocotyl and leaf to produce the maximum number of shoots on all culture media, not taking into account of genotype. The cotyledon explants produced 36.48 hoots (Fig. 1a and d), 28.76 shoots in hypocotyl explants (Fig. 1b and e), and 23.54 shoots in leaf explants of the Arka Vikas genotype (Fig. 1c and f). The PED genotype induces 24.68 shoots per cotyledon, 19.44 shoots per hypocotyl, and 17.64 shoots per leaf explants (Tables 1–3).

3.3. Shoot elongation and rooting

Successful plant regeneration protocol of tomato depends upon the elongation of shoots and subsequent rooting of elongated shoots. The multiple shoot buds initiated in three explants were unable to elongate on the same medium fortified with ZEA (2.0 mg l^{-1}) alone or supplemented with IAA (0.1 mg l^{-1}) and on shoot induction media amended with diverse concentrations and combinations of PGRs after two successive subcultures. All three explants were induced with tiny shoot buds (less than 5 mm in length as observed under stereomicroscope) on different shoot induction media fortified with PGR combinations and concentrations. The shoot buds were transferred onto a shoot elongation medium strengthened with different ZEA, IAA, and Gibberellic acid (GA3) concentrations. The diverse combinations and concentrations have significantly improved the elongation of healthy shoots in three explants of both genotypes of tomato. The MS medium amended with GA_3 (1.0 mg l^{-1}), ZEA (0.5 mg l^{-1}), and IAA (0.05 mg l^{-1}) showed maximum shoot elongation in cotyledon explants with 96.78% response. The hypocotyl explant showed a 92.56% response and leaf explants with 89.74% response with shoot length of 7.6 cm in the Arka Vikas cultivar (Fig. 1g). In the PED cultivar, the cotyledon explants showed 92.74% response. The hypocotyl with 86.42% response and leaf explants with 82.64% response with an average shoot length of 5.6 cm within 15 d of culture after subculture with 7-d interval.

The rooting efficiency was evaluated using proliferated shoots from three explants of both genotypes. The shoots were separated from the shoot bunch then shifted to a rooting medium amended with diverse individual concentrations of three auxins such as NAA, IBA, and IAA (0.1, 0.5, 1.0, and 2.0 mg l^{-1}). The rooting efficiency and the nature of roots depend upon the various types of auxins used in the rooting medium. Among the different three auxins concentrations evaluated, medium fortified with IBA was observed to best auxin (84.64% in PED genotype and 99.62% in Arka Vikas genotype) followed by IAA (84.52% in PED genotype and 86.36% in Arka Vikas genotype) for induction root frequency in both genotypes (Fig. 1h). NAA augmented medium recorded the lowest frequency of root induction in both genotypes of tomato (76.82% in PED genotype and 84.68% in Arka Vikas genotype). The maximum number of roots per shoot was recorded as 22.36 roots in the Arka Vikas genotype, whereas in the PED genotype, shoots produced 16.42 roots on a medium amended with a low concentration of IBA (0.5 mg l^{-1}). The average root length was 14.25 cm in PED and 15.25 cm in Arka Vikas genotype on medium amended with IBA (0.5 mg l^{-1}). The media fortified with IAA or NAA induced fewer roots in both genotypes.

3.4. Hardening and acclimatization

The complete plantlets were obtained after the induction roots to the shoots within two weeks on the rooting medium in both genotypes. The rooted plantlets were moved to paper cups containing soilrite™ and cocopeat for hardening. The hardened plants were successfully established under greenhouse conditions (Fig. 1i). After transplantation, the survival rate was observed as 88% for the Arka Vikas genotype and 85% for the PED genotype after four weeks. The plantlets regenerated from various explant types were phenotypically similar to their mother plants.

3.5. Factors affecting transformation efficiency

3.5.1. The sensitivity of cotyledon explants to hygromycin

The regeneration response was evaluated on different hygromycin concentrations (0 to 25 mg l^{-1}). The cotyledon explants were turned into browning on periodical sub-culture (each sub-culture for 10 d) onto the fresh medium fortified with the same concentration of hygromycin (Fig. 2a). After three successive sub-cultures, the hygromycin at 20 mg l^{-1} of the explants was completely bleached. The increased levels of hygromycin severely decreased the regeneration response and survival of the cotyledon explants.

3.5.2. Effect of pre-culture period

The pre-culture period played an essential role in increasing the transformation efficiency in the cotyledon explants of tomato. The transformation efficiency progressively improved with increased pre-culture duration, and a 2-day pre-culture period was evident to be optimum (Fig. 2b). The cotyledon explants pre-cultured for 2 d increased the maximum transformation frequency in Arka Vikas (36.8%) and PED (23.6%).

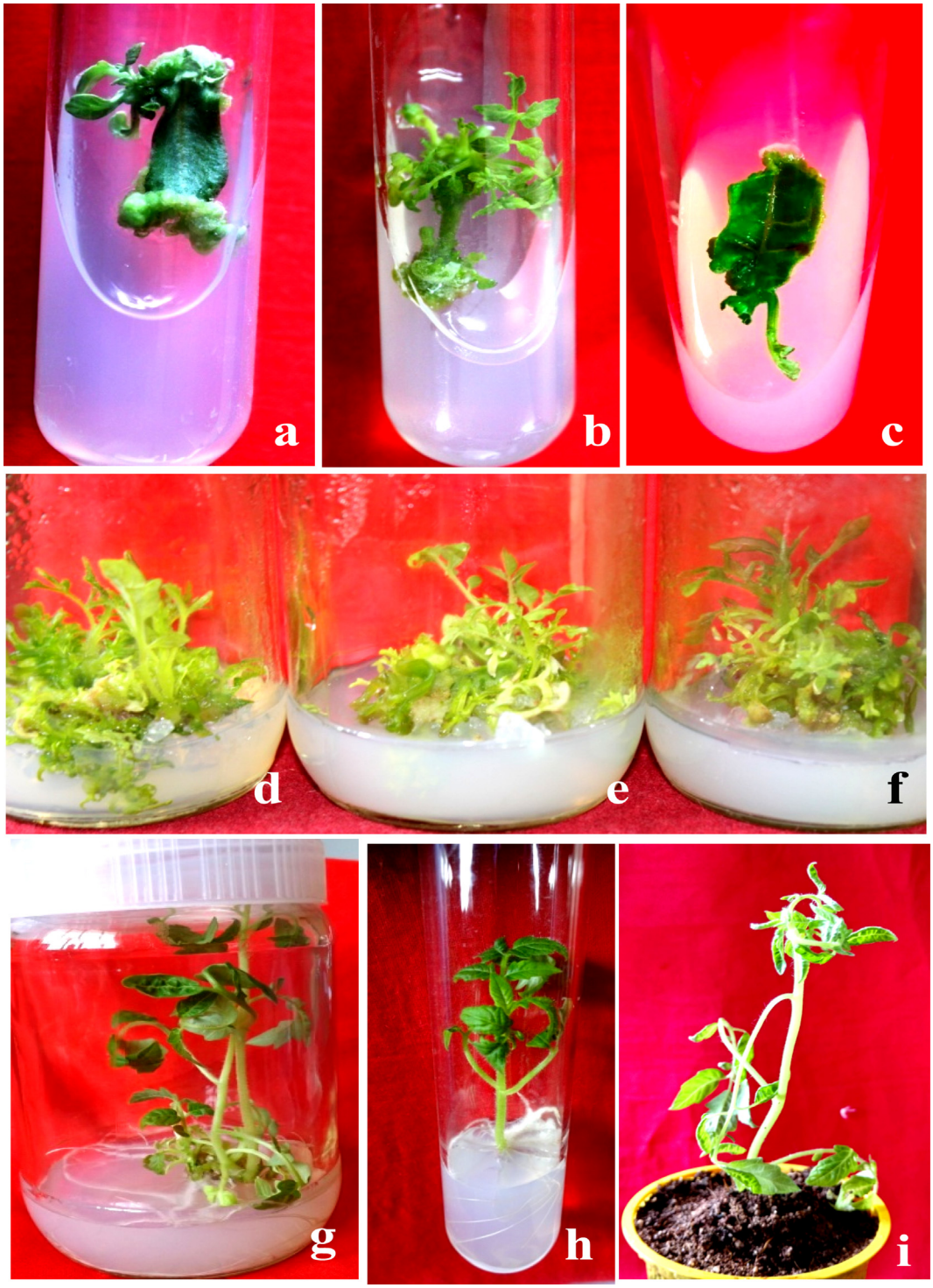

Fig. 1. Regeneration from various explants of tomato cv. Arka Vikas. (a) shoot induction from cotyledon explant, (b) shoot induction from hypocotyl explant, (c) shoot induction from leaf explants on MS medium amended with ZEA (2.0 mg l^{-1}) and IAA (0.1 mg l^{-1}), d) multiple shoot proliferation from cotyledon explant, (e) multiple shoot proliferation from hypocotyl, (f) multiple shoot proliferation from leaf explant, (g) multiple shoots elongated on MS medium fortified with GA_3 (1.0 mg l^{-1}), ZEA (0.5 mg l^{-1}), and IAA(0.05 mg l^{-1}), (h) elongated shoots on MS medium supplemented with IBA (0.5 mg l^{-1}), and (i) acclimatized plantlet under greenhouse conditions.

3.5.3. Effect of bacterial cell density

The histochemical assay was performed by co-cultivated explants infected with different bacterial cell densities. The histochemical *gus* assay revealed that the transformation efficiency considerably improved with increased bacterial cell density up to 0.6 OD and decreased noticeably after that (Fig. 2c).

3.5.4. Effect of the bacterial infection period

The cotyledon explants were infected in an *Agrobacterium* suspension (OD_{600} = 0.6) for various time intervals (5 to 30 min). The cotyledon explants were infected with *Agrobacterium* suspension at 0.6 OD significantly improved transformation efficiency compared to those infected for various time durations (Fig. 2d).

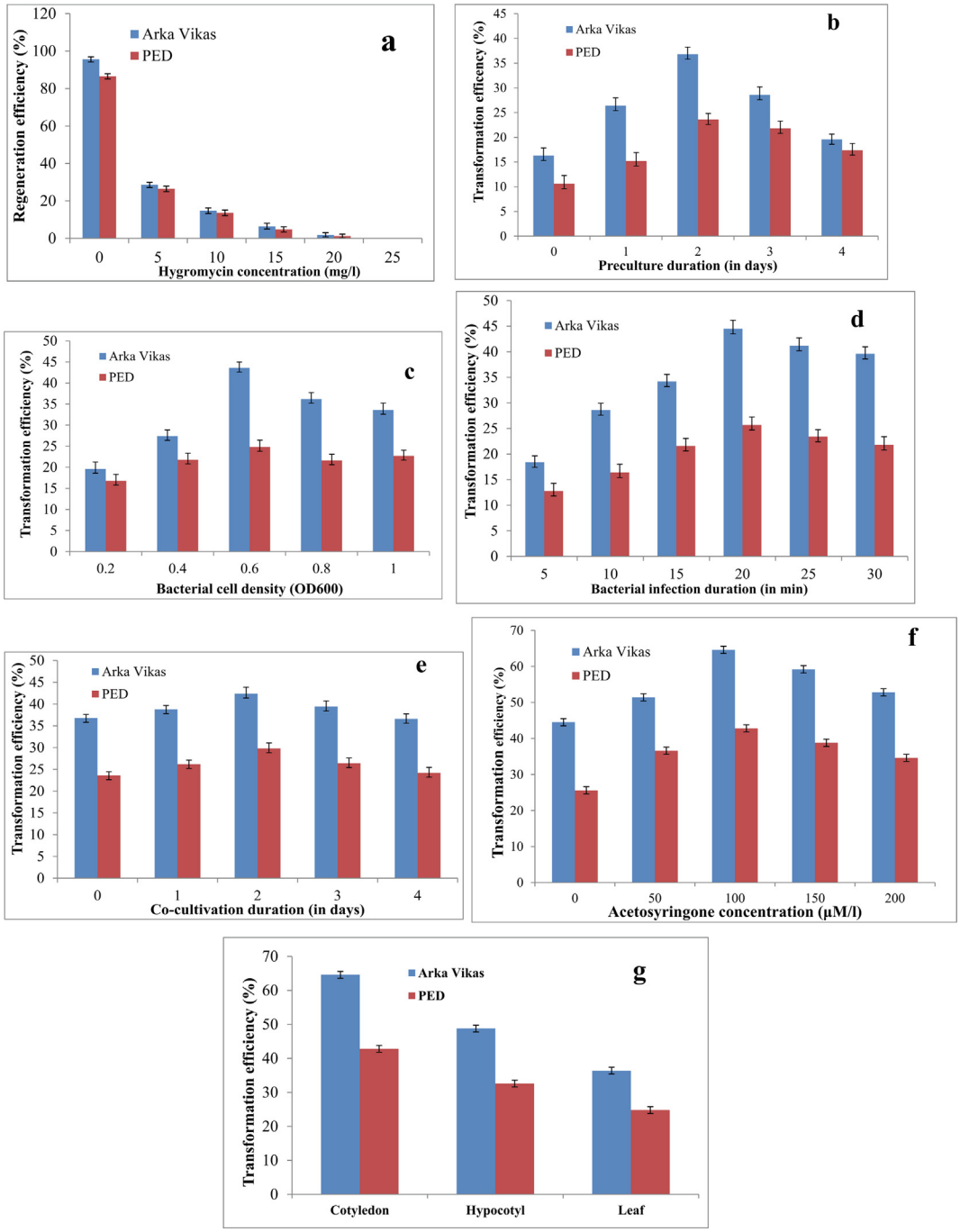

Fig. 2. Effect of different parameters on transformation efficiency (%) in cotyledon explants of tomato genotypes Arka Vikas and PED. (a) The sensitivity of cotyledon explants of tomato cvs. Arka Vikas and PED on MS medium containing 2.0 mg l^{-1}ZEA and 0.1 mg l^{-1} IAA with various concentrations of hygromycin after 4 weeks of culture, (b) effect of pre-culture duration (in days), (c) effect of bacterial density (OD at 600 nm), (d) effect of bacterial infection duration (in minutes), (e) effect of co-cultivation duration (in days), (f) effect of acetosyringone concentration (in µM), and (g) effect of explant type. Each experiment was repeated three times with thirty explants. The bar represents the mean ± standard error.

3.5.5. Effect of co-cultivation period

The co-cultivation duration significantly influences the transformation efficiency in cotyledon explants of two tomato genotypes. The cotyledon explants co-cultivated for 2-days enhanced the transformation efficiency considerably.

3.5.6. Effect of acetosyringone concentration

The different concentrations of acetosyringone (0 to 200 µM) were employed to evaluate the transformation efficiency using cotyledon explants of two genotypes of tomato. The infected explants were co-cultivated for 2 days. Among various concentrations examined, acetosyringone concentration at 100 µM observed the maximum transformation efficiency in both tomato genotypes (Fig. 2f). The overall transformation efficiency was recorded with 64.6% in Arka Vikas and 42.8% in PED genotypes.

3.5.7. Effect of explants

The present study showed considerable differences in the transformation efficiencies within the explants. The cotyledon explants

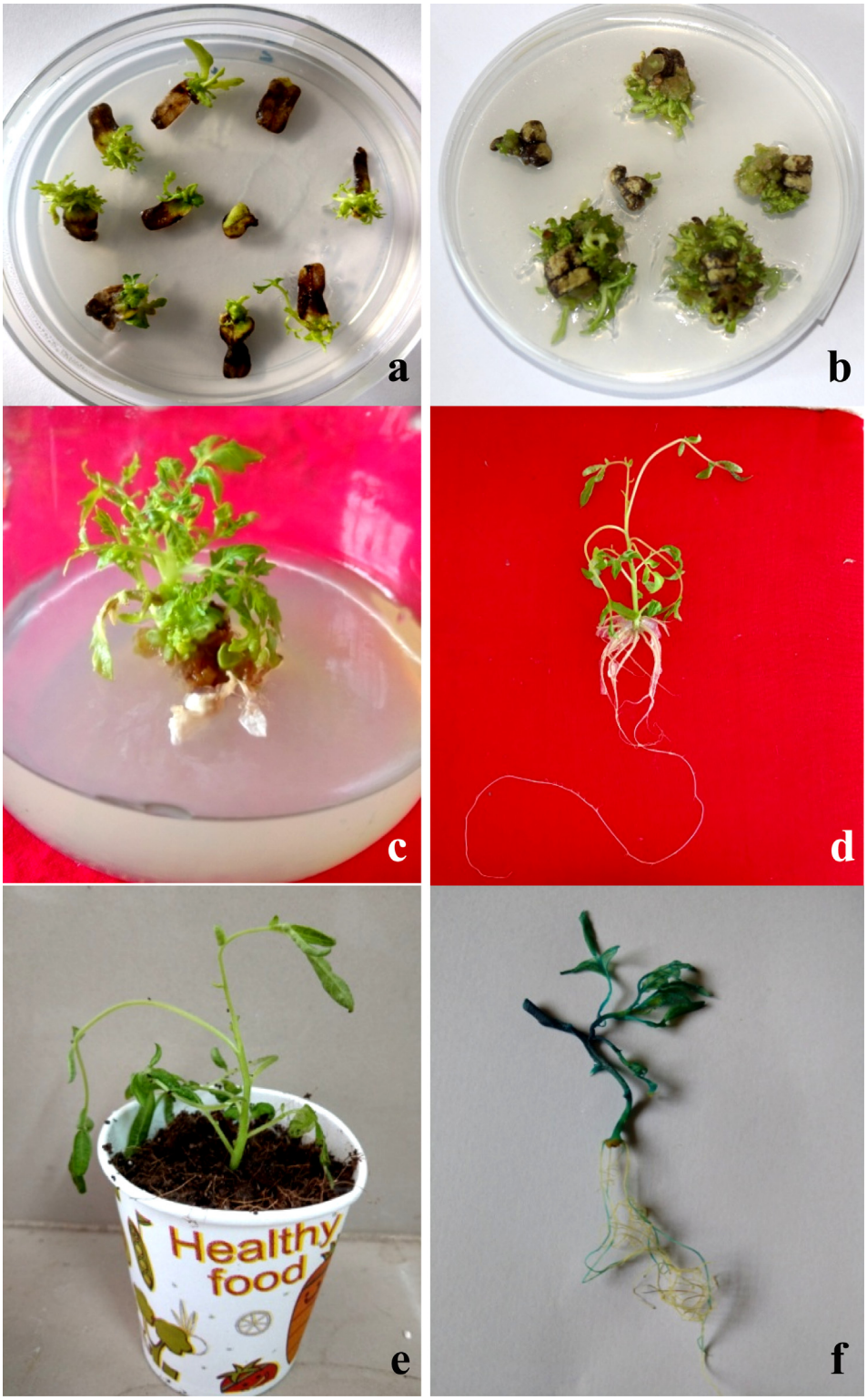

Fig. 3. *Agrobacterium*-mediated genetic transformation of cotyledon explants tomato cv. Arka Vikas with LBA4404 strain harbouring pCAMBIA 1305.1. (a) Shoot bud initiation from cotyledon explants on MS medium supplemented with 2.0 mg l^{-1} ZEA and 0.1 mg l^{-1} IAA, cefotaxime (400 mg l^{-1}) and hygromycin (10 mg l^{-1}) after two weeks, (b) multiple shoot buds proliferation from cotyledon explant after four weeks on shoot induction medium fortified with cefotaxime (400 mg l^{-1}) and hygromycin (20 mg l^{-1}), (c) elongation of putative transgenic shoots on shoot elongation medium supplemented with GA3 (1.0 mg l^{-1}), ZEA (0.5 mg l^{-1}), IAA (0.05 mg l^{-1}), cefotaxime (250 mg l^{-1}) and hygromycin (10 mg l^{-1}), (d) root induction from the shoots on rooting medium amended with IBA (0.5 mg l^{-1}), cefotaxime (200 mg l^{-1}) and hygromycin (10 mg l^{-1}), (e) hardening of the putatively transformed plant in a paper cup, and (f) stable expression of *gus* gene in the transformed plant after eight weeks.

of both genotypes were found to be more suitable for explant tissue than hypocotyl and leaf explants (Fig. 2g).

3.5.8. Regeneration of putative transgenic plants

The co-cultivated explants were cultured on shoot induction medium amended with ZEA (2.0 mg l^{-1}), IAA (0.1 mg l^{-1}), cefotaxime (400 mg l^{-1}), and hygromycin (10 mg l^{-1}) for 14 d to induce putatively transformed shoot buds from the cut ends of the explants (Fig. 3a). The explants containing multiple shoot buds were subcultured onto 20 mg l^{-1} hygromycin for two to three times to eliminate non-transformed shoots with 10 to 14 d intervals. The repeated subculturing facilitated the production of stable transformed shoots. The non-transformed (hygromycin-sensitive) cotyledon explants were completely bleached. It was observed that the increased hygromycin concentrations (10 to 20 mg l^{-1}) were helpful in the efficient selection of transformants from non-transformed cells (Fig. 3b). The hygromycin induced the rapid death of non-transformed cells (sensitive cells) and improved the induction and proliferation of healthy shoot buds resistant to hygromycin. The cotyledon explants with several tiny putatively transformed shoots were subcultured on shoot elongation medium fortified with 10 mg l^{-1} hygromycin, 250 mg l^{-1} cefotaxime, ZEA (1.0 mg l^{-1}), IAA (0.05 mg l^{-1}), and GA_3 (1.0 mg l^{-1}) (Fig. 3c). The putatively transformed shoots were shifted to root induction medium fortified with IBA 0.5 mg l^{-1}, 10 mg l^{-1} hygromycin, and 200 mg l^{-1} cefotaxime to obtain complete plantlets (Fig. 3d). The

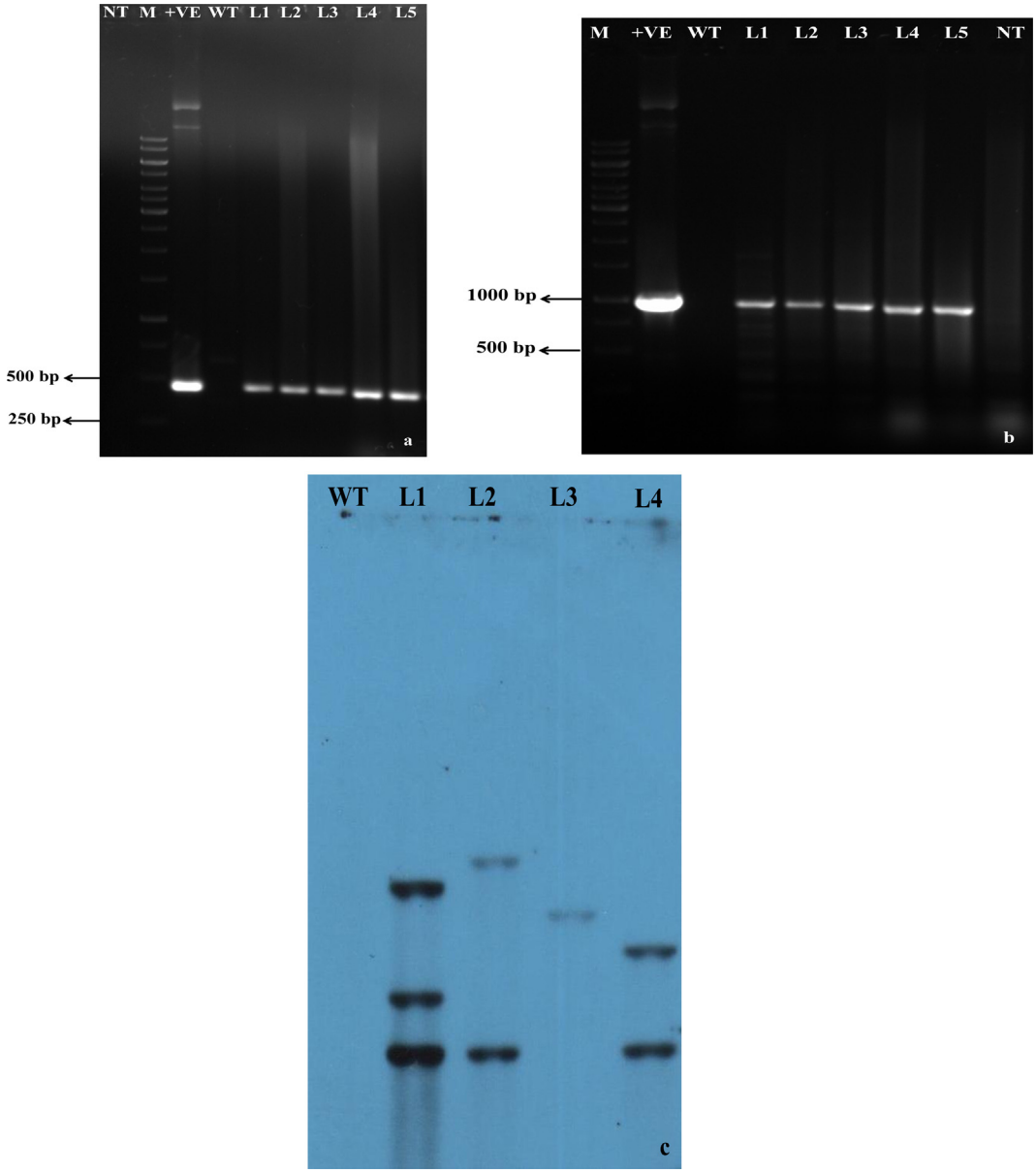

Fig. 4. Molecular confirmation of putatively transformed plants of tomato cv. Arka Vikas. (a) PCR analysis of putatively transformed tomato plants using *hptII* gene, Lane NT No template, Lane M DNA ladder (1 Kb), Lane + ve Plasmid DNA, Lane WT DNA sample from a non-transformed plant, L1–L5 DNA sample from putatively transformed plants. (b) PCR analysis of putatively transformed tomato plants using *gus* A gene, Lane M DNA ladder (1 Kb); Lane + ve Plasmid DNA, Lane WT DNA sample from the non-transformed plant, L1–L5 DNA sample from putatively transformed plants, Lane NT No template. (c) Southern blot hybridization of PCR-positive putatively transformed tomato plants using *hptII* gene. WT (Wild type) non-transformed tomato plant genomic DNA as a negative control, L1 – L4, genomic DNA from putatively transformed tomato plants.

putatively transformed plants were shifted to paper cups for acclimatization (Fig. 3e). The putatively transformed plants were flowered normally.

3.5.9. Molecular analysis of putatively transformed plants

After successfully establishing putatively transformed plantlets, the expression of blue colour in the plant exhibited the stable integration of the gusA gene (Fig. 3f). The non-transformed (wild type) plantlets do not show any blue colour, which validates the absence of *gus*A. The PCR technique validated the presence of transgene in the putatively transformed plants of the Arka Vikas genotype. The amplified fragments of 448 bp (Fig. 4a, L1–L5) of the *hptII* gene and 995 bp (Fig. 4b, L1–L5) of the *gus*A gene have validated the presence of transgenes in the genomic DNA of putatively transformed plants. In contrast, the control plant genomic DNA did not show any amplification (Fig. 4a and b, lanes WT-wild type). The Southern blotting validated transgene integration and its copy number in the genome of transformed plants. The *hptII* gene integration was observed as three copies (lane 1), two copies (lanes 2 and 4), and a single copy (lane 3) in the genomes of putatively transformed plants (Fig. 4c). In contrast, the non-transformed (wild type) plants genome does not indicate a hybridization and serves as a negative control (Fig. 4c, lane 1 wt).

4. Discussion

4.1. Effect of cytokinins on multiple shoot induction

A highly efficient and repeatable plant regeneration protocol is a prerequisite for genetic modification of tomato genotypes that could improve tomato genotypes with the help of different biotechnological methods, including the development of new genome editing techniques (Reem and Van Eck, 2019; Van Eck, 2020). Cytokinins in plants stimulate cell division, bud initiation, and proliferation (Van Staden et al., 2008). *In vitro* regeneration of tomato has been significantly affected by the explants, genotypes, and PGR concentrations and combinations used in the culture medium (Alatar et al., 2017; Prihatna et al., 2019; Saeed et al., 2019; Vinoth et al., 2019).

The results confirmed that ZEA was a superior cytokinin concerning multiple shoot induction among three plant growth regulators tested. Successful regeneration procedures have been reported using different explant types of many tomato genotypes on medium augmented with ZEA (Gupta and Van Eck, 2016; Saeed et al., 2019). In shoot induction medium containing ZEA, all the explants generated the optimum number of shoots (from 2.86 to 26.76 shoots) after two successive subcultures (Tables 1–3). Among different concentrations of ZEA, ZEA at 2.0 mg l^{-1} was a suitable concentration for the initiation of the maximum number of shoots from three explants of both genotypes; our observations confirm with the previous reports on the successful application of ZEA to induce efficient shoot regeneration in tomato (Bamishaiye et al., 2017). The shoot bud initiation was observed in two genotypes when cultured on the concentration of cytokinins. Still, the response percentage differed with diverse PGRs (BAP, TDZ, and ZEA) tested. The number of explants that responded to multiple shoot production increased with increasing concentrations of cytokinins (Tables 1–3). The ZEA was superior to BAP and TDZ in numerous shoot buds initiation and proliferation from different explant types of various genotypes of tomato. Our observations confirm that ZEA was the most suitable cytokinin than BAP and TDZ for shoot bud initiation and proliferation from various explants of tomato (Koul et al., 2014; Rajesh et al., 2016).

4.2. Effect of cytokinins with auxins on multiple shoot induction

Auxins and cytokinins interactions can play critical morphological processes during the in vitro multiplication stage. High concentrations of cytokinins and low auxins stimulate shoot buds, proliferation, and enhancement of shoot production (Gupta and Rashotte, 2012; Phillips and Garda, 2019). The media supplemented with ZEA and IAA was the most efficient cytokinin and auxin combination for shoot bud initiation and proliferation and plant regeneration in several genotypes of tomato (Senapati, 2016; Gupta and Van Eck, 2016; Saeed et al., 2019; Lee et al., 2020).

In contrast, the PED genotype showed fewer shoots in three explants (Tables 1–3). Different explants of several tomato genotypes induced multiple shoots on media amended with IAA and BAP (Koul et al., 2014; Sun et al., 2015; El-Shafey et al., 2017; Timerbaev et al., 2019), BAP and IBA (Alatar et al., 2017), and BAP and NAA (Kumar et al., 2017). Similarly, the medium augmented with TDZ (2.0 mg l^{-1}) and IAA (0.1 mg l^{-1}) did not improve the multiple shoots in cotyledon, hypocotyl, and leaf explants types of two genotypes of tomato (Tables 1–3). Successful plant regeneration systems have been reported from different explant types of several genotypes of tomato on medium augmented with TDZ and auxin (IAA/IBA/NAA) (Ashakiran et al., 2011; Vinoth et al., 2019). Plant growth and developmental processes are influenced by cytokinins and auxins (Gupta and Rashotte, 2012; Gupta and Van Eck, 2016; Phillips and Garda, 2019). The interactions between auxins and cytokinins are essential for developing shoot apex (Tran et al., 2021). ZEA supplemented with auxins (IAA/IBA/NAA) was found to be superior over other cytokinins (BAP/TDZ) and auxins combinations for shoot initiation, proliferation, and plant regeneration in several genotypes of tomato (Saeed et al., 2019). The addition of either IBA or NAA combined with ZEA/BAP/TDZ, these combinations did not show any significant improvement in shoot bud induction and plant regeneration in three explants and two genotypes of tomato.

4.3. Shoot elongation and rooting

The presence of cytokinin alone or amended with auxin and/or GA_3 in the shoot elongated medium plays a significant role in elongating normal and healthy shoots. The addition of various cytokinin concentrations significantly influenced shoot elongation by affecting cell division and cell expansion. Low levels of such as ZEA (Bamishaiye et al., 2017; Vinoth et al., 2019), BAP (Rashid and Bal, 2010), and 2iP (Vinoth et al., 2012) significantly increased shoot elongation from the bunch of shoot buds induced from various explants of tomato. Successful shoot elongation was observed in several explants types with numerous shoot buds of tomato on medium amended with different concentrations of GA_3 alone (Koul et al., 2014) or GA_3 in combination with BAP (Mamidala and Nanna, 2011), GA_3, and KIN (Banu et al., 2017), GA_3, and ZEA (Godishala et al., 2012). Banu et al. (2017) observed enhanced shoot elongation on medium fortified with BAP (1.0 mg 1^{-1}), KIN (0.5 mg 1^{-1}), IAA (0.5 mg l^{-1}) and GA_3 (0.1 mg l^{-1}) in different genotypes of tomato. Vinoth et al. (2019) reported the successful shoot elongation on medium amended with 2iP, GA_3, and 30% algal (*Gracilaria edulis*) extract in shoot buds obtained from leaf explants of tomato. Successful shoot elongation was achieved from explants with multiple shoots on shoot elongation medium amended with ZEA, IAA, and GA_3.

The Arka Vikas genotype gave the best response to rooting compared to the PED genotype. Auxins are known to play a significant role in plant growth and developmental processes, especially in root apical meristem development (Gupta and Van Eck, 2016). Among the different auxins evaluated for root induction, IBA was observed as the most suitable auxin to increase the percentage of

rooting and number of roots per shoot than IAA and NAA. Rooting was induced when shoots derived from different explants were transferred to rooting medium augmented with various concentrations of IBA. IBA has been reported as the most suitable auxin for rooting of shoots of tomato (Koul et al., 2014; Vinoth et al., 2012; 2019). IAA was an efficient auxin in inducing roots in different genotypes of tomato (Sivankalyani et al., 2014).

4.4. Factors affecting transformation efficiency

Among various concentrations of hygromycin, 20 mg l^{-1} was observed as the most suitable concentration for the successful recovery of transformants of tomato (Koul et al., 2014; Li et al., 2013). Diverse hygromycin concentrations have been successfully applied as a selection agent to recover transgenic plants in tomato (Koul et al., 2014; Prihatna et al., 2019; Gadir et al., 2018). Pre-culturing of explants was successfully enhanced the transformation efficiency in different explants of several tomato genotypes (Koul et al., 2014; Shah et al., 2016; Gadir et al., 2018; Stavridou et al., 2019). Bacterial cell density was recorded as one of the most critical factors in improving tomato transformation efficiency (Koul et al., 2014; Gadir et al., 2018). The infection time for 20 min was recorded maximum transformation efficiency in cotyledon explants, in contrast, more than 20 min was recorded to decrease transformation efficiency. Our observations are consistent with earlier reports in tomato (Arshad et al., 2014; Koul et al., 2014; Gadir et al., 2018). Explants co-cultivated for two days recorded maximum transformation efficiency as 42.4% in Arka Vikas and 29.8% in PED in cotyledon explants (Fig. 2e). Our results agree with previous observations in tomato (Koul et al., 2014; Sun et al., 2015; Gadir et al., 2018 Stavridou et al., 2019). Acetosyringone, a natural plant phenolic compound released during plant wounding and it stimulate bacterial attachment and viral genes transcription (Atkinson and Gardner, 1991; Gelvin, 2003). Our results suggest that 100 μM acetosyringone amended in the co-cultivation medium greatly improved the efficiency of transformation, which is essential for the transformation of tomato (Arshad et al., 2014; Koul et al., 2014; Gadir et al., 2018).

Transgenic tomato plants were successfully produced from various explants like cotyledon, hypocotyl, and leaf explants using *Agrobacterium*-mediated genetic transformation (Koul et al., 2014; Sun et al., 2015; Gadir et al., 2018). Various transformation efficiencies and transgenic plants recovery were observed from different tomato explants and genotypes (Koul et al., 2014; Gadir et al., 2018; Prihatna et al., 2019).

5. Conclusions

The present study reports an efficient and repeatable regeneration protocol from cotyledon, hypocotyl, and leaf explants of tomato cultivars Arka Vikas and PED. Among three different explants evaluated, cotyledons were more pronounced than hypocotyl and leaf explants of both cultivars of tomato. Furthermore, we have optimized various factors for an efficient *Agrobacterium*-mediated transformation procedure using cotyledon explants of two tomato cultivars. The established genetic transformation protocol will help to introduce novel agronomic traits and apply precise genome editing techniques like CRISPR/Cas to improve tomato genotypes.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author Contribution

DS and PJ performed experiments and wrote the manuscript. AKV and PS participated in the experiments. VP participated in data analysis and revision of the manuscript. VRA and SA designed the experiments and supervised them. All authors read and approved the final manuscript.

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