

Optimization for *Agrobacterium*-Mediated Transformation of Reporter Gene in Tomato

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ABSTRACT: In tropical and subtropical region tomato (*Solanum lycopersicum*) has economical vegetable crop and extensively cultivated. The current study purpose is to optimize the conditions for *in vitro* germination of various tomato seeds cultivars and develop an efficient *Agrobacterium*-mediated transformation procedure using the GUS reporter gene. Half-strength MS medium supplemented with 2 mg/L NAA and 2 mg/L BAP were used to culture seeds, which shows reduced time required for germination. Four different cultivars of tomato using hypocotyl and cotyledonary leaf as explants and subjected to transformation using *Agrobacterium tumefaciens* strain EHA101 with vector pZY102 holding the GUS gene. For infection bacterial culture with OD₆₆₀ of 0.3–0.4 was used with acetosyringone for 15 minutes. Co cultivation of explants was done at 22°C in the dark for 2 days, after pre selection of 7 days followed by histochemical GUS assay at 37°C. The results of present study showed that optimized conditions for germination significantly increased seed emergence and both explant types showed successful transformation with difference among cultivars. The optimized infection conditions and presence of acetosyringone enhanced T-DNA delivery and expression of GUS. Higher efficiency of transformation was observed under controlled infection parameter and co-cultivation. This study reveals that type of explant and conditions for culture plays an important role in improving transformation efficiency in tomato. It is concluded that a protocol which is reliable and efficient for *Agrobacterium*-mediated transformation has been developed; still, to achieve higher rate of transformation and stable expression of gene continuous optimization is required.

Key words: *Agrobacterium*, Acetosyringone, Transfer DNA, Betaglucuronidase, Optical Density

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INTRODUCTION

Solanum lycopersicum (tomato) as a recalcitrant crop for transformation is used for improving dicotyledonous plants using genetic modelling (Prihatna et al., 2019). This transformation has time constraints with frequency ranging 6.2 - 10.4% which depends on species genotype, regeneration protocol and transformation procedure (Ellul et al., 2003). Similar protocol has been used to change in genotype which has reduced the efficiency upto 1.8% with partial success rate (Cortina and Culianez-Macia, 2004). The major hindrance in obtaining the transgenic tomato is the lack of vastly effective transformation method. Numerous factors are involved in transformation of tomato such as plant genotype, explants type, *agrobacterium* strain, its density addition of phenolic compounds and infection and co-cultivation duration (Kapoor 2020). Literature has reported to reveal the influence of high transformation efficiency for tomato leaf disks which are a good explant (Park et al., 2011).

For biotechnology tool tomatoes breeding programs can be highly beneficial, like the technology of gene transfer, which allows the integration of foreign genes in the plant's chromosome, without changing

genetic basis of varieties. Though, an efficient *in vitro* plant regeneration system is directly depended on a breeding program linked to biotechnological tools. Gerszberg, et al. (2015) has reported tomato regeneration (*In vitro*) using protocols in which cotyledons were used for regeneration of adventitious shoot. It is based on the three culture phases first one is the initiation of bud, second one (Compton and Veillux (1991) explant was cultured in a medium supplemented with cytokinin; (Dong and Jia, (1991) third is the elongation phase when plant was transferred to the media containing less amount of cytokinin; and at last rooting phase when auxin was added in the media.

For genetic transformation most important step is the regeneration of the plant through organogenesis or somatic embryogenesis to multiply the genetically identical clones. The important limiting factor to achieve transgenic plant is the frequency of the frequency of transformation. On the other hand, a dependable protocol of regeneration for plant used for transformation is also important parameter. So, prior to transformation, an optimized protocol for given plant regeneration and type of explant is needed (Santos et al., 2020). Regeneration of tomato has been formerly reported through organogenesis

in numerous articles by using different type of explants, like, cotyledon, and leaf (Rai et al., 2020).

There is a great need to optimize conditions for successful transformation of the local cultivars. Keeping in view the importance of tomato as a crop as well as a model plant, present study deals with the optimization of various conditions for transformation of local tomato cultivars. Thus, a variety of tomato has been optimized considering factors such as time of co-cultivation, incubation period and concentrations for transformed shoot regeneration (Park *et al.*, 2011).

During the early 2010s, research efforts primarily focused on refining *Agrobacterium tumefaciens*-mediated transformation protocols for different tomato cultivars. Cotyledonary leaves and hypocotyl segments were the most commonly used explants due to their high regeneration potential (Van Eck et al., 2006; Rai et al., 2012).

Optimization of different factor like time of infection, optical density (OD600), time of co-cultivation, and selection of antibiotic has been done during this period. After co cultivation and regeneration of stable shoots to check transient gene expression histochemical GUS assays were regularly employed. Rai et al. (2012) has optimized the duration of pre-culture and conditions of co-cultivation which significantly enhanced the number of GUS-positive explants in tomato. Likewise, Park et al. (2011) reported that vigilant adjustment of kanamycin concentration enhanced regaining of stable GUS-expressing transformants. Therefore, it is confirmed that the GUS staining provided quick visual approval of positive T-DNA transfer earlier molecular validation.

Among 2014 and 2017, research extended to assess genotype-specific transformation efficiency and hormonal optimization for shoot regeneration. Tomato has a large number of cultivars and transformation efficiency is different significantly so its protocol customization is needed (Kaur et al., 2015).

During this period binary vectors such as pBI121, which have both selectable marker gene npt II and GUS, were extensively utilized. GUS histochemical assays reliably revealed blue stain in transformed tissues, confirmative expression of the introduced gene construct. Further studies involve PCR, Southern blotting after GUS expression to check stable genomic integration (Sun et al., 2014).

Afterward 2018 to onward researches was done to improve efficiency of transformation and reproductivity and for the confirmation advanced molecular techniques were used. Dan et al. (2018) reported the better transformation frequency by optimizing the concentration of acetosyringone and conditions of co-cultivation. After histochemical GUS assays positively, stained explants were increased as compared to the earlier protocols.

Recent studies combined Southern blot analysis and quantitative PCR (qPCR) with GUS assays to confirm stable integration and copy number (Yarra and Kirti, 2019). In some of the cultivars transformation efficiencies were improved up to 40% by optimized infection condition and regeneration media (Ali et al., 2020).

By 2021–2022, CRISPR/Cas-mediated gene editing systems with GUS assays were frequently used confirm transformation former to mutation analysis (Brooks et al., 2014; Vu et al., 2020). GUS is highly recommended and valuable because of its less cost even though fluorescent markers are progressively used, especially in developing countries.

There is a great need to optimize conditions for successful transformation of the local cultivars. Keeping in view the importance of tomato as a crop as well as a model plant, present study deals with the optimization of various conditions for transformation of local tomato cultivars. Hence various transformation factors like incubation period, time of co-cultivation, concentrations for acetosyringone, bacteria; density, vacuum filtration transformed shoot regeneration have been optimized in different varieties of tomato.

MATERIALS AND METHODS

Plant Material: Different cultivars of seeds of tomato (*Solanum lycopersicum* L.) cultivar Rio Grade, Roma, Raja, Anokhi were obtained from Punjab seed Corporation. Uniform and healthy seeds were carefully chosen for in vitro germination and following transformation experiments.

Seed Sterilization and In Vitro Germination: Under flowing water, the tomato seeds were sterilized by washing for 10–15 minutes, after that 70% (v/v) ethanol were added for half a minute. Then seeds were soaked for 10–15 minutes with 1–2% sodium hypochlorite solution with few drops of Tween-20. Further, seed were rinsed with distilled water to avoid any contamination.

Treated seeds were further inoculated using test tubes which contains full strength MS medium with 5.76 pH at $25 \pm 1^\circ\text{C}$ and 70% of the relative humidity under 16 hours photoperiod at $50 \mu\text{mol m}^{-2} \text{s}^{-1}$. The seeds were placed at 25°C and 20°C during day and night respectively. Cotyledonary leaves excised from 3-week-old seedlings (cut into 2-3 transverse segments) were used as explants (Brooks et al. 2014).

Preparation of Explants: The seeds were placed at 25°C and 20°C during day and night respectively. Cotyledonary leaves excised from 3-week-old seedlings (cut into 2-3 transverse segments) were used as explants.

Before infection the explants were carefully pre-cultured on MS medium having with appropriate concentrations of plant growth regulators for 24–48 hours.

Bacterial Strain and Plasmid Vector: The glycerol stock of *Agrobacterium* with standard binary vector Pzy102 containing the β -glucuronidase (GUS) transformed in *A. tumefaciens* strains EHA101 was streaked out onto an LB agar plate (Sambrook et al.,1989) containing appropriate antibiotics

Agrobacterium Culture Preparation: *Agrobacterium tumefaciens* was inoculated into LB liquid medium containing selective antibiotics and incubated at 28°C. The bacterial culture was centrifuged (180–200 rpm) and resuspended in liquid MS medium to obtain an optical density (OD 600) of 0.5–0.8. Acetosyringone (100–200 μ M) was added to enhance vir gene activation.

Agrobacterium-Mediated Transformation: The cotyledonary leaves were excised and infected with the suspension of *Agrobacterium* in VIM for ~15-20 min. Infected explant was inoculated in plates which contain Medium 1 plus 200mg/L Acetosyringone (AS) and placed in dark with a climatic condition of 20°C for 2 days [MS+BAP 1mg/L + NAA 0.1 mg/L + AS 20~60mg/L]. For incubation of 2 days, explants were washed using MS media plus cefotaxime 200mg/ml and then shifted to Media 2 [MS + IAA 0.1 mg/L+ Zeatin 2mg/L + kanamycin 100~150mg/L +Clavamox 250mg/L] and afterward cultured at room temperature with a photoperiod of 16-hr. Explants were shifted to a fresh Medium 3 with and kanamycin and Clavamox after every 2-3 weeks. leaf discs and hypocotyls which have been selected from selection media were subjected to β -glucuronidase assay.

Shoot Regeneration and Rooting: Emerging shoots were expunged and moved to shoot extension medium with reduced concentrations of growth regulators followed by transferring well-developed shoots to rooting medium (MS supplemented with auxins such as IBA or NAA). Rooted plantlets were acclimatized gradually by transferring them to soil-containing pot dishes under controlled greenhouse conditions.

GUS Histochemical Assay: Leaf disk of treated tomato was dipped in the phosphate buffer (Triton X-100.50 mM NaPO₃, pH 6.8 with 0.1 % (v/v)) incubated at 37°C for

45 min. while on the other hand, 5-Bromo-4--chloro- 3-indoyl β -D-glucuronide sodium i.e X-gluc 3.0 mg was dissolved in 150 μ L of the dimethyl formamide. A solution B which contains 5.0 mM potassium ferrocyanide, 5.0 mM potassium ferricyanide, 0.1% (v/v) Triton X-100 and 0.1 M sodium phosphate, eight hundred and fifty μ L was supplemented in to 150 μ L the X-gluc solution. To the leav disks Phosphate buffer was added and incubated for one hour and then buffer was discarded. Leaves disks were dipped into the solution B with Xgluc. Overnight incubation was given for overnight at room temperature. Later they were dipped in destaining solution containing ethanol 50% (v/v), formaldehyde 10% (v/v) and glacial acetic acid in a ratio of 5% (v/v) (Zheng *et al.*, 1993).

Experimental plan and Statistical Analysis: After inoculation the explant was observed every day and the data was documented relating to the contamination, transformation efficiency and frequency of regenerated plants. ANOVA was used to analyze GUS expression. Means were compared using LSD test at 5% probability level.

RESULTS AND DISCUSSION

Effect of Sodium Hypochlorite on Sterilization and Germination of Seeds: For the surface sterilization of tomato seeds different concentrations (3–30%) of sodium hypochlorite (NaOCl) were analyzed. Percentage of germinated seeds and contaminated seeds were varied among treatments and cultivars (Table 1).

Lowest contamination and shows highest germination rate were 8% NaOCl recorded at with lowest contamination and at the same time lowest concentration results in contamination and higher concentration reduced the germination rate of the seeds due to phytotoxic effects. In different cultivars, *Rio Grande* have better sterilization tolerance as compared to *Raja* (Figure a,b,c and d).

In higher concentration of NaOCl rate of germination reduced due to oxidative damage to the embryonic tissue. However Similar tendency was observed in tomato and other solanaceous crops showing normal moderate NaOCl concentrations enhanced aseptic germination without upsetting viability of seed.

Table 1. Effect of seed sterilization on percentage of seed germination in all four cultivars

Genotype	Non-germinated	Germinated		Germination %
		Contaminated	Non-Contaminated	
Rio Grande	2	19	280	93
Roma	8	14	278	92
Anokhi	5	10	285	95
Raja	9	15	276	92

Optimization of MS Medium Strength for Seedling Growth: Strength of MS medium significantly affected germination of seed and seedling vigor. Full and double strength of MS medium half-strength MS medium produced uniform and healthy seedlings with improved root development. Reduced concentration of salt likely minimized ion toxicity and osmotic stress and early seedling growth (Table 2).

These results are aligned with previous reports which shows that reduced MS salt concentration increases in vitro development of seedling in tomato.

Table 2. Effect of diverse strength of MS basal media on seed germination

Strength of MS media	Days for germination			
	Rio Grande	Roma	Anokhi	Raja
MS 2/3	11	9.3	10.7	11
MS ¼	10.3	9.7	11.3	11
MS ½	8	8.3	9	8.7
MS	12.3	13	13.3	13
MS 2	17.7	18.3	17	15

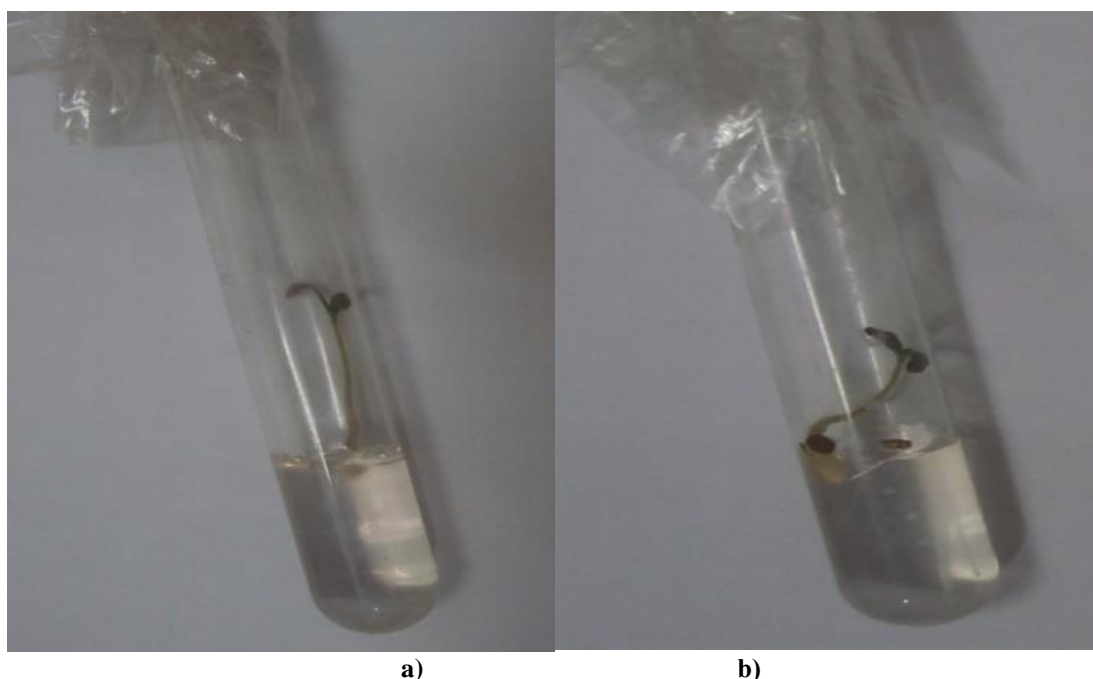
Influence of Plant Growth Regulators on Seed Germination: The application of isolated BAP (0.5–3.0 mg L⁻¹) and in combination with NAA significantly influenced shoot initiation and seedling development. The blend of 1.0 mg L⁻¹ BAP + 0.5 mg L⁻¹ NAA has shown superior shoot proliferation compared to other treatments (Table 3).

Abnormal morphology of shoot and reduced elongation with high concentration of BAP (>2.0 mg L⁻¹) was observed which indicates imbalance hormones. Interaction of Cytokinin–auxin plays an important role in maintaining morphogenesis, and regeneration efficiency enhanced with optimal balance.

The germination in all four cultivars was recorded when inoculated in MS half strength supplemented with BAP and NAA within 6 to 7 days (Figure a, b, c and d). The effect of varying concentration of BAP and NAA was recorded. When BAP 2mg/l and NAA 2mg/l were used germination started after inoculation in 6 to 10 days. Different range of plant growth regulator in varying concentration has been used for the in vitro regeneration of different cultivars of tomato to obtain the cotyledonary stage for the transformation (Safitr et al., 2023).

Table 3. Influence of concentration of BAP (mg/l) on germination of seeds

Treatment	Concentration of BAP (mg/l)	Days to Germination			
		Rio Grande	Roma	Anokhi	Raja
MS1	0.5	15	16.3	15.7	15
MS2	1	13	12.7	13	12
MS3	2	7	6.3	8	6.7
MS4	3	11.3	12.3	13.3	13



a)

b)

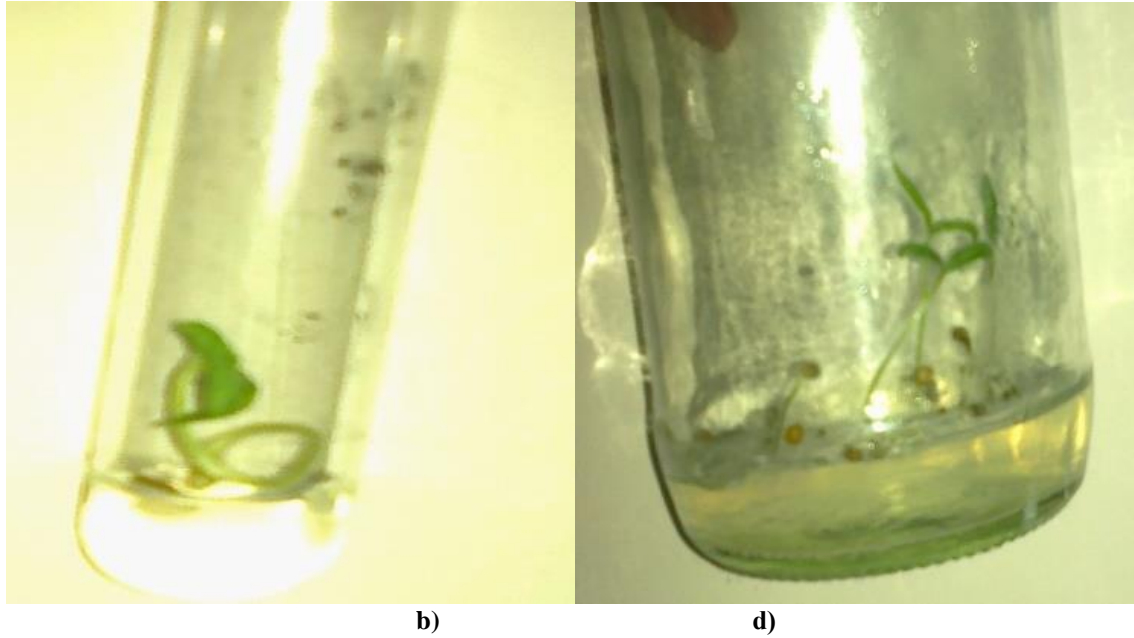


Figure 1. a) Progress of cotyledonary leaves of Raja on semi strength MS medium supplemented with 2 mg/L BAP and 2 mg/L NAA, b) Growth of cotyledonary leaves of Roma on partial strength MS medium supplemented with 2mg/L BAP and 2mg/L NAA, c) Growth of cotyledonary leaves of Rio Grande on half strength MS medium supplemented with 2mg/L BAP and 2mg/L NAA, d) Growth of cotyledonary leaves of Anokhi on half strength MS medium supplemented with 2mg/L BAP and 2mg/L NAA after 7 days of inoculation

Consequence of Explant Age on Transformation Efficiency: Age of the explants removed from seedlings (10–30 days) displayed noteworthy variation in expression of GUS. In 15 days, old explant maximum transient GUS expression was recorded, as explant gets

older reduced response was recorded. Younger tissues have higher meristematic activity and have greater capacity to *Agrobacterium*-mediated gene transfer (Figure 2) (Table 4 and 5).

Table 4. Outcome of seedlings age on transformation efficiency of leaf discs as explant

Seedling Age (Days)	Percentage of GUS assay			
	Rio Grande (%)	Roma (%)	Anokhi (%)	Raja (%)
10	65	55.3	65.3	45.3
15	81	84.7	84.7	87.3
20	74	77.3	77.8	75.7
25	66.3	61.7	51	78
30	50.2	47.2	51.3	48

Table 5. Consequence of seedlings phase on transformation efficiency of hypocotyls as explant

seedling Age (Days)	Percentage of GUS assay			
	Rio Grande (%)	Roma (%)	Anokhi (%)	Raja (%)
10	67	63	69	59.3
15	83	85	86.3	89.2
20	78	79	69	75
25	62	64	65	63

In highest gene transformation efficiency, the most important step is the selection of explant at proper

age. Explant derived from 15 days old *in-vitro* grown seedlings exhibited considerably stronger expression of

GUS (80.07%), explants after 10, 28 and 30 days did not show good expression of GUS. With the increasing days of *in vitro* grown seedling effectiveness of transformation was decreased. Effectiveness of transformation verified by activity of GUS concentrated in Rio Grande shadowed by Raja, Anokhi and Roma in the explants of the same age. Raj *et al.* (2005) has derived explants for co cultivation after 14 days and observed 14.2% transformation proficiency in tomato. Shahriari *et al.*, (2006) has used *in vitro* grown seedling of 11 day both of tomato explants hypocotyls and leave discs. Thus, the transformation efficiency is higher in the young and the cells with high dividing activity than the older explants.

Optimization of Agrobacterium Density and Infection

Duration: Maximum GUS expression was recorded at OD600 between 0.3–0.6 of bacterial suspension as compared to higher or lower densities. If bacterial density is low, it will affect transformation efficiency and high density caused tissue necrosis because of overgrowth, whereas lower density reduced transformation frequency (Table 6).

Likewise, infection time of 15 minutes showed maximum GUS-positive explants. Whereas longer exposure (20–25 minutes) create bacterial toxicity and reduced survival rate (Table 7).

Results for transient expression of GUS assay was recorded on the bases of transformed explant after 2 days co cultivation. For the production of transformed plants regeneration of transformed tissue is an important step. Numerous direct DNA transmission methods have been developed (Makhzoum *et al.*, 2015).

Optimization of Acetosyringone Strength and Co-

cultivation Period: Acetosyringone concentration was affected greatly on transformation efficiency, where, 200

μM acetosyringone showed highest transformation GUS expression. Decreased concentrations lower vir gene activation, although increased levels exhibited no extra benefit (Table 8). Co-cultivation of 2 days at 20°C meaningfully improved transformation efficiency. Lengthier durations (3–4 days) directed to overgrowth of bacteria and browning of tissue (Table 9). Acetosyringone played an important role in activation of virulence gene, and enabling T-DNA transfer (Figure 3).

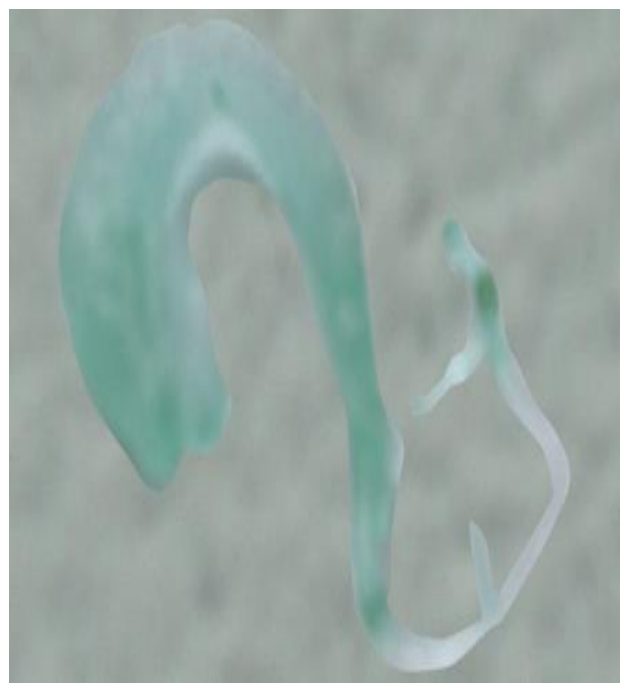


Figure 2. Stable GUS expression with 200 μM Acetosyringone concentration in hypocotyl

Table 6. Outcome of acetosyringone on GUS expression in leaf discs

Acetosyringone μM	Percentage of GUS assay			
	Rio Grande (%)	Roma(%)	Anokhi(%)	Raja(%)
50	8.7	7.7	5.3	11
100	34.3	31.7	46.3	35.7
150	50.3	55.3	64.3	49.7
200	81.7	81.3	80.3	86

Table 7. Influence of Acetosyringone on GUS expression in hypocotyls

Acetosyringone μM	Percentage of GUS assay			
	Rio Grande (%)	Roma (%)	Anokhi (%)	Raja (%)
50	20.3	28	27.9	29.2
100	39.4	40.5	45.9	47
150	67.2	67.9	60.2	63.7
200	91.3	93.4	91.3	93.3

Table 8. Result of acetosyringone on GUS expression in leaf discs

Acetosyringone μM	Percentage of GUS assay			
	Rio Grande (%)	Roma (%)	Anokhi (%)	Raja (%)
50	8.7	7.7	5.3	11
100	34.3	31.7	46.3	35.7
150	50.3	55.3	64.3	49.7
200	81.7	81.3	80.3	86

Table 9. Effect of acetosyringone on GUS expression in hypocotyls

Acetosyringone μM	Percentage of GUS assay			
	Rio Grande (%)	Roma (%)	Anokhi (%)	Raja (%)
50	20.3	28	27.9	29.2
100	39.4	40.5	45.9	47
150	67.2	67.9	60.2	63.7
200	91.3	93.4	91.3	93.3

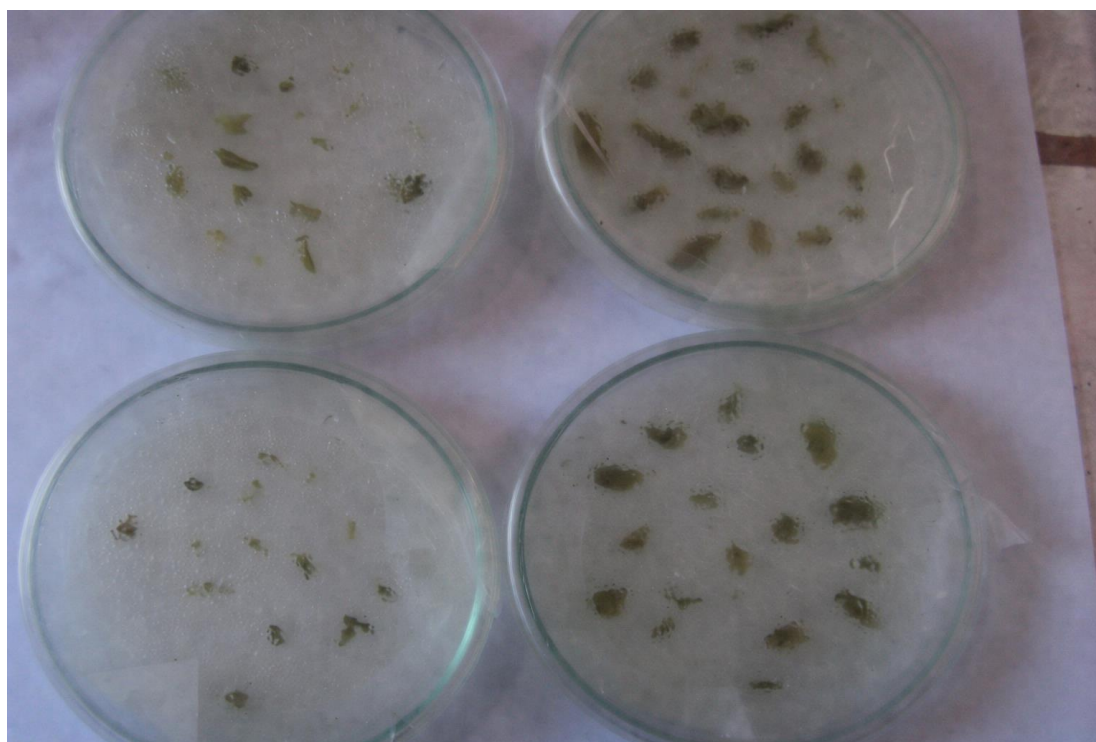


Figure 3. Co cultivation for two days in dark after infection of four Tomato Cultivars.

Acetosyringone added in the co cultivation media of dicotyledonous plants will improve the efficiency of transformation Du, et al., (2022) by numerous additions of T-DNA. Best response of GUS expression was observed at 200μM of acetosyringone. to enhance the efficiency of transformation acetosyringone was used for dicots tea (Lopez et al., 2004), tomato (Cortina and Culianez-Macia, 2004. Raj et al. (2004) conveyed that great transformation productivity was

attained by adding 200 μM acetosyringone in co-culture media. 100 μM of acetosyringone was added in the co cultivation media for transformation by (Reda et al., 2004). Cortiana and Culianez-Macia (2004) has used 200 μM of acetosyringone in *L. esculentum* UC82B Lopez et al., (2004) have used different concentration of acetosyringone and for tea plant and best response was at 400 μM acetosyringone. In monocots acetosyringone was also used for transformation like rice (Lin and Zhang,

2005), has stated that if the media for pre-culture supplied with 200 μ Macetosyringone could show the best results for transformation, much developed than formerly reports for indica rice (Aldemita and Hodges, 1996; Lin and Zhang, 2005; Lopez *et al.*, 2004; Matsumoto and Fukui, 1999). Hence the results of present study indicate that factors affecting transformation in the selected cultivars of tomato. Though so far only transient expression has been analyzed but these results may be helpful for stable gene expression.

Effect of Vacuum Infiltration and Wounding: Vacuum pressure 50 mm Hg for 10 minutes pointedly increased GUS expression compared to non-vacuum treatments. Though, increased vacuum (600 mm Hg) produced lesser transformation frequency and tissue damage (Table 10). Improved expression of GUS recorded if explants are subjected to cutting and wounding prior to infection. Release of phenolic compounds from wounds likely improves stimulation of vir gene improving and activation T-DNA transfer efficiency (Figure a, b, c, d and e).

Table 10. Influences of Vacuum Pressure Strength and Duration

Vacuum Pressure mmHg	Time	Percentage of GUS assay			
		Rio Grande (%)	Roma (%)	Anokhi (%)	Raja (%)
50	5	37	45	62	71.3
	7	71.7	74.7	77.7	81.3
	9	40.3	43.7	63.7	52.3
	11	56.7	59.7	56	44.7
	15	52.7	60.3	43.3	48
600	0.25	63.3	47	42.7	39
	0.5	84.7	87.3	87.7	82.7
	0.75	74	78.7	71.7	69.3
	1	71.7	80.3	74	73.7
	1.25	74.3	72.3	75.7	73.3



(a)



(b)



(c)



(d)



(e)

Figure 5. (a) Constant GUS appearance for a couple of weeks after selection with 200 μ M acetosyringone concentration in *Raja*. (b) Stable GUS appearance a couple weeks after selection with 200 μ M acetosyringone concentration in *Rio Grande*. (c) Stable GUS appearance a couple weeks after selection with 200 μ M acetosyringone concentration in *Raja*. (d) Stable GUS appearance a couple of weeks after selection with 200 μ M acetosyringone concentration in *Anokhi*. (e) Stable GUS appearance a couple weeks after selection without acetosyringone

Effect of Pre-selection Duration and Histochemical GUS Expression Analysis: Duration of pre-selection have a significant effect on recovery of transformed tissues. Optimal results were:

- Pre-selection of 5 days for leaf discs
 - Pre-selection of 7 days for hypocotyls
- Reduced regeneration frequency was recorded when immediately exposed to antibiotics, while non transformed tissues survived if delayed selection increased.

In infected explants transient GUS expression was recorded as blue coloration. After incubation of 7 days maximum staining intensity was recorded at 37°C. reduced enzymatic activity was recorded at higher incubation temperatures 47°C.

Transformation efficiency (%) was calculated based on GUS-positive explants. Among cultivars, *Rio Grande* and *Roma* showed relatively higher transformation frequency compared to *Anokhi* and *Raja*, indicating genotype-dependent response.

Table 12: Effect of incubation days after GUS test using leaf discs as explant

Incubation Time	Percentage of GUS assay			
	Rio Grande	Roma	Anokhi	Raja
Days	(%)	(%)	(%)	(%)
2	34.5	45.2	41.4	42.4
5	54.3	54.2	56.9	59.3
7	79.3	72.3	79.2	81.3
10	32.5	31.5	39.2	38.4

Table 13: Effect of incubation days after GUS test using hypocotyls as explant

Incubation Time	Percentage of GUS assay			
	Rio Grande	Roma	Anokhi	Raja
Days	(%)	(%)	(%)	(%)
2	45.3	42.4	41.4	47.2
5	62.4	61.4	62.4	61.1
7	80.1	82.1	85.2	83.1
10	23.5	45.1	46.1	42.5

After infecting the explant with *Agrobacterium* time for the co cultivation is an important factor for the successful transformation. Eight to fifteen days old *in vitro* grown seedling were used to derive the leaf discs and hypocotyl and co cultivated for different days in different experiments *i.e.*, 1, 2, 3 and 4 days, maximum transient expression of GUS activity was observed in the explants which were co cultivated for two days Joyce *et al.*, (2010). Roy *et al.* (2006) has used the 2 days for the co cultivation but on the other hand (Reda *et al.*, 2004) has used 3 days for co cultivation after infecting the explant and detect expression of GUS.

Conclusion: This study successfully developed a protocol for an efficient and stable transformation of agrobacterium mediated transformation with GUS as a reporter gene in tomato. Transformation is influenced by environmental and physiological parameters. Different parameter analysis confirmed that sterilization of seeds, strength of MS media, concentration of acetosyringone, seedling age, duration of co cultivation, bacterial density,

temperature and pre selection regime have significant effects on efficiency of transformation. Minimal contamination with maximum germination was on 8 %sodium hypochlorite was recorded. Half strength MS media had shown best *in vitro* growth of tomato seedlings. Explants excised after 15 days of germination showed best competency for transformation. Infecting explant with bacterial density (OD₆₀₀ 0.3–0.6) for 15 minutes and acetosyringone with strength of 200 µM after 15 min cocultivation and placing in dark for 2 days are the optimized conditions for transformation. Wounding and applying vacuum filtration have improved results of transformation. To confirm transformation histochemical GUS assay proved to be the most reliable method. Among four cultivars Rio Grand and Roma show highest transformation rate as compared to the Anokhi and Raja which indicates genetic variation. This study an optimized protocol was developed which provides a reproducible system for transformation of different tomato cultivars under Pakistani laboratory conditions.

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