



Precision microneedle-assisted wounding enhances regeneration and enables efficient *Agrobacterium*-mediated transformation in carnation (*Dianthus caryophyllus* L.)

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Abstract

Carnation (*Dianthus caryophyllus* L.) is an important ornamental crop, but its molecular breeding is limited by low regeneration competence and inefficient *Agrobacterium*-mediated transformation. This study aimed to establish a reproducible transformation framework for carnation by integrating optimized regeneration conditions with precision microneedle-assisted wounding and infection strategies. Significant genotype-dependent variation in regeneration capacity and auxin responsiveness was observed among six cultivars. Targeted microneedle-assisted wounding at the leaf-base vein junction markedly enhanced direct shoot regeneration, resulting in a 2.27-fold increase in shoot number per explant and earlier regeneration compared with non-wounded controls. Among three infection strategies tested, immersion-wounding with sterile microneedles followed by bacterial immersion produced the highest regeneration frequency (60.0%) and shoot formation. Optimization of non-ionic surfactants revealed that Tween 20 (0.01%) provided a broad and stable operational window, whereas Triton X-100 showed strong concentration-dependent phytotoxicity. Using the optimized protocol, five independent *acdS*-transgenic carnation lines were successfully recovered, corresponding to a PCR-verified transformation efficiency of 16.67% on an explant basis. PCR and qRT-PCR analyses confirmed stable transgene integration and expression without adverse effects on plant morphology. These results demonstrate that precision microneedle-assisted wounding is a key enabling factor for efficient regeneration and facilitates *Agrobacterium*-mediated transformation in carnation, providing a practical platform for molecular breeding of regeneration-recalcitrant ornamental crops.

Keywords Carnation · Microneedle-assisted wounding · Direct shoot regeneration · *Agrobacterium tumefaciens* · Transformation efficiency · *acdS*

Introduction

Carnation (*Dianthus caryophyllus* L.) is a commercially important ornamental crop worldwide; however, progress in its molecular breeding remains limited. This constraint is primarily attributed to low and inconsistent regeneration capacity and poor efficiency of *Agrobacterium*-mediated transformation. These constraints restrict the routine application of transgenic and genome-editing technologies

in carnation improvement (Jeong et al. 2021; Aalami et al. 2023).

Efficient and reproducible plant regeneration is a prerequisite for successful genetic transformation. In *Dianthus* species, regeneration efficiency is strongly influenced by genotype, explant source, and plant growth regulator composition, often resulting in highly variable outcomes among cultivars and protocols (Zhang et al. 2021; Campol et al. 2024). Although direct shoot organogenesis is generally preferred over callus-mediated pathways because it shortens culture duration and reduces somaclonal variation, achieving stable and robust direct regeneration remains a major technical bottleneck in carnation tissue culture systems (Aalami et al. 2023).

Mechanical wounding is known to play a critical role in activating regeneration competence and facilitating

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Agrobacterium infection by triggering wound-responsive signaling pathways and enhancing bacterial access to internal tissues. Wounding rapidly induces jasmonate-, cytokinin-, and auxin-responsive transcriptional networks that promote cell-cycle re-entry and organogenic reprogramming (Ikeuchi et al. 2017; Iwase et al. 2021; Zhang et al. 2023). However, conventional wounding methods such as scalpel incision or pinpricking are difficult to standardize and often cause uneven damage, resulting in variable regeneration responses and tissue necrosis. In contrast, microneedle-assisted wounding enables the delivery of uniform and reproducible micro-injuries while minimizing excessive tissue disruption. Although microneedle-based systems have recently been validated for precise biomolecule delivery in model and crop plants (Viswan et al. 2022), their potential application as a regeneration- and transformation-enhancing strategy in ornamental crops has not yet been systematically evaluated.

In addition to physical conditioning, efficient *Agrobacterium* infection requires optimized chemical conditions that enhance bacterial infiltration without compromising tissue viability. Non-ionic surfactants such as Tween 20 and Triton X-100 are widely used to reduce surface tension and facilitate bacterial entry; however, their effects are highly concentration dependent and can become phytotoxic above critical thresholds (Underwood et al. 2007; Liu et al. 2019). Recent studies emphasize the need for empirical optimization of surfactant type and dosage, particularly in regeneration-sensitive species such as carnation (Demirkurt et al. 2025).

Ethylene accumulation during in vitro culture imposes an additional constraint on carnation regeneration, as Dianthus tissues are especially sensitive to ethylene-mediated stress responses (Naing et al., 2021). The bacterial *acdS* gene encodes 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which degrades ACC—the immediate precursor of ethylene—thereby lowering endogenous ethylene levels. Expression of *acdS* has been shown to enhance shoot regeneration and stress tolerance in multiple plant species, making it a suitable functional transgene for evaluating transformation systems aimed at alleviating ethylene-related inhibition (Glick 2014; Naing et al. 2022).

The objective of this study was to establish a practical and reproducible *Agrobacterium*-mediated transformation framework for carnation by integrating optimized direct regeneration conditions with controlled microneedle-assisted wounding and empirically optimized infection chemistry. By systematically examining the effects of microneedle-induced wounding, infection protocols, and surfactant conditions, and validating the optimized system through the generation of *acdS*-transgenic plants, this study provides a robust platform for improving genetic

transformation efficiency in carnation and offers a transferable strategy for other regeneration-recalcitrant ornamental species.

Materials and methods

Plant material

All six carnation cultivars (*D. caryophyllus* L. ‘Pegasus’, ‘Red Beauty’, ‘Lira’, ‘Kumbyeol’, ‘Eunbyeol’, and ‘Dreambyeol’) were propagated in vitro. Explants were maintained on hormone-free Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962) supplemented with 30 g L⁻¹ sucrose and 8 g L⁻¹ agar, adjusted to pH 5.8 prior to autoclaving. Cultures were incubated at 25 ± 2 °C under a 16 h light/8 h dark photoperiod with a photosynthetic photon flux density (PPFD) of 40–50 μmol m⁻² s⁻¹ provided by cool-white fluorescent lamps. Culture media were sterilized by autoclaving at 121 °C for 16 min at 15 psi. Plant growth regulators, thidiazuron (TDZ) and indole-3-butyric acid (IBA), were prepared as filter-sterilized stock solutions (1 mg mL⁻¹ in distilled water) and added to the autoclaved medium after cooling to approximately 60 °C to obtain the desired final concentrations.

Explant Preparation and hormone optimization for direct shoot regeneration

Fully expanded leaves were excised from the second node of 4-week-old in vitro-grown plantlets. Only the basal region of each leaf, including the vein junction area, was used as the explant source. Leaf-base segments approximately 7–10 mm in length were aseptically excised using a sterile scalpel and used for subsequent experiments.

Based on prior optimization studies in our laboratory (Thu et al. 2020), a combination of 0.2 mg L⁻¹ thidiazuron (TDZ) and 0.1 mg L⁻¹ indole-3-butyric acid (IBA) was identified as optimal for direct shoot regeneration in carnation, whereas TDZ concentrations above 0.3 mg L⁻¹ induced excessive callus formation. Accordingly, 0.2 mg L⁻¹ TDZ was fixed as the cytokinin component in all experiments.

To determine cultivar-specific auxin responses, leaf-base explants from all six cultivars were cultured on MS medium containing 0.2 mg L⁻¹ TDZ supplemented with varying concentrations of IBA (0, 0.05, 0.1, 0.2, and 0.4 mg L⁻¹). Cultures were maintained for 4 weeks at 25 ± 2 °C under standard photoperiod conditions. Each treatment consisted of 30 explants (10 explants per Petri dish × three independent replicates). Regeneration frequency (%) and the mean number of shoots per responsive explant were recorded. Statistical significance of cultivar effects, IBA concentration

effects, and their interaction was evaluated using two-way ANOVA followed by Tukey's HSD post hoc test ($\alpha=0.05$).

Microneedle-assisted wounding for enhanced shoot regeneration

Microneedle-assisted wounding was performed using a stainless-steel microneedle array (Jevomec model 1418U, China) consisting of 14 microneedles (0.18 mm diameter, 15 mm length) arranged in a semicircular configuration. Wounding was specifically applied to the leaf-base vein junction region, where the primary and secondary veins converge, by positioning the microneedle array horizontally over this area immediately prior to culture initiation to generate standardized micro-injuries. Non-wounded explants without physical treatment served as controls.

Both microneedle-wounded and non-wounded leaf-base explants were cultured on Murashige and Skoog (MS) medium supplemented with 0.2 mg L⁻¹ thidiazuron (TDZ) and 0.1 mg L⁻¹ indole-3-butyric acid (IBA). Cultures were maintained at 25±2 °C under a 16/8-h light/dark photoperiod with a photosynthetic photon flux density of 40–50 μmol m⁻² s⁻¹. Each treatment consisted of 30 explants (10 explants per Petri dish × 3 independent replicates).

Direct shoot organogenesis was defined as the emergence of shoot primordia directly from the explant surface without intervening callus formation. After 4 weeks of culture, regeneration frequency (%) and the mean number of shoots per responsive explant were recorded. Statistical differences between microneedle-wounded and non-wounded treatments were evaluated using an independent samples t-test at a significance level of $P<0.05$.

Agrobacterium-mediated genetic transformation

Binary vector and bacterial strain

Agrobacterium tumefaciens strain GV3101 harboring the binary vector pCB302-3 (Jung et al., 2018) was used for genetic transformation. The vector (13,805 bp) contains a T-DNA region flanked by left and right border sequences. Within the T-DNA, the *acdS* gene encoding 1-aminocyclopropane-1-carboxylate (ACC) deaminase from *Pseudomonas* sp. (DVS1 StaA) is driven by the CaMV 35 S promoter and terminated by the nos polyadenylation signal. A bar selectable marker gene, driven by the nos promoter, confers resistance to phosphinothricin. The vector backbone includes a pBR322 origin of replication and a spectinomycin resistance gene for bacterial selection.

Agrobacterium cultures were maintained on Luria–Bertani (LB) agar supplemented with rifampicin (25 mg L⁻¹) and kanamycin (50 mg L⁻¹) at 28 °C. For transformation

experiments, bacteria were grown in liquid LB medium with antibiotics at 28 °C with shaking at 180 rpm for 16 h, harvested by centrifugation (5,000 × g, 10 min), and resuspended in sterile distilled water to an OD₆₀₀ of approximately 0.6.

Optimization of *Agrobacterium* infection strategies

Three infection protocols were evaluated using leaf-base explants of 'Pegasus'. In the non-wounded control treatment, intact explants were immersed in bacterial suspension for 15 min. In the dipped-wounding treatment, explants were perforated using microneedles pre-soaked in bacterial suspension. In the immersion-wounding treatment, explants were first perforated with sterile, dry microneedles and then immersed in bacterial suspension for 15 min. After infection, explants were co-cultivated on MS medium supplemented with 100 μM acetosyringone in darkness at 25±2 °C for 4 days. Explants were subsequently transferred to regeneration and selection medium (MS+0.2 mg L⁻¹ TDZ+0.1 mg L⁻¹ IBA+1 mg L⁻¹ phosphinothricin) and cultured under standard photoperiod conditions for two sequential 4-week selection cycles ($n=30$ explants per treatment; 10 explants per dish × 3 replicates). Transformation efficiency was assessed as selection efficiency (%) and mean number of shoots per responsive explant. Statistical analysis was performed using one-way ANOVA followed by Tukey's HSD test ($\alpha=0.05$).

Optimization of surfactant type and concentration

Based on the optimal infection strategy identified above, immersion-wounding was combined with non-ionic surfactants to further improve transformation efficiency. Microneedle-wounded leaf-base explants were infected with *Agrobacterium* suspension supplemented with Tween 20 or Triton X-100 at concentrations of 0, 0.0025, 0.005, 0.01, 0.02, or 0.04% (v/v).

Following a 15-min infection, explants were co-cultivated for 4 days on MS medium containing 100 μM acetosyringone in darkness and then transferred to selection medium for two sequential 4-week culture periods ($n=30$ explants per treatment; 10 explants per dish × 3 replicates). Regeneration frequency (%) and mean number of shoots per responsive explant were recorded. Statistical differences were analyzed using one-way ANOVA with Tukey's HSD post hoc test ($\alpha=0.05$).

Molecular verification of transgene integration and expression

Genomic DNA was extracted from regenerated shoots after two sequential selection cycles using a commercial plant genomic DNA extraction kit (BIOFACT, Seoul, Korea) according to the manufacturer's instructions. DNA quantity and purity were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA).

For PCR-based verification of T-DNA integration, *acdS*-specific primers (forward: 5'-CGAAAGTGCTTTTACGCGGAC-3'; reverse: 5'-AGCCGTTGCGAAACAAGAA G-3') were used to amplify an expected ~1,000 bp fragment (Jeong et al. 2021). PCR was performed in 20 µL reactions containing 10 µL of 2× Taq PCR Pre-Mix (Solgent, Korea), 10 pmol of each primer, and 100 ng genomic DNA. Cycling conditions were: 95 °C for 2 min; 35 cycles of 95 °C for 20 s, 60 °C for 40 s, and 72 °C for 1 min; and a final extension at 72 °C for 5 min. Integration was further confirmed by PCR amplification of the bar selectable marker using bar-specific primers under the same reaction composition and cycling conditions, with the extension time adjusted to the expected amplicon size where necessary. PCR products were resolved on 1% (w/v) agarose gels in 1× TAE buffer at 100 V for 40 min and visualized under UV illumination. Plasmid DNA (pCB302-3) was used as a positive control, and genomic DNA from non-transformed plants served as a negative control.

Using leaf-base explants, pronounced cultivar-dependent differences in shoot regeneration were detected under the standard hormone regime (0.2 mg L⁻¹ TDZ combined with IBA). Regeneration frequency varied markedly among cultivars, ranging from low responsiveness in 'Lira' and 'Eunbyeol' to consistently high regeneration in 'Pegasus'. Two-way ANOVA confirmed that cultivar identity exerted a significant main effect on regeneration frequency ($P < 0.05$), highlighting the strong influence of genetic background on organogenic competence in carnation.

Systematic variation of IBA concentration further revealed distinct cultivar-specific hormone response profiles. While moderate IBA levels promoted shoot regeneration in responsive cultivars, increasing IBA concentrations did not uniformly enhance regeneration across genotypes. In several cultivars, differences among IBA treatments within the same genetic background were not statistically significant, indicating that auxin sensitivity was secondary to cultivar-dependent regenerative capacity. Together, these results demonstrate that direct shoot regeneration in carnation is primarily governed by genotype and explant positional competence, with the leaf-base vein-junction region serving as a consistent and reliable source of regeneration-competent tissue.

Statistical analysis

All data were analyzed using SAS software version 9.4. Independent samples t-tests were applied to two-treatment comparisons, and one-way ANOVA was used for optimization experiments involving multiple treatments. Where significant differences were detected ($P < 0.05$), Tukey's HSD test was applied for post hoc comparisons. Results are presented as mean ± standard error of the mean (SEM).

Results

Cultivar-dependent variation in shoot regeneration and hormone responsiveness

Direct shoot organogenesis was consistently observed from the basal region of leaf explants across all carnation cultivars examined (Supplementary Fig. S1). Shoot primordia predominantly initiated near the vein-junction area of the leaf base, whereas explants derived from the middle and apical regions mainly produced non-organogenic callus or failed to respond. These reproducible spatial patterns justified the exclusive use of leaf-base explants for subsequent regeneration assays.

Using leaf-base explants, pronounced cultivar-dependent differences in shoot regeneration were detected under a fixed thidiazuron (TDZ) concentration (0.2 mg L⁻¹) combined with varying levels of indole-3-butyric acid (IBA) (Fig. 1). Regeneration frequency varied markedly among cultivars, with 'Pegasus' consistently exhibiting the highest responsiveness, whereas 'Lira' and 'Eunbyeol' showed low regeneration frequencies across most IBA treatments. Two-way ANOVA revealed significant main effects of cultivar and IBA concentration, as well as a significant cultivar × IBA interaction ($P < 0.05$), indicating genotype-dependent differences in auxin responsiveness.

Analysis of shoot proliferation further highlighted qualitative differences among cultivars (Fig. 2). Although moderate IBA concentrations promoted shoot formation in highly responsive cultivars, increases in IBA concentration did not uniformly enhance the mean number of shoots per explant across genotypes. In several low-responsive cultivars, shoot number remained limited and showed no significant differences among IBA treatments, reflecting low intrinsic organogenic competence. Notably, regeneration frequency and shoot number did not always correlate, demonstrating that these parameters represent distinct aspects of regenerative performance.

Collectively, the results presented in Figs. 1 and 2 indicate that direct shoot regeneration in carnation is primarily governed by genetic background, with auxin concentration

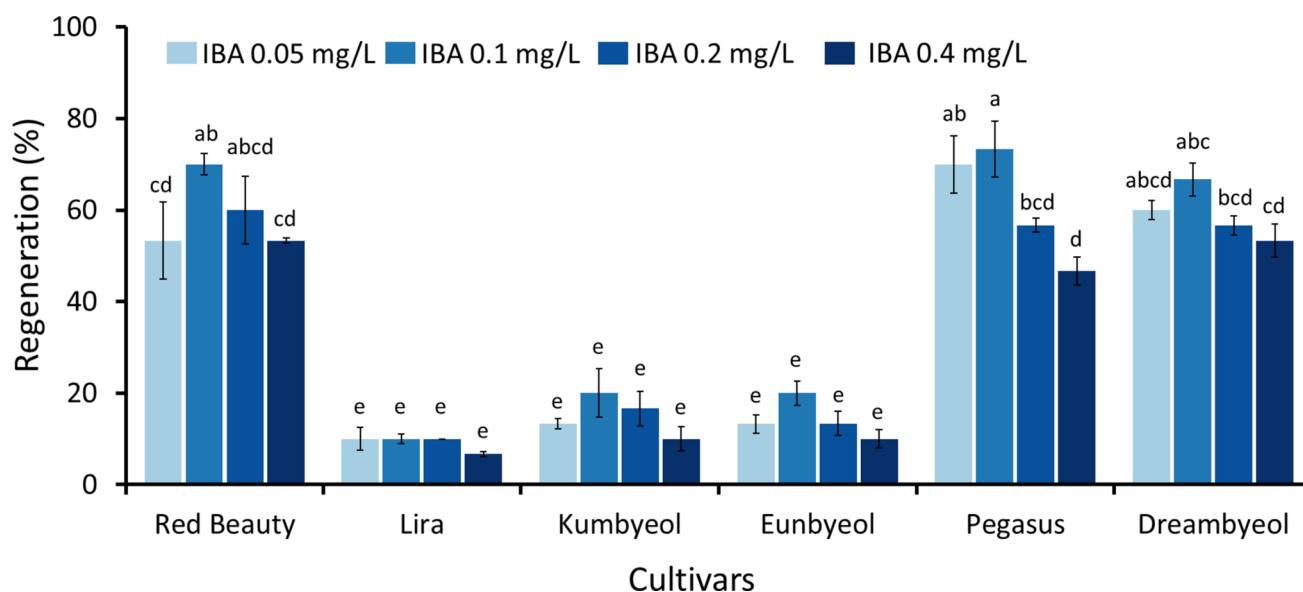


Fig. 1 Effects of indole-3-butyric acid (IBA) concentration on shoot regeneration frequency in six carnation (*D. caryophyllus* L.) cultivars under a fixed thidiazuron (TDZ) level. Leaf explants were cultured for 4 weeks on Murashige and Skoog (MS) medium supplemented with 0.2 mg L⁻¹ TDZ in combination with varying concentrations of IBA

(0.05, 0.1, 0.2, or 0.4 mg L⁻¹). Shoot regeneration frequency (%) was recorded after 4 weeks of culture. Bars represent mean ± SEM ($n=3$ independent replicates). Different letters indicate statistically significant differences among treatments within cultivars, as determined by two-way ANOVA followed by Tukey's HSD test ($P<0.05$)

Fig. 2 Heatmap illustrating the effects of indole-3-butyric acid (IBA) concentration on shoot regeneration in six carnation (*D. caryophyllus* L.) cultivars. Leaf explants were cultured for 4 weeks on Murashige and Skoog (MS) medium supplemented with a fixed concentration of thidiazuron (TDZ; 0.2 mg L⁻¹) in combination with varying IBA concentrations (0, 0.05, 0.1, 0.2, or 0.4 mg L⁻¹). Color intensity represents the mean number of shoots per explant, ranging from light blue (0 shoots) to dark blue (maximum value, 5 shoots per explant). Each treatment consisted of 30 explants (10 explants per Petri dish × three independent replicates). Values represent mean ± SEM of three independent replicates. Numerical values within cells indicate mean shoot numbers, and different letters denote statistically significant differences among IBA concentrations within each cultivar, as determined by two-way ANOVA followed by Tukey's HSD test ($P<0.05$)

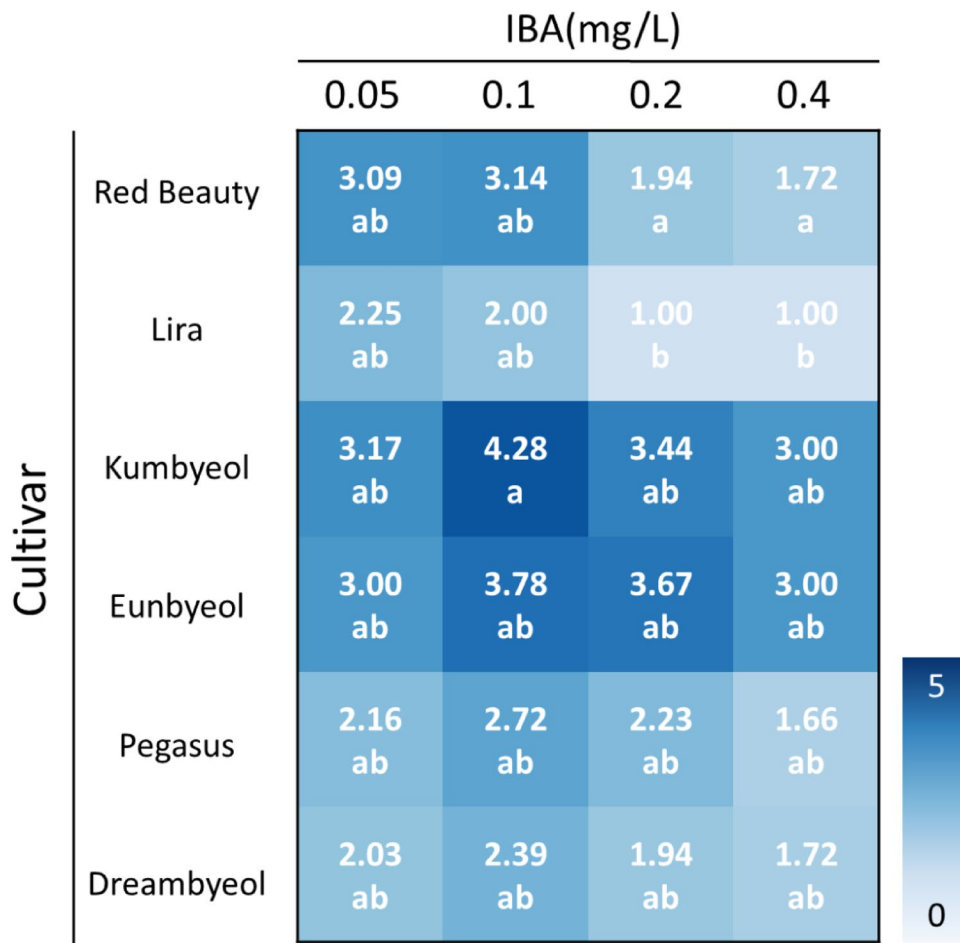


Fig. 3 Effect of microneedle-induced wounding on shoot regeneration from leaf-base explants of carnation. Microneedle-mediated wounding significantly enhanced direct shoot regeneration in *D. caryophyllus*. Data for ‘Pegasus’ are shown in the upper panel, and those for ‘Lira’ in the lower panel. Bars represent mean $\pm$ SEM ($n=30$; 10 explants $\times$ 3 replicates). *** $P<0.001$ (independent samples t-test)

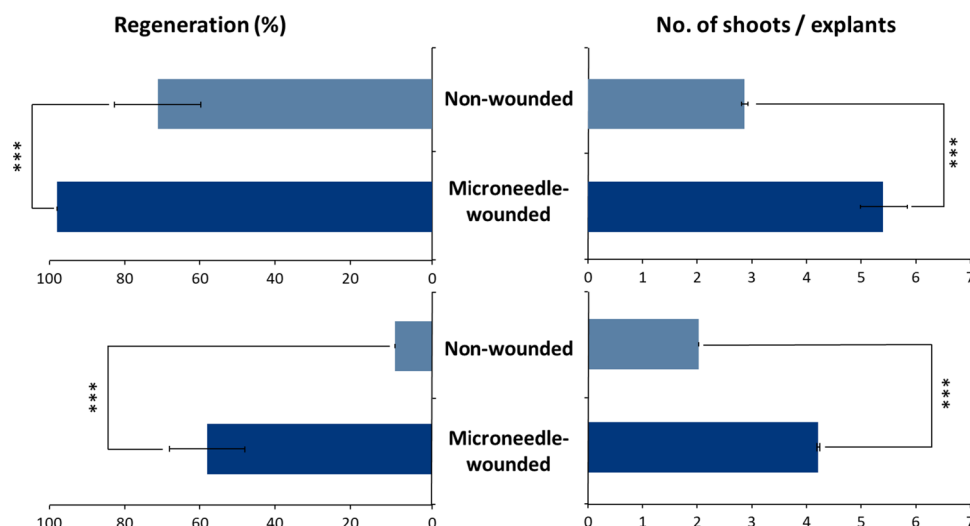


Table 1 Effects of microneedle-mediated wounding on *Agrobacterium*-mediated shoot regeneration from leaf-base explants of carnation ‘Pegasus’

Treatments	Regeneration (%)	No. of shoots / explants
Non-wounded control	26.7 $\pm$ 3.3 b	2.88 $\pm$ 0.11 b
Dipped-wounding	56.7 $\pm$ 3.3 a	3.40 $\pm$ 0.17a
Immersion-wounding	60.0 $\pm$ 0 a	3.61 $\pm$ 0.72 a

All explants were co-cultivated for 4 days and cultured on selection medium for 4 weeks. Values are means $\pm$ SEM ($n=30$). Different letters within a column indicate significant differences (one-way ANOVA, Tukey’s HSD test, $P<0.05$)

exerting a secondary, genotype-dependent effect. The leaf-base vein-junction region thus represents a consistent and reliable source of regeneration-competent tissue for comparative regeneration and transformation studies.

Microneedle-assisted enhancement of direct shoot regeneration

Microneedle-induced wounding significantly enhanced direct shoot regeneration from leaf-base explants of carnation (Fig. 3). Based on the preceding auxin optimization experiment, the highly responsive cultivar ‘Pegasus’, which exhibited superior regeneration across indole-3-butyric acid (IBA) concentrations, and the low-responsive cultivar ‘Lira’, which showed consistently poor regeneration, were selected to evaluate the effect of microneedle-induced wounding. In ‘Pegasus’ (upper panel), microneedle-wounded explants showed a marked increase in both regeneration frequency and the mean number of shoots per explant compared with non-wounded controls ($P<0.001$). In contrast, although ‘Lira’ (lower panel) exhibited an overall reduced regeneration capacity, microneedle treatment still resulted in a significant improvement in shoot regeneration relative to non-wounded explants ($P<0.001$). These results demonstrate that microneedle-assisted wounding

enhances regeneration across genotypes with contrasting intrinsic organogenic competence, with a stronger absolute effect observed in the highly regenerative cultivar.

Optimization of microneedle-assisted *Agrobacterium* infection strategies

Three *Agrobacterium* infection protocols were compared in carnation ‘Pegasus’ leaf-base explants: non-wounded immersion, dipped-wounding, and immersion-wounding (Table 1). Both wounding-based treatments significantly improved regeneration compared with the non-wounded control. The regeneration efficiencies reported in Table 1 represent the proportion of phosphinothricin (PPT)-positive explants relative to the total number of inoculated explants and therefore indicate selection efficiency rather than PCR-verified transformation efficiency.

Immersion-wounding (C) resulted in the highest regeneration frequency (60.0%) and shoot number (3.61 ± 0.72), while dipped-wounding (B) also showed significantly enhanced regeneration (56.7%, 3.40 ± 0.17) relative to the non-wounded control (26.7%, 2.88 ± 0.11). No significant differences were detected between treatments B and C. One-way ANOVA confirmed a significant treatment effect ($P<0.05$), and Tukey’s HSD test separated treatment A from treatments B and C ($P<0.05$).

To visualize the optimized transformation workflow and subsequent regeneration process, the microneedle-assisted infection protocol and representative stages of plant recovery are shown in Fig. 4. Following microneedle-induced wounding at the leaf-base vein junction and immersion in *Agrobacterium* suspension, explants underwent two sequential selection cycles, during which shoot primordium initiation, shoot elongation, rooting, and acclimatization were consistently observed. This visual progression confirms that the optimized infection strategy supports stable

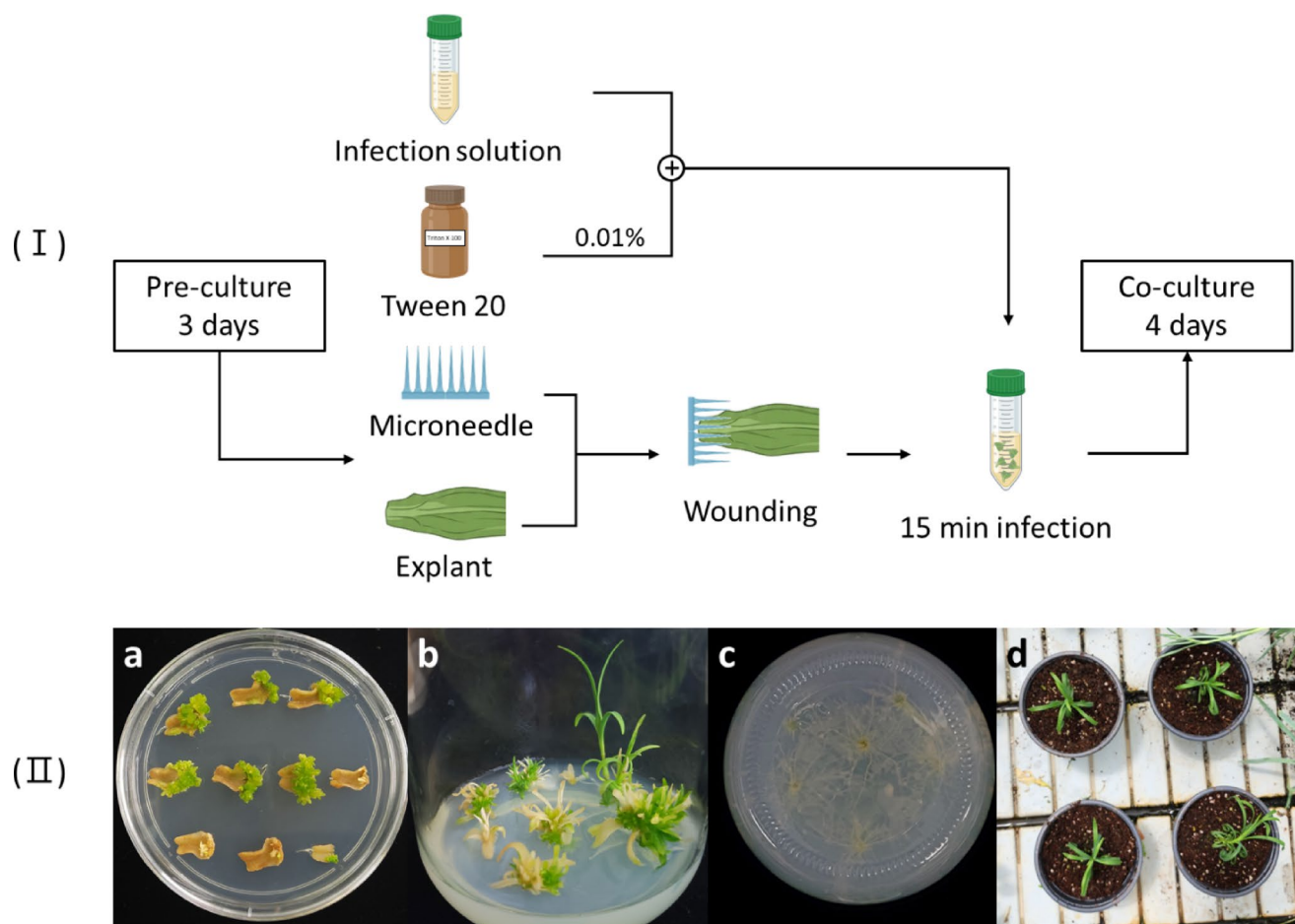


Fig. 4 *Agrobacterium*-mediated genetic transformation of carnation leaf explants. (I) Optimized infection protocol. Leaf-base explants were microneedle-wounded at the vein-junction region and immersed for 15 min in an *A. tumefaciens* suspension ($OD_{600} = 0.6$) containing 0.01% (v/v) Tween 20, followed by 4-day co-cultivation and transfer

to selective regeneration medium. (II) Representative stages of regeneration and plant recovery: (a) first selection with shoot primordium initiation, (b) second selection with shoot elongation, (c) rooting of regenerated shoots, and (d) acclimatized transgenic plants in soil

regeneration and recovery of transformed carnation plants under selection conditions.

Optimization of non-ionic surfactants for improved transformation efficiency

Two non-ionic surfactants, Tween 20 and Triton X-100, were evaluated at concentrations ranging from 0 to 0.04% to optimize *Agrobacterium*-mediated transformation efficiency in carnation (Table 2). Both surfactants enhanced transformation relative to unsupplemented controls but exhibited distinct concentration-dependent responses.

Tween 20 achieved maximal transformation efficiency (60.0%) at 0.01%, producing 4.22 ± 0.07 shoots per explant. Efficiency remained relatively stable between 0.01% and 0.02% before declining at higher concentrations. In contrast, Triton X-100 displayed a narrow effective range, with peak efficiency (60.0%, 4.78 ± 0.03 shoots per explant) at

0.0025%, followed by a sharp decline, resulting in complete loss of transformation at concentrations $\geq 0.01\%$.

One-way ANOVA revealed significant effects of surfactant type and concentration ($F = 31.59$, $P < 0.05$). Although no significant difference was detected between the optimal concentrations of Tween 20 (0.01%) and Triton X-100 (0.0025%) ($P = 0.182$), Tween 20 provided a broader operational window, offering greater robustness against concentration-dependent phytotoxicity.

Overall, these results indicate that while both surfactants can enhance transformation efficiency, Tween 20 is more suitable for routine application due to its wider effective concentration range and lower risk of tissue damage.

PCR- and qRT-PCR-based molecular verification of *AcdS* transgene integration and expression

PCR-based screening of regenerated shoots after two sequential 4-week selection cycles on PPT-containing

Table 2 Effects of surfactant type and concentration on regeneration efficiency and shoot formation in carnation ‘Pegasus’ leaf-base explants following *Agrobacterium*-mediated transformation

Surfactant type	Concentration (%)	Regeneration (%)	No. of shoots / explants
control	-	46.7±3.3 abc	3.86±0.07 abc
Tween 20	0.0025	26.7±6.7 d	3.25±0.57 bc
	0.005	33.3±3.3 cd	4.00±0.27 abc
	0.01	60.0±10.0 a	4.22±0.26 ab
	0.02	53.3±6.7 ab	3.13±0.16 bc
	0.04	40.0±0.0 bcd	3.17±0.36 bc
Triton X-100	0.0025	60.0±10.0 a	4.78±0.35 a
	0.005	46.7±3.3 abc	2.57±0.03 c
	0.01	33.3±3.3 cd	3.20±0.25 bc
	0.02	0 e	-
	0.04	0 e	-

Values are means±SEM ($n=30$ explants per treatment). Explants were infected with *A. tumefaciens* using an optimized immersion-wounding method with Tween 20 or Triton X-100 (0.0025–0.04%). After 4-day co-cultivation on MS medium with 100 μM acetosyringone, explants were cultured on selection medium (MS+0.2 mg L^{-1} TDZ, 0.1 mg L^{-1} IBA, 1 mg L^{-1} PPT) for two 4-week phases. Different letters within columns indicate significant differences ($P<0.05$, Tukey’s test)

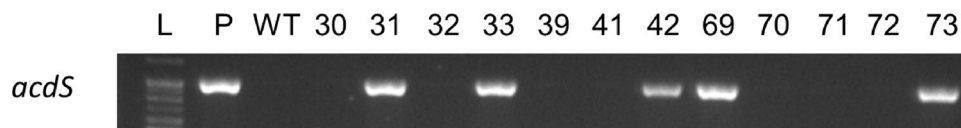
medium identified five independent transgenic lines with confirmed T-DNA integration (Fig. 5). These lines were all recovered from leaf-base explants of carnation cv. ‘Pegasus’, which was used for transformation optimization due to its high regeneration capacity. All PCR-positive lines showed

the expected amplification of both the *acdS* gene (1,000 bp) and the *bar* selectable marker (449 bp), whereas no amplification was detected in non-transformed controls. Plasmid DNA used as a positive control produced the expected band patterns, confirming the reliability of the PCR assays.

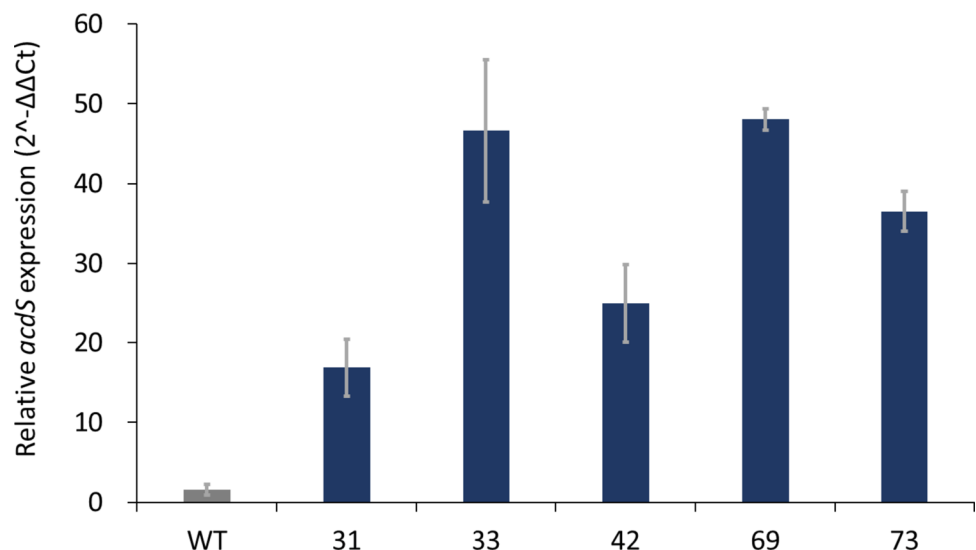
Quantitative RT-PCR analysis further confirmed transcriptional activation of the *acdS* transgene in all PCR-positive lines (Fig. 6). Relative *acdS* expression levels varied among transgenic lines but were consistently and significantly higher than those of wild-type plants, in which *acdS* transcripts were undetectable or present at basal background levels. This variation among lines is indicative of independent transgene integration events and is consistent with positional effects commonly observed in *Agrobacterium*-mediated transformation.

Based on PCR screening, the overall transformation efficiency was calculated as 5.95% (5 transgenic plants from 84 regenerated shoots), corresponding to a recovery efficiency of 16.67% when calculated on an explant basis (5 independent transgenic events from 30 inoculated explants). All transgenic lines regenerated morphologically normal *To* plantlets that were indistinguishable from non-transformed controls, indicating that *acdS* integration and expression did not adversely affect plant development under *in vitro* conditions.

Collectively, the combined PCR and qRT-PCR analyses confirm stable integration and active expression of the *acdS*

**Fig. 5** PCR-based verification of *acdS* transgene integration in carnation. Genomic DNA from regenerated plants was amplified using *acdS*-specific primers. Lane M, DNA size marker; WT, non-transformed control; P, plasmid positive control; lanes 31, 33, 42, 69, and

73 represent independent transgenic lines. The expected amplicon (~1,000 bp) was detected in all transgenic lines and the positive control but not in the WT control

Fig. 6 Relative expression of the *acdS* transgene in wild-type (WT) and independent transgenic carnation lines. Transcript levels of *acdS* were quantified by quantitative real-time PCR (qRT-PCR) in WT plants and five independent transgenic lines (31, 33, 42, 69, and 73). Relative expression was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method, with WT used as the calibrator. Data represent the mean±SEM of three biological replicates (WT: 1.598±0.653; line 31: 16.856±3.557; line 33: 46.609±8.866; line 42: 24.960±1.921; line 69: 48.063±1.345; line 73: 36.531±2.487)

transgene in carnation, demonstrating that the optimized transformation system enables reliable recovery of functional transgenic plants.

Discussion

Efficient regeneration competence is a fundamental prerequisite for successful *Agrobacterium*-mediated transformation, particularly in regeneration-recalcitrant ornamental crops such as carnation. In this study, microneedle-assisted micro-wounding significantly enhanced direct shoot regeneration from leaf-base explants, as evidenced by increased regeneration frequency, higher shoot numbers per explant, and accelerated, synchronized shoot initiation. These observations support the concept that regeneration efficiency constitutes a primary bottleneck in carnation transformation systems and that improving morphogenic competence directly translates into improved transformation outcomes.

Mechanical wounding is known to activate regeneration-associated signaling cascades involving jasmonates, cytokinins, and auxin redistribution, thereby promoting cell-cycle re-entry and organogenic competence (Ikeuchi et al. 2017; Iwase et al. 2021; Zhang et al. 2023). In *Dianthus* species, where regeneration responses are highly genotype- and explant-dependent, uncontrolled or excessive wounding often leads to tissue necrosis and inconsistent outcomes. The microneedle approach used here provides a standardized and reproducible means of delivering uniform micro-injuries while minimizing collateral damage. Similar microneedle platforms have recently been validated as precise plant–tissue interfaces for biomolecule delivery and localized tissue conditioning in *Arabidopsis* and maize (Viswan et al. 2022), supporting the broader applicability of this strategy. The earlier onset and improved synchrony of regeneration observed in microneedle-treated explants suggest that controlled micro-injury effectively accelerates the transition from differentiated leaf tissue to a morphogenically competent state.

Importantly, the enhanced regeneration competence conferred by microneedle treatment was directly reflected in improved *Agrobacterium*-mediated transformation performance. Among the three infection strategies evaluated, immersion-wounding—where explants were wounded with sterile microneedles prior to bacterial immersion—was clearly superior to both non-wounded and dipped-wounding approaches. The complete failure of dipped-wounding, despite its apparent mechanical advantage, indicates that excessive or poorly controlled bacterial entry can be detrimental, leading to bacterial overgrowth, tissue necrosis, and loss of regenerative capacity. This finding is consistent with previous reports in *Dianthus*, which emphasize

that transformation efficiency is often constrained by post-infection tissue survival rather than T-DNA delivery per se (Zhang et al. 2021). By temporally separating physical wounding from bacterial exposure, immersion-wounding appears to balance bacterial accessibility with tissue preservation, thereby maintaining regeneration-competent cells during the critical window of T-DNA transfer.

Beyond physical conditioning, chemical facilitation of *Agrobacterium* infection proved to be a critical determinant of transformation success. Both Tween 20 and Triton X-100 enhanced transformation efficiency relative to surfactant-free controls; however, their effective concentration ranges differed markedly. Triton X-100 exhibited a narrow optimal window and pronounced phytotoxicity at slightly elevated concentrations, resulting in rapid loss of transformation efficiency. This contrasting behavior is likely attributable to differences in detergent strength and interactions with cellular membranes. Triton X-100, a stronger membrane-disrupting agent, can compromise plasma membrane integrity and induce tissue necrosis in sensitive carnation leaf-base explants, whereas the milder non-ionic surfactant Tween 20 better preserves tissue viability. In contrast, Tween 20 maintained high transformation efficiency across a broader concentration range, providing greater robustness against minor variations in surfactant preparation. Given the inherent sensitivity of carnation tissues to chemical perturbations (Aalami et al. 2023), this broader operational window makes Tween 20 more suitable for routine application in reproducible transformation systems. These results align with recent optimization studies highlighting that surfactant selection should prioritize tissue tolerance and reproducibility rather than maximal short-term efficiency alone (Demirkurt et al. 2025).

Molecular verification further confirmed the effectiveness of the integrated optimization strategy. PCR-based screening identified five independent transgenic lines carrying both *acdS* and *bar* genes, demonstrating stable T-DNA integration. qRT-PCR analysis revealed markedly elevated *acdS* transcript levels in all transgenic lines relative to wild-type plants, confirming transcriptional activity of the introduced gene. The observed variation in expression levels among independent lines is consistent with positional effects, copy-number variation, or partial transcriptional silencing, which are commonly reported in *Agrobacterium*-mediated transformation systems. Notably, all transgenic lines exhibited normal morphology during the T₀ generation, indicating that *acdS* integration and expression did not adversely affect in vitro plant development.

While the overall transformation efficiency achieved in this study (5.95%) remains modest, it represents a clear improvement over previously reported efficiencies for carnation, which often remain below 2% in commercial

cultivars (Jeong et al. 2021; Aalami et al. 2023). This outcome underscores the intrinsic recalcitrance of carnation but also demonstrates that systematic optimization of regeneration competence, infection strategy, and chemical conditions can yield reproducible gains.

Several limitations and avenues for further improvement merit consideration. First, although microneedle-assisted wounding clearly enhanced regeneration, additional experiments comparing wounded and non-wounded explants across anatomically distinct leaf-base regions would help disentangle the relative contributions of tissue competence and mechanical stimulation. Second, the failure of dipped-wounding suggests excessive bacterial load as a causal factor; this interpretation could be strengthened by quantitative assessment of bacterial proliferation or tissue necrosis during co-cultivation. Finally, although transcriptional activation of *acdS* was confirmed, future studies should incorporate copy-number estimation, T₁ segregation analysis, and functional assays—such as ACC deaminase activity, ethylene emission measurements, or stress-response phenotyping—to further validate transgene stability and functionality.

In summary, this study demonstrates that microneedle-assisted wounding serves as a central enabling step linking efficient regeneration with improved *Agrobacterium*-mediated transformation in carnation. When combined with optimized infection timing and robust surfactant conditions, this approach provides a practical and reproducible framework for genetic transformation. Beyond carnation, the principles established here—standardized physical conditioning, controlled bacterial exposure, and emphasis on operational robustness—are likely transferable to other regeneration-recalcitrant ornamental species, thereby broadening the toolkit available for functional genomics and molecular breeding in ornamental crops.

Conclusion

This study demonstrates that microneedle-assisted wounding markedly enhances direct shoot regeneration and improves *Agrobacterium*-mediated transformation efficiency in carnation (*D. caryophyllus* L.). By integrating controlled physical micro-wounding with optimized infection strategies and non-ionic surfactant conditions, a reproducible transformation system was established, enabling the stable recovery of *acdS*-transgenic plants. Although further functional validation of transgene expression and inheritance is required, the results indicate that improving regeneration competence is a key determinant of transformation success in carnation. The framework developed here provides a practical and transferable approach for overcoming

regeneration-associated constraints and may be applicable to other regeneration-recalcitrant ornamental crops.

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Author contributions JSY performed the experiments and analyzed the data. JSY and CKK wrote the manuscript. CKK designed the study and revised manuscript.

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Declarations

Conflict of interest The authors report no conflicts of interest.

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