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Original Research Paper

Agrobacterium-mediated transformation of bell pepper (*Capsicum annuum* L.) using a binary vector system

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ABSTRACT

This study established an efficient *Agrobacterium tumefaciens*-mediated transformation system for *Capsicum annuum* L. cv. California Wonder. Cotyledon and hypocotyl explants were co-cultivated with *Agrobacterium* strain LBA 4404, harboring the binary vector pCAMBIA2301, carrying the npt-II (neomycin phosphotransferase II) and gus (â-glucuronidase) genes. The effects of bacterial cell density and co-cultivation time on transformation efficiency were evaluated. Optimal transformation efficiencies of 43.3% for cotyledon and 33.3% for hypocotyl explants were achieved using an *Agrobacterium* concentration of OD_{600} = 0.6 and a 48-h co-cultivation period. As a pre-requisite, *in vitro* regeneration media were optimized: cotyledon explants exhibited shoot organogenesis on Murashige & Skoog (MS) medium supplemented with 8 mg/L 6-benzylaminopurine (BAP) and 6 mg/L indole-3-acetic acid (IAA), while hypocotyls produced shoots on 6 mg/L meta-topolin (mT) and 4 mg/L IAA. Shoot elongation was maximized on MS medium containing 2 mg/L mT, 1 mg/L gibberellic acid (GA_3), and 0.5 mg/L IAA. Root induction on MS medium with 1 mg/L IBA. Bacterial overgrowth was controlled with 500 mg/L cefotaxime, and transformants were selected on 60 mg/L kanamycin. PCR analysis verified stable gus gene integration in putative transgenic plantlets. This optimized protocol offers a valuable tool for introducing agronomically important genes to enhance biotic and abiotic stress tolerance in *C. annuum*, facilitating genetic improvement of this challenging crop.

Keywords: Agrobacterium, California Wonder, Capsicum, regeneration, transformation

INTRODUCTION

Capsicum annuum L., encompassing chilli peppers (pungent) and bell peppers (non-pungent), is India's most widely cultivated *Capsicum* species. It serves as a spice, a natural red food and a vegetable in a variety of culinary preparations. Its key bioactive compounds include capsanthin, responsible for its red colour, and capsaicin, which imports pungency. These compounds offer significant nutritional and medicinal benefits. *Capsicum* is also rich in vitamin C, vitamins A, B6, and K, and minerals such as calcium, magnesium, folate, potassium, thiamine, iron, and copper (Chakrabarty et al., 2017).

The susceptibility of *C. annuum* genotypes to abiotic and biotic stresses significantly constrains yield. Conventional breeding has improved *Capsicum* genotypes but is limited by a narrow genetic pool (Kothari et al., 2010). Biotechnological techniques, including tissue culture and recombinant DNA technologies, complement breeding efforts, accelerating genetic improvement. Among Solanaceae, *C. annuum* L. is highly recalcitrant to *in vitro* morphogenesis and genetic transformation (Heidmann et al., 2011). Challenges include poor morphogenesis, rosette shoots, and genotype dependence, hindering tissue culture and genetic transformation (Kothari et al., 2010).

Over the last decade, researchers have optimized regeneration and transformation techniques for pepper. Li et al. (2003) achieved 40.8% transformation efficiency using cotyledons in F_1 hybrids, while protocols have been developed for cultivars such as Pusa Jwala (Kumar et al., 2012), California Wonder (Verma et al., 2013), Pusa Jwala and Pusa Sadabahar (Mahto et al., 2018), and CO-4 (Vinodhini et al., 2024). However, bell pepper transformation remains inefficient and genotype-dependent. This study aims

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to develop a robust *Agrobacterium*-mediated transformation protocol for *Capsicum annuum* cv. California Wonder.

MATERIALS AND METHODS

Plant material, media and growth condition

Seeds of *Capsicum annuum* L. cv. California Wonder were procured from the Division of Vegetables Science, ICAR-Indian Agricultural Research Institute, New Delhi. Surface sterilization was done with 4% NaClO for 10 min, followed by three sterile water washes. The sterilized seeds were placed on autoclaved blotting sheets to remove excess moisture. Subsequently, the seeds were cultured on a steam-sterilized (121ºC for 20 mins) $^{1}/_{2}$X Murashige & Skoog (1962) medium with vitamins (Himedia, India), 3% sucrose, and 2.6% gelrite with pH adjusted to 5.8. Approximately 15 seeds were cultured in each bottle containing 50 mL of the medium. Bottles were incubated at 25±2ºC for 4 days in the dark, then transferred to a 16 h light/8 h dark photoperiod for germination. Cotyledon and hypocotyl explants were excised from 2-week-old seedlings for Kanamycin sensitivity assay and genetic transformation experiments.

Kanamycin sensitivity assay

To determine the optimal kanamycin concentration for selection of genetically transformed cells, the sensitivity of cotyledon and hypocotyl explants to kanamycin was assessed. The explants were cultured on Murashige and Skoog (MS) regeneration medium with varying concentrations of kanamycin (0, 15, 30, 45, 60, 75, 90 and 105 mg/L). For each concentration, three plates were prepared each containing ten hypocotyl and cotyledon explants. Plates were incubated in growth chamber at 25±2ºC and 16 h light/ 8 h dark photoperiod to induce callus formation, in each concentration of kanamycin was recorded at 0, 30 and 45 days after culture and the average was calculated. Data were statistically analysed by analysis of variance (ANOVA), using statistical analyses with R package software (Version 4.3.2 for Windows).

Agrobacterial inoculum preparation

The *Agrobacterium tumefaciens* strain LBA4404, harbouring the plasmid pCAMBIA2301 (Fig. 1), was used for plant transformation. The binary vector pCAMBIA2301 contains the *uidA* (β-glucuronidase) reporter gene and *npt-II* marker gene under the control of the Cauliflower mosaic virus 35S (CaMV35S) promoter and nopaline synthase (Nos) terminator. To prepare the *Agrobacterium* strain, a single colony was inoculated in 5 mL of Luria Bertani broth with 50 mg/L kanamycin and 50 mg/L rifampicin, incubated overnight at 28°C with shaking at 200 rpm. The following day, 500 µL of the overnight culture was inoculated into 50 mL of LB medium containing the same antibiotics and incubated at 28°C until the culture reached an OD_{600} of 0.6. Cells were then harvested by centrifugation at 5000 rpm for 5 minutes. The resulting pellet was resuspended in liquid MS medium to obtain final OD_{600} values of 0.2, 0.4, and 0.6. These varying optical densities were used to evaluate the effect of bacterial concentration on transformation efficiency.

Plant transformation and regeneration

The composition of pre-culture (PCM), co-cultivation (CCM), shoot induction (SIM), shoot elongation (SEM), and rooting (RM) media and the duration of

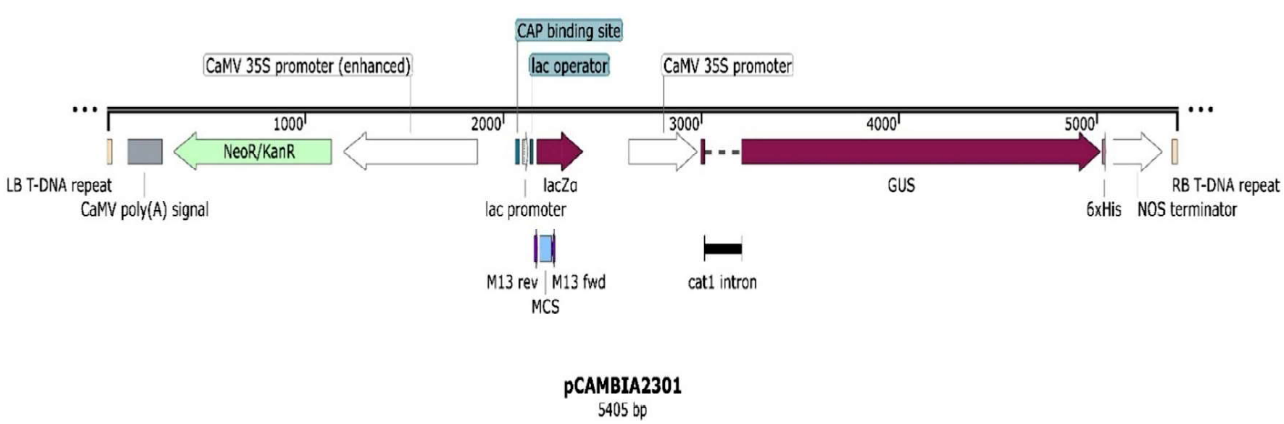


Fig. 1 : T-DNA region of binary vector pCAMBIA2301

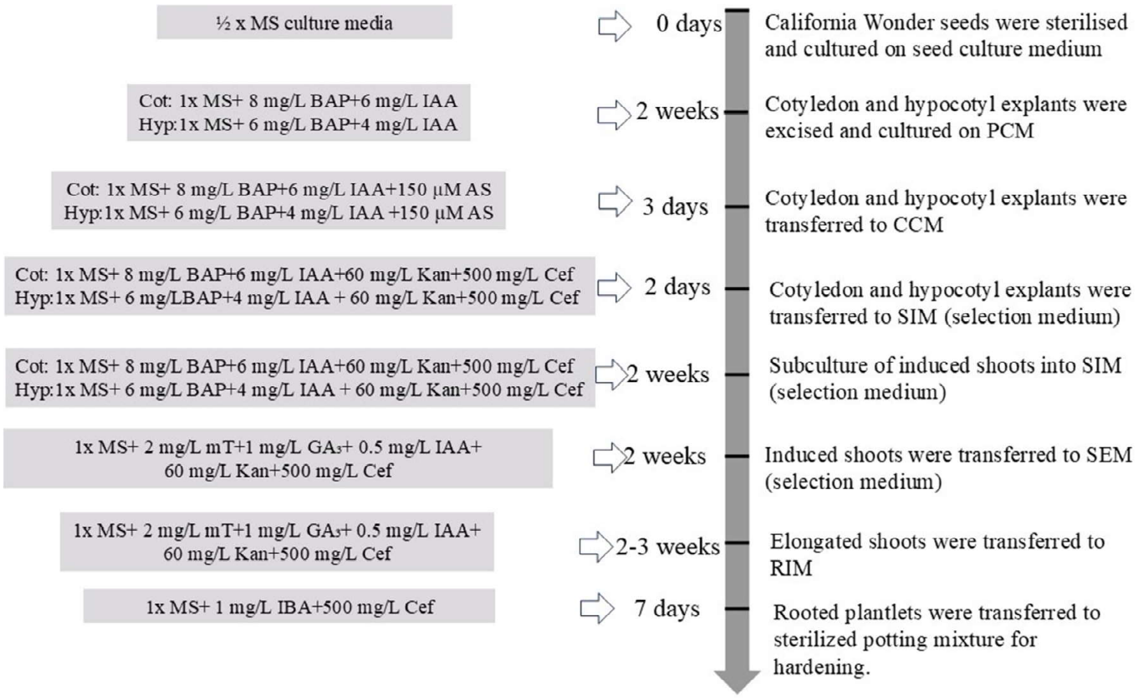


Fig. 2 : Schematic representation of *Agrobacterium tumefaciens*-mediated genetic transformation in var. California Wonder. The media compositions used are listed on the left side, while the timeline and sequence of each step are shown on the right (BAP: 6-benzylaminopurine; IAA: indole-3-acetic acid; mT: meta-topolin; GA_3: gibberellic acid; IBA: indole-3-butyric acid)

each step are given in Fig. 2. The growth regulators used in the study were 6-benzylaminopurine (BAP), indole-3-acetic acid (IAA), meta-topolin (mT), gibberellic acid (GA_3) and indole-3-butyric acid (IBA). Cotyledon and hypocotyl explants from 15-day-old seedlings were excised into ~1 cm segments and preconditioned on PCM for 72 h at 26±2°C under a 16 h photoperiod. Cultures were maintained under the same thermal and photoperiod conditions throughout regeneration. The explants showing no signs of necrosis were co-cultivated with *Agrobacterium tumefaciens* LBA4404 (pCAMBIA2301) prepared at OD_{600} values of 0.2, 0.4, or 0.6 in MS liquid medium supplemented with 150 µM acetosyringone. A total of 30 cotyledon and 30 hypocotyl explants were used in each treatment, i.e. 10 in each replication. Each set of explants treated with a specific bacterial density was incubated at room temperature for 15 minutes, blotted dry, and then transferred to co-cultivation medium (CCM) in the dark for 24, 48, or 72 h to assess the effect of both bacterial concentration and co-cultivation duration on transformation efficiency. Post-co-cultivation, explants were rinsed with MS medium containing cefotaxime (500 mg L^{-1}), blotted, and transferred to SIM. Explants forming shoots were sub-cultured onto fresh selection medium biweekly. Developing shoots were subsequently transferred to SEM and RIM. Rooted plantlets were acclimatized in a sterile potting mix before transfer to the Phytotron.

Molecular confirmation of transformants

Genomic DNA was extracted from the leaves of two randomly selected putative transformants (T) and non-transgenic (NT) California Wonder plants using the CTAB method (Doyle & Doyle, 1990). The GUS gene (538 bp) was amplified using primers 5′-ATCAGTT CGCCGATGCAG-3′ and 5′-CCGCTAGTGCCTT GTCCAGT-3′. The PCR mixture contained 2× PCR master mix (G Biosciences, USA), 10 pmol of each primer, and 50 ng/µL template DNA in a 10 µL total volume. PCR was performed in a thermal cycler (Bio-Rad, USA) with initial denaturation at 94°C for 4 min, followed by 35 cycles of 40 sec at 94°C, 45 sec at 58°C, 40 sec at 72°C, and a final extension at 72°C for 8 min. Amplicons were separated on 2% agarose gel in 1×TAE buffer, sizes of amplicons were calculated using a 50 bp ladder (G Biosciences, USA), and analyzed under UV light using a gel doc system (Alphaimager HP, USA).

Table 1 : The impact of varying concentrations of kanamycin on the relative growth, based on fresh weight, of cotyledon and hypocotyl explants from cv. California Wonder over different time intervals

Kanamycin concentration (mg/L)	Average fresh weight of callus (mg)					
	No. of days					
	Cotyeldon			Hypocotyl		
	0	30	45	0	30	45
0	3.07	382.5	382.5	3.38	396.57	396.57
15	3.22	178.22	142.1	3.44	249.57	156.67
30	3.3	122.76	131.2	4.01	108.23	134.43
45	3.19	88.98	117.5	3.73	86.73	161.23
60	2.98	84.92	111.1	3.9	76.87	124.93
75	3.29	74.08	104.7	3.34	75.6	86.7
90	2.95	58.16	76.64	3.61	40.33	34.7
105	2.95	34.18	48.38	3.93	28.37	49.1
Factors	$CD_{0.05}$	SE±		$CD_{0.05}$	SE±	
Kanamycin concentration (K)	59.63	30.09		66.61	33.34	
Effect of no. of days (T)	37.43	18.90		42.00	21.05	
Effect of (K)*(T)	22.17	11.17		27.98	13.92	

RESULTS AND DISCUSSION

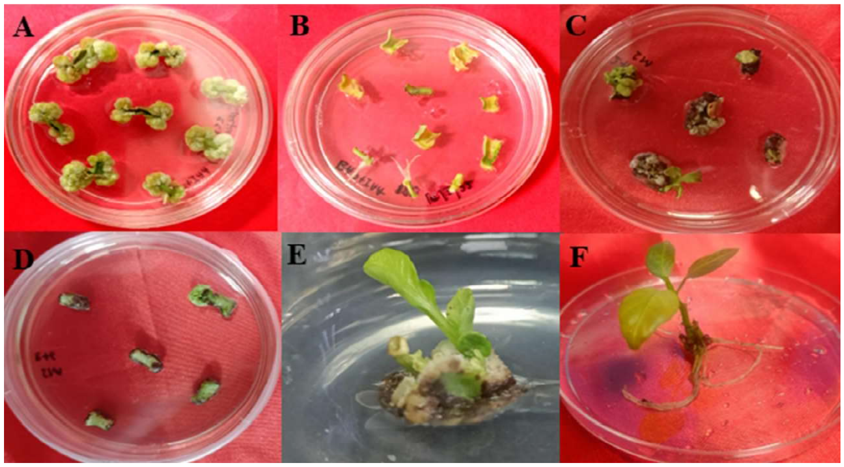
Kanamycin sensitivity assay

The selection of optimum kanamycin concentration for effective screening of transformed explants is critical in *Agrobacterium*-mediated transformation. In this study, we tested the sensitivity of explants using MS medium with kanamycin concentrations of 0, 15, 30, 45, 60, 75, 90, and 105 mg/L at 0, 30, and 45 days after culture. Both Kanamycin concentration (K) and time (T) significantly influenced the callus growth (Table 1).

Kanamycin sensitivity testing demonstrated a pronounced impact on the growth of cotyledon and hypocotyl explants, even at 15 mg/L. Fresh weight decreased progressively with higher kanamycin concentrations at 30- and 45-days post-inoculation. Without kanamycin, callus growth peaked at 382.5 mg in cotyledon and 396.57 mg in hypocotyl explants after 30 days (Table 1 & Fig. 4A). At 15 mg/L (K2), growth dropped to ~178 mg (cotyledon) and ~249 mg (hypocotyl) at 30 days, further reducing to ~142 mg and ~156 mg at 45 days. At 105 mg/L (K8), growth was nearly suppressed to ~34 mg (cotyledon) and ~28 mg (hypocotyl). Callus growth progressively increased over time at lower concentrations of kanamycin (0-30 mg/L), whereas higher concentrations (60-105 mg/L) induced severe necrosis after 30 days. Based on these observations the kanamycin concentration of 60 mg/L (K5) was determined as optimal (Fig. 4B). The response of *Capsicum* explants to kanamycin varied with genotype, as previously reported. For instance, Manoharan et al. (1998) used 50 mg/L kanamycin for Pusa Jwala, while, Kim et al. (2009) applied 150 mg/L for the P915 Chilli pepper variety.

Plant transformation and regeneration of transgenic plants

Capsicum annuum L. var. California Wonder was used to optimize transformation conditions. The *in vitro* regeneration medium for transgenic plants was refined based on protocols developed in our laboratory (manuscript in preparation). Before transformation, cotyledon and hypocotyl explants were pre-cultured in a PCM for 72 h to promote differentiation, with over 90% responding positively. Similar findings were reported for cotyledon explants in California Wonder (Verma et al., 2013), while a 42-h preconditioning period was optimal for Xiangyan 10, Zhongjiao, and Zhongjiao hybrids (Li et al., 2003).



A. Non-transformed explants cultured on MS medium without kanamycin at 45 days, B. Non-transformed explants cultured on MS medium supplemented with 60 mg/L Kanamycin at 45 days, C&D. Transformed cotyledon and hypocotyl explants on shoot induction medium (SIM), E. Regenerated shoots on shoot elongation medium (SEM), F. *In vitro* regenerated putative transgenic plantlet

Fig. 4(A-F) : *In vitro* regeneration of California Wonder transgenic plantlets

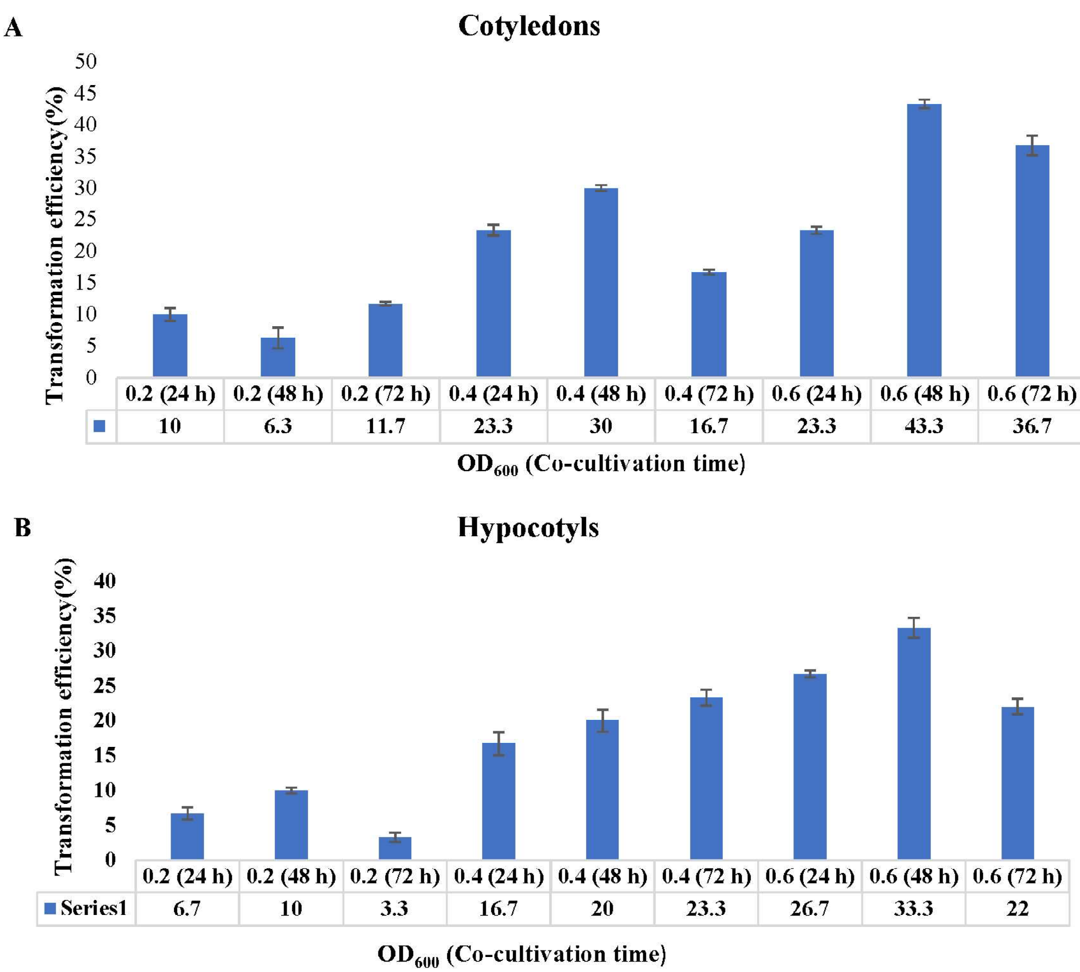


Fig. 3 : Effect of days of co-cultivation and bacterial cell density on % of transformation in A. Cotyledon and B. Hypocotyl explants of California Wonder

Significant variation in transformation efficiency was observed across different *Agrobacterium* cell densities (OD_{600}) and cocultivation durations in both cotyledon and hypocotyl explants of California Wonder (Fig. 3A and 3B). Maximum efficiency was recorded at OD_{600} = 0.6 with 2 days of cocultivation in both the explants, 43.3% in the cotyledon and 33.3% in the hypocotyl, indicating optimal bacterial density and exposure duration. Lower or higher values for either parameter resulted in reduced transformation, likely due to insufficient infection or tissue damage, respectively. Similar observations were reported by Kumar et al. (2012), who found that undiluted *Agrobacterium* cultures (OD_{600} = 0.5–1.0) caused necrosis in red pepper tissues, thereby hindering regeneration. To overcome this, they used a lower OD_{600} range of 0.05–0.1 with 48 h of co-cultivation for successful hypocotyl transformation. In another study, Mahto et al. (2018) optimized transformation in Pusa Jwala and Pusa Sadabahar using an OD600 range of 0.2–0.5 combined with a 72-h co-cultivation period, achieving around 30% transformation efficiency.

Cotyledon and hypocotyl explants of 'California Wonder' were cultured on SIM supplemented with hormones under selection pressure (60 mg/L kanamycin) and a bacteriostatic agent (500 mg/L cefotaxime) (Fig. 4C & 4D). Cotyledon explants exhibited efficient shoot regeneration on 8 mg/L BAP and 6 mg/L IAA, while hypocotyls regenerated optimally on 6 mg/L mT and 4 mg/L IAA. BAP and IAA synergistically promote cell division and organogenesis in *Capsicum* (Sanatombi & Sharma, 2006; 2008). Won et al. (2021) also achieved successful shoot induction with 8 mg/L BAP and 6 mg/L IAA, producing well-developed shoot buds within four weeks. Meanwhile, mT (meta-Topolin), a relatively newer cytokinin, has been shown to enhance regeneration efficiency in solanaceous crops such as *Solanum tuberosum* L. (Char et al., 2023) and *Solanum melongena* L. (Gande et al., 2024).

Following shoot induction, regenerated shoots were transferred to SEM (Fig. 4E). While BAP, alone or with GA_3, is widely used for shoot elongation in *Capsicum,* this study used 2 mg/L mT, 1 mg/L GA_3, and 0.5 mg/L IAA, facilitating elongation. GA_3 has been extensively reported for shoot elongation in *Capsicum*. Dabauza & Pena (2001) found that supplementing SEM with 10 mg/L GA_3 and 0.5 mg/L TDZ produced 3.45 elongated shoots per cotyledon explant in Agridulce sweet pepper. Similarly, Zeatin (2 mg/L) and GA_3 (2 mg/L) promoted elongation in the Dempsey variety (Won et al., 2021).

Once elongated, shoots were transferred to root induction medium (RIM) supplemented with 1 mg/L IBA, which promoted robust root formation. Acclimatized plantlets exhibited well-developed root systems and showed over 90% survival under controlled greenhouse conditions (Fig. 4F). Our observations clearly demonstrate that IBA is a potent auxin for rooting, particularly in recalcitrant species like *Capsicum annuum* (Gunay & Rao, 1978).

Molecular confirmation of transformation

PCR is widely used to confirm T-DNA presence in transgenic plants. Two randomly selected 'California Wonder' transgenic plantlets were analyzed alongside non-transgenic plants (negative control) to verify gus gene integration. PCR amplification using gus-specific primers yielded a distinct 538 bp band in transgenic plants, while no amplification was observed in the negative control, confirming successful transformation (Fig. 5). An earlier study with the pBI121 vector reported a 484 bp gus gene fragment in 40.8% of regenerated *Capsicum* plants (Li et al., 2003). Similarly, Verma et al. (2013) also found that among five transgenic *Capsicum annuum* L. cv. California Wonder plantlets, only two exhibited amplified bands of 750 bp and 800 bp for the nptII and gus genes, respectively.

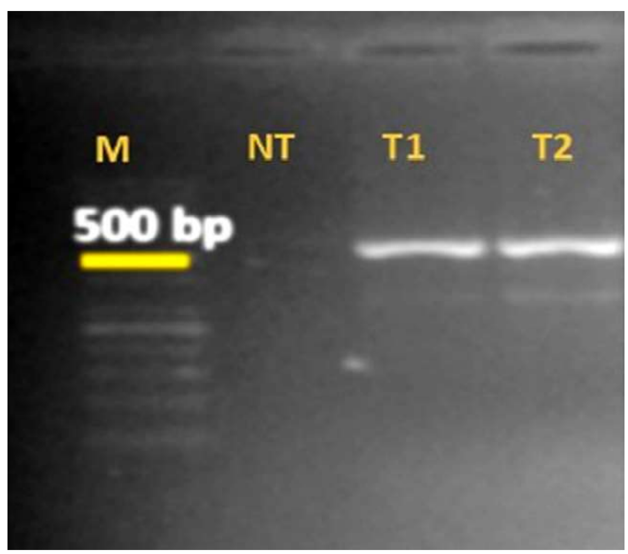


Fig. 5 : Molecular characterization of transgenic plants (M=marker, NT=non-transgenic control, T1/T2=transformants)

CONCLUSION

This study establishes an efficient protocol for *Agrobacterium*-mediated transformation in *Capsicum annuum* using the pCAMBIA 2301 vector. The optimized kanamycin concentration, regeneration media, and molecular confirmation techniques provide a robust framework for genetic transformation studies in *Capsicum*. Future research focusing on overcoming existing limitations and exploring new biotechnological approaches could further expand the utility of this transformation system in crop improvement programs.

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