

Enhanced hairy root induction in garlic (*Allium sativum* L.) via *Agrobacterium rhizogenes*: Targeting the basal plate of intact sprout

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Abstract

Hairy root induction in garlic represents a promising approach to actively exploit biomass from a familiar plant species rich in antibacterial compounds. Previously, the stem disc region of garlic was identified as a potential site for morphogenesis; however, few studies have focused on utilizing this tissue for hairy root induction and optimizing the conditions to enhance transformation efficiency. Histological analysis revealed that the basal plate contains soft parenchyma tissue conducive to root and vascular development, making it a suitable target for transformation in monocot species. In this study, we investigated the factors influencing hairy root induction from this recalcitrant tissue. Our results indicated that maintaining the integrity of the basal plate of stem disc in 10-day-old garlic seedlings significantly enhanced induction efficiency compared to sectioning methods. Through preservation of the primary root system and inner clove tissue and addition of acetosyringone, roots exhibited further improved stability in genetic, morphological, and growth traits, with fresh biomass accumulation reaching 1 g per induced explant and the hairy-root induction rate achieved 100%. PCR analysis confirmed the presence of *rolB* and *rolC* genes in induced roots, verifying successful gene transfer by *Agrobacterium rhizogenes* ATCC 15834. Overall, this study provides important insights into improving hairy root induction in garlic—a monocotyledonous species typically considered recalcitrant—and highlights the stem disc as a reliable and efficient target tissue for generating bioactive root biomass. These findings not only advance transformation strategies in monocots but also open broader opportunities for metabolic engineering, enhanced production of valuable secondary metabolites, and the development of sustainable biotechnological platforms for pharmaceutical and agricultural applications.

Keywords: *Agrobacterium*-mediated transformation; *Allium sativum*; *in vitro* garlic hairy roots; monocotyledonous species; stem disc

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Introduction

Hairy root induction by *Agrobacterium rhizogenes* has emerged as a pivotal tool in plant biotechnology, enabling the establishment of stable root cultures for secondary metabolite production, genetic transformation, and gene function analysis. This technique offers an efficient platform for producing valuable phytochemicals and for functional genomics studies due to the genetic stability and high growth rate of hairy roots (Giri and Narasu, 2000; Guillon *et al.*, 2006; Veena and Taylor, 2007, Faraz *et al.*, 2024).

While *A. rhizogenes*-mediated transformation is well-established and highly efficient in many dicotyledonous plants (dicots), its application in monocotyledonous plants (monocots) remains challenging. Monocots generally exhibit very low or negligible rates of hairy root induction, posing a fundamental biological question about the anatomical and physiological traits that underlie their recalcitrance to *A. rhizogenes* infection (Veena and Taylor, 2007). The differences in vascular bundle structure between dicots and monocots provide key insights into this disparity. In dicots, vascular bundles are arranged in a ring with a distinct pith and abundant parenchymatous cells surrounding the bundles, which have high regenerative potential. The presence of a vascular cambium layer enables secondary growth and facilitates cellular dedifferentiation and root formation upon *A. rhizogenes* infection (Evert and Eichhorn, 2006).

In contrast, monocots possess scattered vascular bundles (atactostele) without a clear pith or vascular cambium, resulting in “closed” vascular bundles surrounded by sclerenchyma tissues that lignify early. This anatomical arrangement leads to early cell wall hardening and a reduced capacity for regeneration (Evert and Eichhorn, 2006). The vascular cambium in dicots creates a conducive microenvironment for root induction by allowing perivascular cells to divide and differentiate into adventitious roots in response to bacterial signals. Monocots lack this cambial layer, and the surrounding tissues are often heavily lignified or have lost regenerative competence, which physically and physiologically impedes root formation (Veena and Taylor, 2007).

Moreover, physiological and immune responses of monocot tissues further restrict *A. rhizogenes*-mediated transformation. Many monocots do not secrete sufficient acetosyringone, a phenolic compound essential for activating the bacterial virulence (*vir*) genes. Upon wounding, monocot tissues typically respond by rapid lignification and cell wall closure rather than callus formation or adventitious root differentiation as observed in dicots. This strong defensive reaction effectively isolates the bacteria and prevents the cellular reprogramming necessary for hairy root induction (Li *et al.*, 2015).

Despite these challenges, successful induction of hairy roots in garlic (*Allium sativum* L.), a monocot with a single cotyledon, presents a valuable model system for further exploration. Garlic hairy root cultures offer promising opportunities for studying monocot root biology and for developing scalable biotechnological platforms for secondary metabolite production.

Garlic is well-known for its potent antibacterial, antifungal, and antiviral activities, primarily attributed to sulfur-containing compounds such as allicin and ajoene (Ankri and Mirelman, 1999; Lanzotti, 2006). Establishing a hairy root system in garlic would open new possibilities for enhancing the yield of its bioactive compounds and for functional gene analysis in monocots.

In our previous study, we successfully induced hairy roots in garlic using sprout tissues as the induction material, achieving a hairy root induction rate of 64.4%. The resulting *A. sativum* hairy roots exhibited strong antibacterial activity against *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*, as reflected by MBC/MIC ratios ≤ 2 (Phuong *et al.*, 2023). These results highlight the biotechnological potential of garlic hairy roots for producing bioactive compounds and motivate further optimization of transformation efficiency. Building upon this foundation, we hypothesized that targeting the basal plate region of intact garlic sprouts would improve hairy root induction efficiency.

In this context, the objectives of this study were to develop an effective method for inducing hairy roots in garlic, a recalcitrant monocot. To our knowledge, this is the first study to systematically evaluate the basal

plate region of garlic for hairy root induction. Importantly, this study aimed not only to establish a more efficient protocol for hairy root induction in garlic, but also to contribute a methodological framework that may be applicable to other monocotyledonous species. As such, the findings could serve as a foundation for expanding hairy root biotechnology beyond dicots and into a broader range of economically and medicinally important monocots. We compared root induction efficiency between intact and sectioned garlic sprouts, supplemented with acetosyringone, and validated transformation via PCR analysis of rol genes.

Materials and Methods

Comparative histological analysis of the basal stem disc parenchyma and upper tissue regions in garlic sprouts

Garlic bulbs (*Allium sativum* L.) were purchased from Huy Vu Trading and Service Co., Ltd. (District 5, Ho Chi Minh City, Vietnam). Garlic sprouts approximately 2 cm in length were excised from individual cloves, and the surrounding storage tissues were removed. Thin transverse and longitudinal sections of the garlic sprouts were manually prepared using sterile surgical blades. The sections were immersed in 10% sodium hypochlorite (NaOCl) for 30 minutes, followed by thorough rinsing with distilled water. Samples were then treated with 10% acetic acid for 10 minutes. Subsequently, the tissue sections were stained with iodine green solution (Vert Iod, Merck, Germany) for 15 minutes and rinsed again with distilled water.

After staining, the samples were examined under a light microscope (Olympus CX23, Olympus Corporation, Japan) using a 4× objective lens to identify and compare meristematic regions.

Effect of basal disc cutting methods on hairy root induction efficiency in garlic sprouts

The *Agrobacterium rhizogenes* strain ATCC 15834 was obtained from the RIKEN BioResource Center (Japan) through the MEXT project. To determine an effective method for hairy root induction from the stem disc region of garlic sprouts, whole sprouts (~2 cm in length) were excised from individual cloves, and the surrounding storage tissues were carefully removed. Necrotic tissue at the basal end (~0.5-1 mm) was removed, and the explants were rinsed with distilled water.

Surface sterilization was performed under aseptic conditions in a laminar flow hood. The explants were treated with 1% sodium hypochlorite (NaOCl) for 10 minutes, followed by 70% ethanol for another 10 minutes, and then rinsed three times with sterile distilled water. Sterilized explants were cultured on B5 medium supplemented with 20 g L⁻¹ sucrose and 10 g L⁻¹ agar. The pH of the medium was adjusted to 5.7 ± 0.1 prior to autoclaving at 121 °C and 1 atm for 15 minutes.

A. rhizogenes ATCC 15834 was cultured in Yeast Mannitol Broth (YMB) at 28 °C until the optical density at 600 nm (OD₆₀₀) reached 0.5-0.8. For infection, garlic sprouts were wounded using three different methods: (1) intact sprouts were wounded at the basal end using a sterile needle (~1 mm in diameter); (2) the basal disc was transversely sliced into sections approximately 2 mm thick; and (3) longitudinal slices of the basal disc, also approximately 2 mm thick, were prepared. Localized wounding can enhance transformation efficiency by increasing bacterial access to the plant cells and triggering wound-responsive signaling pathways. The explants were pre-treated with 100 μM acetosyringone for 10 minutes and then immersed in the *A. rhizogenes* suspension (OD₆₀₀ = 0.6) for 5 minutes. After inoculation, explants were blotted dry on sterile filter paper and transferred to B5 medium for co-cultivation in the dark for 48 hours (Phuong *et al.*, 2023).

Following co-cultivation, the explants were transferred to B5 medium supplemented with 250 mg L⁻¹ cefotaxime to eliminate any residual *A. rhizogenes*. Cultures were maintained under a 16h light/8h dark photoperiod. Non-inoculated explants were used as negative controls. The frequency of hairy root induction was evaluated after two weeks of culture. Each treatment included 30 explants per replicate, with three independent replicates performed to ensure reproducibility.

Effect of garlic sprout age on the susceptibility of stem disc to A. rhizogenes

To determine the appropriate developmental stage of the basal stem disc for efficient hairy root induction in garlic, three types of sprout-derived materials were evaluated: (1) Internal garlic sprouts (~2 cm in length) excised from dormant cloves, with all surrounding storage tissues carefully removed to expose the basal stem disc; (2) Garlic sprouts cultured *in vitro* for 10 days, prepared by excising internal sprouts (~2 cm) from surface-sterilized cloves, removing storage tissue, and culturing on B5 medium under standard growth conditions; (3) Garlic sprouts cultured *in vitro* for 20 days, prepared in the same manner.

For 10- and 20-day-old plantlets, roots that had developed during *in vitro* culture were carefully removed prior to *A. rhizogenes* inoculation to standardize the wound site and ensure accurate assessment of hairy root formation from the basal stem disc.

In all cases, explants containing the basal stem disc were subjected to surface sterilization and inoculation with *A. rhizogenes* ATCC 15834 following the procedure described in Section “Effect of basal disc cutting methods on hairy root induction efficiency in garlic sprouts”. The frequency of hairy root induction was evaluated after two weeks of culture under a 16 h light / 8 h dark photoperiod. Each treatment included 30 explants per replicate, with three independent replicates.

Effect of garlic clove tissue on hairy root induction at the stem disc of 10-day-old sprouts

To compare the efficiency of hairy root induction in 10-day-old *in vitro* garlic plantlets prepared under different conditions, two types of explants were used: (1) garlic sprouts (~2 cm in length) excised from cloves with surrounding storage tissue removed prior to *in vitro* culture, and (2) garlic sprouts maintained intact within the cloves during *in vitro* culture. In both cases, materials were surface-sterilized and cultured on B5 medium for 10 days under the same growth conditions before being used for *A. rhizogenes* infection.

Any roots that developed during the culture period were carefully removed prior to inoculation to standardize the wound site and ensure accurate assessment of hairy root formation from the basal stem disc.

All explants containing the basal stem disc were surface-sterilized and inoculated with *A. rhizogenes* ATCC 15834 following the procedure described in Section “Effect of basal disc cutting methods on hairy root induction efficiency in garlic sprouts”. The frequency of hairy root induction was evaluated after two weeks of culture under a 16 h light / 8 h dark photoperiod. Each treatment included 30 explants per replicate, with three independent replicates.

Effect of wounding types on hairy root induction at the stem disc region of garlic plantlets

To enhance the efficiency of hairy root induction and increase biomass yield following infection, this experiment was conducted using 10-day-old *in vitro*-grown garlic plantlets. The garlic sprouts (~2 cm in length) were kept intact within the cloves (i.e., not dissected) to support the development of robust seedlings prior to *A. rhizogenes* inoculation.

Surface sterilization and plantlet initiation were carried out as follows: garlic cloves were peeled and initially soaked in 1% sodium hypochlorite (NaOCl) for 15 minutes. They were then transferred to a laminar flow hood and agitated in 70% ethanol for 15 minutes, followed by immersion in 0.5% NaOCl for an additional 15 minutes. Finally, the cloves were rinsed three times with sterile distilled water.

The cloves were cultured on B5 medium supplemented with 20 g L⁻¹ sucrose and 10 g L⁻¹ agar. The medium pH was adjusted to 5.7 ± 0.1 before autoclaving at 121 °C for 15 minutes. After 10 days of culture, healthy and aseptic plantlets were selected for transformation experiments.

To investigate the effects of wounding and root presence on transformation efficiency, three treatments were applied prior to bacterial inoculation: (1) complete removal of the root system, (2) retention of the intact root system, and (3) retention of the root system with additional localized wounds at the basal and root zones using a sterile needle (~1 mm diameter).

Explants were first immersed in 100 μM acetosyringone for 10 minutes and then incubated in a suspension of *A. rhizogenes* strain ATCC 15834 ($\text{OD}_{600} = 0.6$) for 5 minutes. Co-cultivation was performed in the dark for 48 hours on B5 medium. Subsequently, explants were washed with 250 mg L^{-1} cefotaxime to eliminate surface bacteria and transferred to B5 medium containing 250 mg L^{-1} cefotaxime. Cultures were maintained under a 16/8 h light/dark photoperiod to ensure complete bacterial elimination.

After three weeks, root formation was assessed, fresh root biomass (g per explant) was recorded, and root samples were collected for transgene expression analysis. Non-inoculated *in vitro*-grown garlic plantlets were used as negative controls. Each treatment had 30 explants per replicate, with three independent replicates.

Confirmation of transgenic hairy roots by PCR analysis

Genomic DNA was extracted from garlic roots after 4 weeks of hairy root induction using three different methods, as well as from *in vitro* roots (non-transgenic controls), using the CTAB method as previously described (Porebski *et al.*, 1997). Ri-plasmid DNA was isolated from *A. rhizogenes* ATCC 15834 according to the method of Currier and Nester (Currier and Nester, 1976).

PCR amplification was performed using genomic DNA from hairy root-induced samples, non-transgenic roots, and Ri-plasmid DNA, with primers specific to the *rolB*, *rolC*, and *virG* genes. Primer sequences were as follows: F-*rolB* (5'-GCTCTTGCAGTGCTAGATTT-3'), R-*rolB* (5'-GAAGGTGCAAGCTACCTCTC-3'); F-*rolC* (5'-CTCCTGACATCAAACCTCGTC-3'), R-*rolC* (5'-TGCTTCGAGTTATGGGTACA-3'); F-*virG* (5'-TTATCTGAGTGAAGTCGTCTCAGG-3'), and R-*virG* (5'-CGTCGCCTGAGATTAAGTGTC-3'). The expected amplicon sizes were 423 bp (*rolB*), 626 bp (*rolC*), and 1030 bp (*virG*) (Lan and Quan, 2010).

Each 25 μL PCR reaction contained 100 ng of plant genomic DNA (or 40 ng of Ri-plasmid DNA), 5 μL of 5 \times Taq buffer, 0.5 μM of each primer, and 1 U of Taq polymerase (Bioline). PCR was performed using a SimpliAmp Thermal Cycler with the following thermal cycling conditions: initial denaturation at 95 $^{\circ}\text{C}$ for 5 min; 35 cycles of 95 $^{\circ}\text{C}$ for 30 s, 54 $^{\circ}\text{C}$ for 30 s, and 72 $^{\circ}\text{C}$ for 60 s; followed by a final extension at 72 $^{\circ}\text{C}$ for 10 min. PCR products were analyzed after electrophoresis on 2% agarose (Sigma Aldrich, USA) gels and visualized using a GelDoc Go Gel Imaging System (Bio-Rad). Each treatment used 30 random explants, with three independent replicates.

Statistical analysis

All experiments were performed in triplicate with 30 samples per treatment. Data were analyzed using SPSS software (version 26; IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) was used to evaluate differences among treatment means, followed by Duncan's multiple range test or the independent-samples t-test (Student's t-test), depending on the experimental design. Significant differences were determined at $p < 0.05$. Results are expressed as means \pm standard deviation (SD). Graphs were generated using Microsoft Excel 365 (Microsoft Corp., Redmond, WA, USA).

Results and Discussion

Comparative histological analysis of the basal stem disc parenchyma and upper tissue regions in garlic sprouts

Histological observations

The efficiency of hairy root induction by *Agrobacterium rhizogenes* depends significantly on the type of plant tissue used. Actively dividing tissues such as shoot apical meristems, vascular-associated regions, or cells with cambium-like characteristics often yield higher transformation rates than fully differentiated, mature tissues (Gelvin, 2017; Dhiman *et al.*, 2018). This is largely attributed to cellular mechanisms involving DNA repair and homologous recombination, which are strongly activated in proliferating tissues and facilitate the integration of T-DNA into the plant genome (Gelvin, 2017).

In dicotyledonous plants, the vascular cambium is a common and stable meristematic structure that plays a central role in regeneration. In contrast, monocot species like garlic (*A. sativum*) lack a clearly defined cambial layer in mature tissues, which presents substantial challenges for hairy root induction (Christey and Braun, 2005). Therefore, identifying specific tissue regions within garlic sprouts that retain high mitotic potential is a critical prerequisite for improving transformation efficiency.

To investigate the anatomical basis for tissue-specific competence in garlic, we examined three regions along the garlic sprout (0-day *in vitro* culture): the apical tip (position 1), the mid-sheath zone (position 2), and the basal parenchymatous region adjacent to the basal plate of the stem disc (position 3).

As shown in Figure 1, transverse sections at positions 1 and 2 displayed well-organized concentric leaf sheaths with evenly spaced peripheral vascular bundles (red arrows). In contrast, the basal parenchymatous region (position 3) lacked distinct sheath boundaries and consisted of a homogeneous parenchyma-rich zone containing irregularly distributed vascular strands and emerging root primordia. Longitudinal sections further confirmed that both leaf sheaths and sprout roots anchor into this same basal parenchymatous tissue, highlighting its role as a developmental origin for multiple organ types.

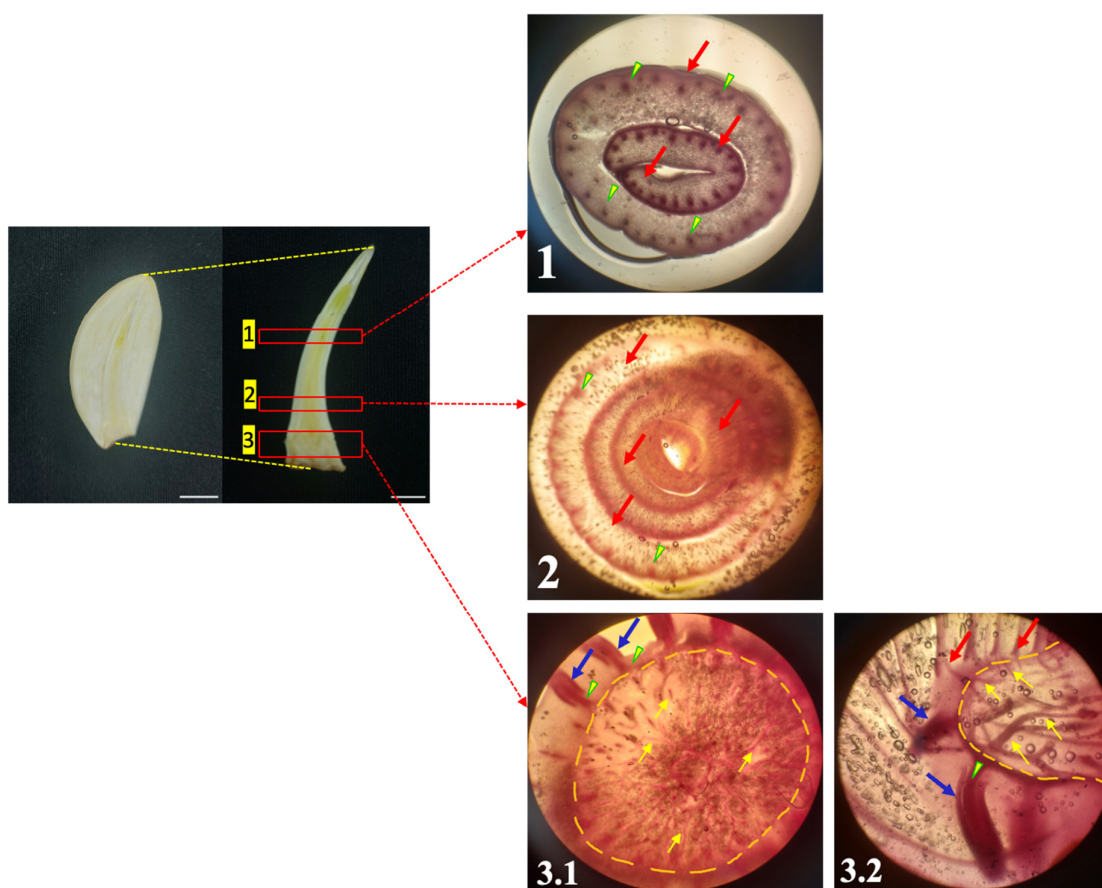


Figure 1. Comparative histological sections of garlic sprout tissues reveal spatial variation in vascular and root initiation zones

Note: A garlic sprout excised from the clove (white scale bar = 0.5 cm) was transversely sectioned at three positions from apex to base (1, 2, and 3) and observed under 40× magnification. At position 3, panel 3.1 represents a transverse section, while panel 3.2 shows a longitudinal section. The region outlined by the yellow dashed line corresponds to the parenchymatous tissue of the basal stem disc. Arrow annotations indicate red arrows - leaf sheaths of the garlic sprout; blue arrows - emergence sites of young roots; solid yellow arrows - vascular strands within the basal parenchyma; and yellow arrows with green outlines - vascular bundles within sprout roots and sheaths

Implications for transformation

These distinct anatomical features provide a structural explanation for the superior responsiveness of the basal stem disc region during hairy root induction. Moradi *et al.* (2018) reported that the basal disc yielded the highest transformation frequency among various explants, but they did not examine its internal anatomy. Our histological analysis clarifies this by showing that the responsive basal region corresponds to a juvenile parenchyma-rich zone with pre-formed root initials and densely packed vascular strands - features aligned with current models of adventitious root formation, where competent founder cells arise from tissues adjacent to the vascular cylinder (Bellini *et al.*, 2014; Verstraeten *et al.*, 2014; Gonin *et al.*, 2019).

Previous studies have also shown that undifferentiated or dedifferentiation-competent tissues are more receptive to *A. rhizogenes* infection. Dhiman *et al.* (2018) noted that *rol* genes in T-DNA can reprogram such tissues for root development, while Giri and Narasu (2000) emphasized the role of tissue plasticity in successful transformation.

Together, these findings identify the basal parenchymatous region of the stem disc as a strategic and biologically competent target tissue for efficient hairy root induction in garlic.

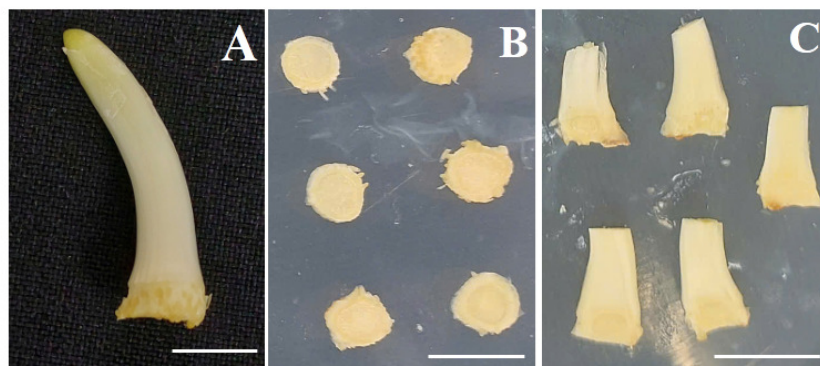
Effect of basal disc cutting methods on hairy root induction efficiency in garlic sprouts

Wounding the stem disc region is a critical factor for inducing hairy roots using *A. rhizogenes*. Mechanical injury not only exposes actively dividing cells but also facilitates direct contact between the bacterium and the target tissue, thereby promoting T-DNA transfer containing *rol* genes from the Ri plasmid (Christey and Braun, 2005; Dhiman *et al.*, 2018). In garlic seedlings at day 0, the parenchymatous tissue in the stem disc is small and located deep within the basal region. To enhance bacterial access, this tissue was exposed by making either longitudinal or transverse cuts, allowing the internal cells to come into direct contact with the bacterial suspension. In the control group, the stem disc remained intact, and only a shallow puncture was made using the tip of a sterile scalpel without removing any tissue. The efficiency of hairy root induction was evaluated and is presented in Table 1 and Figure 2.

Table 1. Effects of garlic sprout treatments on hairy root formation rate two weeks after infection with *A. rhizogenes* ATCC 15834

Garlic sprouts treatments	Intact sprouts (control)	Transverse cut sprouts	Longitudinally cut sprouts
Root formation rate (%)	13.33 ± 5.77 a	0.00 ± 0.00 b	0.00 ± 0.00 b

Different superscript letters within a column indicate significant differences between treatments at $p < 0.05$ (Duncan's multiple range test)



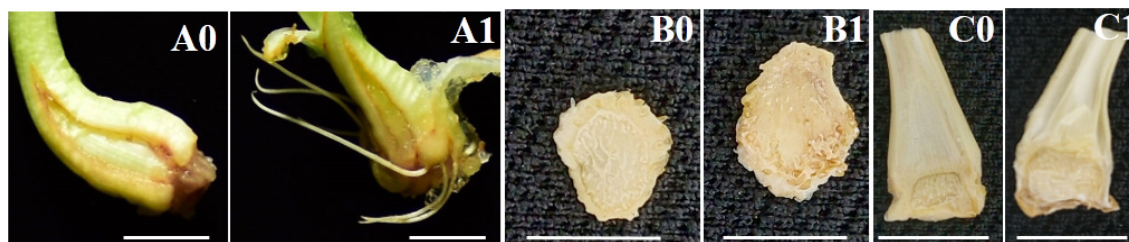


Figure 2. Hair root formation in garlic seedlings subjected to different basal disc treatments after infection with *A. rhizogenes* ATCC 15834

Note: Top row: A - intact garlic sprout, B - sprout cut transversely, C - sprout cut longitudinally, at the time of infection with *A. rhizogenes* (day 0). Bottom row: A0, B0, C0 - corresponding non-infected controls after 2 weeks of culture on B5 medium. A1, B1, C1 - corresponding infected samples after 2 weeks of culture on B5 medium. Each treatment included 30 samples, with all experiments performed in triplicate. Scale bar = 1 cm

The results presented in Table 1 and Figure 2 indicate that cutting the basal disc tissue either transversely or longitudinally did not induce hairy root formation, even after exposure to *A. rhizogenes*. In contrast, intact garlic sprouts that received a deliberate wound at the basal disc region using a scalpel exhibited clear hairy root induction (sample A1). Moreover, in the non-infected control group (A0, B0, C0), no root formation was observed, confirming that hairy root development is specifically induced by bacterial infection and only occurs when the structural integrity of the target tissue is relatively preserved.

These findings suggest that, beyond explant type, the structural integrity of the target tissue at the time of inoculation plays a critical role in the efficiency of hairy root induction. Thin slicing of the basal disc tissue may disrupt tissue continuity, cause widespread damage, or alter the local microenvironment necessary for bacterial attachment and T-DNA transfer. In contrast, samples with preserved tissue structure but with a localized, controlled wound may allow the bacterium to directly access viable, competent cells in an appropriate physiological state.

Previous studies have established that wounding is essential for activating the *vir* gene system in *Agrobacterium*, thereby initiating T-DNA transfer to plant cells (Christey and Braun, 2005; Nester, 2015). Techniques that inflict stronger tissue damage, such as sonication or mechanical crushing, have been shown to enhance transformation efficiency in many plant species (Kumar *et al.*, 2006; Ahsan *et al.*, 2007). However, successful transformation without deliberate wounding has also been reported, for instance, via bacterial entry through stomatal openings (Escudero and Hohn, 1997). These contrasting outcomes indicate that the degree and type of wounding must be optimized for each specific plant-bacterium system, as excessive or physiologically incompatible damage may inhibit rather than promote transformation.

Our findings add a complementary perspective to this issue in garlic (*A. sativum*), a monocot with distinct anatomical features. While Moradi *et al.* (2018) focused on comparing the transformation efficiency across different tissue types, our study centered solely on the basal disc tissue and evaluated how different wound treatments affect transformation success. The results suggest that selecting appropriate wounding strategies is crucial, especially in plant species where tissue integrity is closely linked to regeneration potential, as is the case with garlic.

Effect of garlic sprout age on the susceptibility of stem disc to A. rhizogenes

The developmental stage and physiological age of plant explants are known to influence their susceptibility to *Agrobacterium* infection. Previous studies in maize, soybean, and grapevine have demonstrated that explant maturity, tissue type, and cellular condition can affect transformation efficiency (Colby *et al.*, 1991; Schlappi and Hohn, 1992; Mangena, 2021). In garlic seedlings, it remains unclear whether the sprout age influences the response to *A. rhizogenes*. Therefore, this experiment was conducted to investigate the effect of sprout age on hairy root induction from basal stem discs. Following the previous experiment, three

developmental stages of garlic sprouts were examined: 0-day-old, 10-day-old, and 20-day-old sprouts grown under *in vitro* conditions. The results are presented in Table 2 and Figure 3.

Table 2. Effect of garlic sprout age on the hairy root formation rate two weeks after infection with *A. rhizogenes* ATCC 15834

Age of garlic sprouts	0-day-old	10-day-old	20-day-old
Root formation rate (%)	13.33 ± 5.77 b	46.47 ± 11.55 a	0.00 ^b ± 0.00 b

Different superscript letters within a column indicate significant differences between treatments at $p < 0.05$ (Duncan's multiple range test)



Figure 3. Garlic sprouts at different developmental stages used for *A. rhizogenes* infection and corresponding controls after 2 weeks

Note: A, B, C: Garlic sprouts after 0, 10, and 20 days of *in vitro* culture, respectively, subjected to *A. rhizogenes* infection on day 0. A0, B0, C0: Non-infected control garlic sprouts at 0, 10, and 20 days of *in vitro* culture, respectively, observed after two weeks on medium. A1, B1, C1: Infected garlic sprouts at 0, 10, and 20 days of *in vitro* culture, respectively, observed after two weeks on medium following *A. rhizogenes* inoculation. Scale bar = 1 cm

In Table 2, hairy root formation was first observed in 0-day-old garlic sprouts upon infection with *A. rhizogenes*, indicating that the basal discs were responsive even at an early developmental stage. When the sprout age increased to 10 days, the root induction rate significantly improved, suggesting enhanced susceptibility at this stage. However, in 20-day-old sprouts, no hairy roots were observed, indicating that older tissues may lose their competence for transformation under the given conditions.

As shown in Figure 3, no root formation was observed in the control sprouts (non-infected) at 0 days, and only minimal root elongation was detected in 10-day-old sprouts after two weeks of culture. Interestingly, spontaneous root emergence occurred only in 20-day-old control sprouts. In contrast, *A. rhizogenes*-infected sprouts at both 0 and 10 days displayed clear root induction from the basal disc region after two weeks, with more extensive elongation observed in 10-day-old samples. These observations suggest that garlic sprouts between 0 and 10 days of age respond more rapidly to wounding and bacterial infection at the basal disc, while older sprouts (20 days) appear less responsive. Notably, 10-day-old sprouts demonstrated the most robust response, indicating this stage as optimal for both wounding and *A. rhizogenes* infection. The decline in

responsiveness in older sprouts may be due to progressive tissue differentiation or reduced cellular competence for transformation.

The results of hairy root induction in garlic sprouts at different developmental stages indicate that the physiological condition of the stem disc parenchyma changes over time. The increasing root induction efficiency from day-0 to day-10 sprouts suggests that allowing the sprouts to develop to an appropriate stage enhances their responsiveness to *A. rhizogenes*. At 10 days of age, the basal tissue likely exhibits strong cell division activity, high regenerative potential, and a favorable physiological state for T-DNA integration. Notably, hairy roots formed within two weeks post-infection in the responsive samples.

In contrast, the lack of response in 20-day-old sprouts may be due to advanced tissue differentiation in the basal region, with reduced cell division activity and diminished reprogramming capacity. This highlights the importance of developmental timing, even within the same tissue type, as the maturity level of internal structures may significantly influence the tissue's ability to respond to bacterial infection.

Age-dependent responsiveness to *Agrobacterium*-mediated transformation has also been reported in other species such as maize and grapevine (Colby *et al.*, 1991; Schlappi and Hohn, 1992). In the present study, similar patterns were observed in garlic, a monocot species, further emphasizing the necessity of selecting explants at an appropriate developmental stage to achieve efficient hairy root induction.

Effect of garlic clove tissue on hairy root induction at the stem disc of 10-day-old sprouts

Garlic cloves serve as essential storage tissues, providing both carbohydrates and endogenous phytohormones necessary for early plant development, including root initiation at the stem disc region. However, they also accumulate secondary metabolites such as allicin and phenolic acids, which may interact bidirectionally with *A. rhizogenes* by activating *vir* gene expression while potentially inhibiting bacterial persistence in host tissues (Atif *et al.*, 2021; Yang *et al.*, 2025). To assess the role of clove tissues in hairy root induction, we examined root formation in 10-day-old garlic sprouts under two conditions: with and without the clove tissue attached. The wounding and bacterial inoculation procedures followed the same protocol as in our previous experiment.

The results presented in Table 3 show that garlic plantlets (10 days old) retaining their clove tissue exhibited a significantly higher root formation rate upon infection with *A. rhizogenes* compared to those without clove tissue. Notably, the average number of new roots formed at the stem disc region per plantlet was approximately 2.5 times higher in the clove-retaining group than in the control. Furthermore, as illustrated in Figure 4, the presence of clove tissue enhanced root development after two weeks of *in vitro* culture regardless of bacterial infection, with both C0 and C1 samples showing more roots than B0 and B1. Particularly in C1 - where plantlets retained the clove tissue and were infected with *A. rhizogenes* - root morphology appeared clearly different from C0. Roots tended to form dense clusters around the stem disc, with shorter lengths and lateral or even upward growth directions toward the surface of the medium (Figure 4, C1). This pattern of asymmetrical, clustered, and directionally abnormal growth is characteristic of hairy roots induced by *A. rhizogenes*, as previously described (Giri and Narasu, 2000; Moradi *et al.*, 2018). These morphological features provide preliminary evidence suggesting the occurrence of hairy root induction in sample C1 following bacterial infection.

Table 3. Percentage of roots formed from wound of different organs after 14 days of induction

Organs of <i>A. sativum</i>	Root formation rate (%)	Number of root per plant
Basal plate sprouts (Control)	46.67 ± 11.55	5.33 ± 1.15
Basal plate sprouts with cloves	63.33* ± 6.35	14.67* ± 1.53

(-): Non-detection. The (*) within a column indicate significant differences between treatments at $p < 0.05$ (T-test)

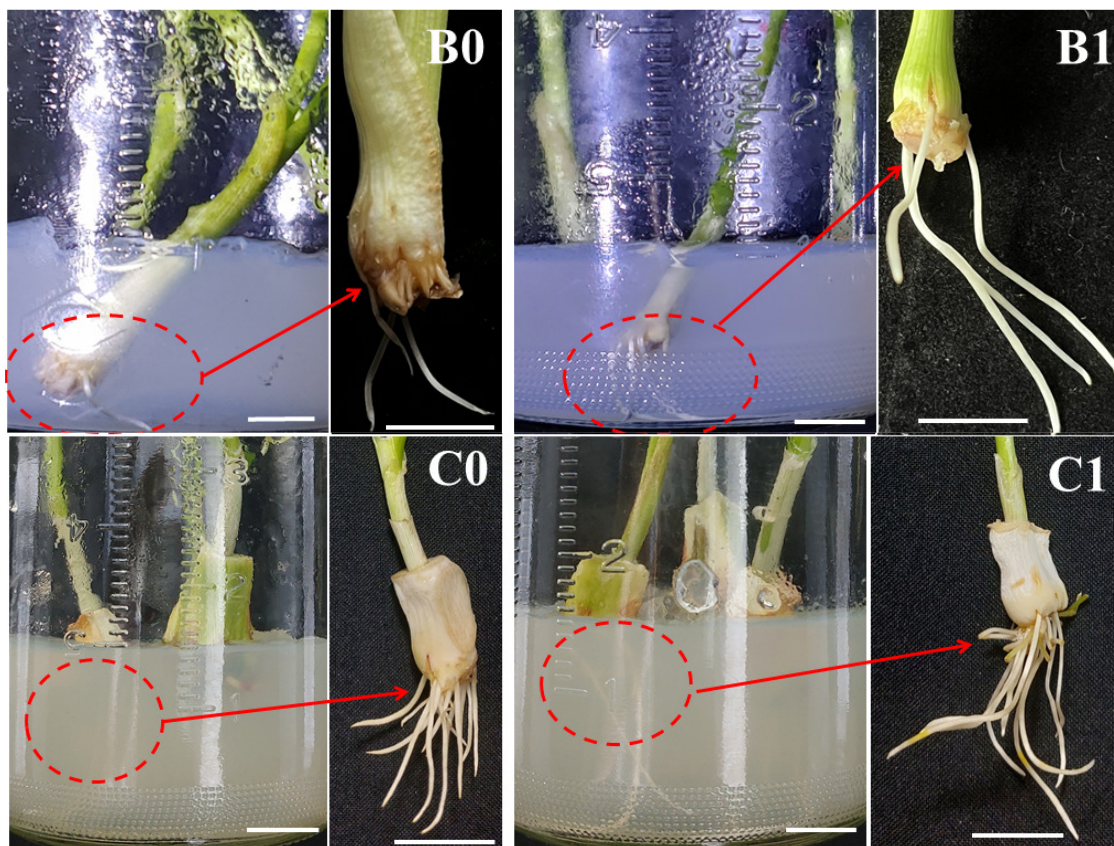


Figure 4. Hairy root induction in 10-day-old *in vitro* garlic plantlets with or without clove tissue, two weeks after infection with *A. rhizogenes* ATCC 15834

Note: B - Plantlets with clove tissue removed; C - Plantlets retaining clove tissue. In each treatment, “0” indicates non-infected control garlic sprouts, and “1” indicates infected garlic sprouts after two weeks. Scale bar = 1 cm

Root formation is a complex process that requires not only a stable energy supply from carbohydrates but also a precise coordination of plant growth regulators, particularly auxins and cytokinins. These hormones have been widely recognized as key factors regulating tissue differentiation and vascular system development during root organogenesis (Liu *et al.*, 2019; Atif *et al.*, 2020; Atif *et al.*, 2021). In this context, garlic clove tissue acts as an important storage organ, providing both carbohydrates and endogenous phytohormones, thereby supporting root development.

In addition to its nutritional role, garlic clove tissue contains secondary metabolites such as phenolic acids and organosulfur compounds (Atif *et al.*, 2021). Previous studies have shown that phenolic compounds can influence the activation of *vir* genes in *A. rhizogenes*, thereby modulating the efficiency of T-DNA transfer (Christey and Braun, 2003). Therefore, retaining the clove tissue during infection may not only promote root development physiologically, but also create a more favorable biochemical environment for hairy root induction.

In sample C1 (clove tissue retained and infected with *A. rhizogenes*), the plantlets not only exhibited a higher average number of roots, but also developed characteristic root morphology - clustered, short roots growing laterally or upward - which is consistent with descriptions of hairy roots in previous studies (Giri and Narasu, 2000). However, since PCR verification of *rolB* gene presence was not performed in this experiment, there remains a possibility of misidentifying normal roots as hairy roots induced by transformation. Therefore, in the next experiment involving garlic plantlets with clove tissue retained, root samples exhibiting similar morphology will be collected and analyzed via PCR to confirm the presence of the *rolB* gene.

Effect of wounding types on hairy root induction at the stem disc region of garlic plantlets

Wounding of plant tissues plays a vital role and is almost a prerequisite for the successful genetic transformation process. Such wounds are not only mechanical injuries but also act as chemical signals that activate the T-DNA transfer system of *A. rhizogenes*, primarily through the perception of phenolic compounds and sugars released from the damaged tissues (Gelvin, 2017; Dhiman *et al.*, 2018). Based on our previous experiment, where 10-day-old garlic sprouts retaining the basal clove tissue were identified as suitable explants for hairy root induction, we employed this same explant type in the this experiment to investigate how different wounding types affect the induction of hairy roots at the stem disc region. The control group consisted of garlic sprouts with intact roots that were not exposed to *A. rhizogenes*. The other treatments included: (1) explants with intact roots inoculated with the bacterium, (2) explants with intact roots inoculated after mechanical wounding of the stem disc using a sterile scalpel, and (3) explants inoculated with the bacterium after complete removal of the original root system.

After three weeks of co-cultivation, successful transformation of *A. sativum* with *A. rhizogenes* strain ATCC 15834 was confirmed across all three inoculation treatments. The induced roots exhibited morphological characteristics consistent with the previously suspected hairy root phenotype. PCR analysis revealed the presence of *rolB* and *rolC* genes in all root samples tested, while the *virG* gene was absent (Figure 5), indicating effective elimination of the bacterium following cefotaxime treatment. No amplification of transformation-related genes was detected in the non-inoculated control group, confirming the specificity of the transformation process. These results demonstrate that all three wounding methods could induce hairy roots with characteristic morphology upon exposure to the bacterium (Figure 5 and Figure 6).

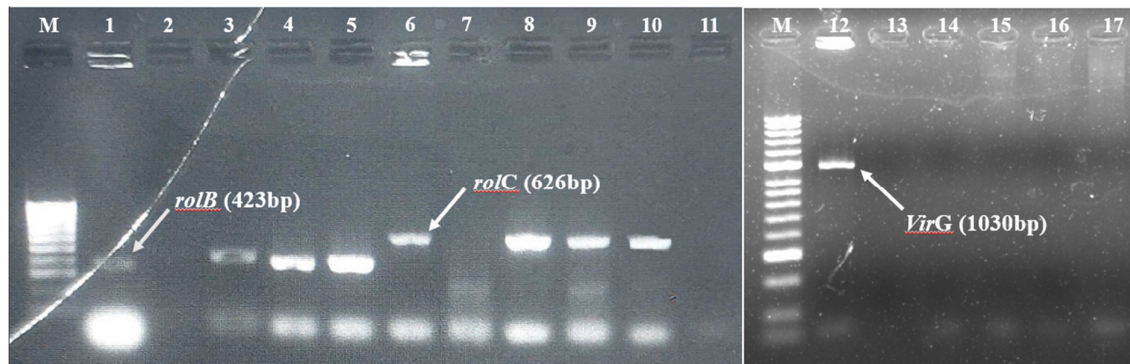


Figure 5. PCR amplification of *rolB*, *rolC*, and *virG* genes from garlic root samples under different inoculation conditions

Note: M: 100 bp plus DNA ladder. Lanes 1, 6, 12: Positive control - *A. rhizogenes* ATCC 15834 genomic DNA amplified with *rolB*, *rolC*, and *virG* primers. Lanes 11, 17: PCR negative controls (no DNA template). Lanes 2, 7, 13: Negative control - DNA from non-inoculated garlic roots. Lanes 3, 8, 14: DNA from garlic roots with intact root system during *A. rhizogenes* inoculation. Lanes 4, 9, 15: DNA from garlic roots with intact root system and localized wounding at the stem disc. Lanes 5, 10, 16: DNA from garlic roots with the entire root system removed prior to inoculation

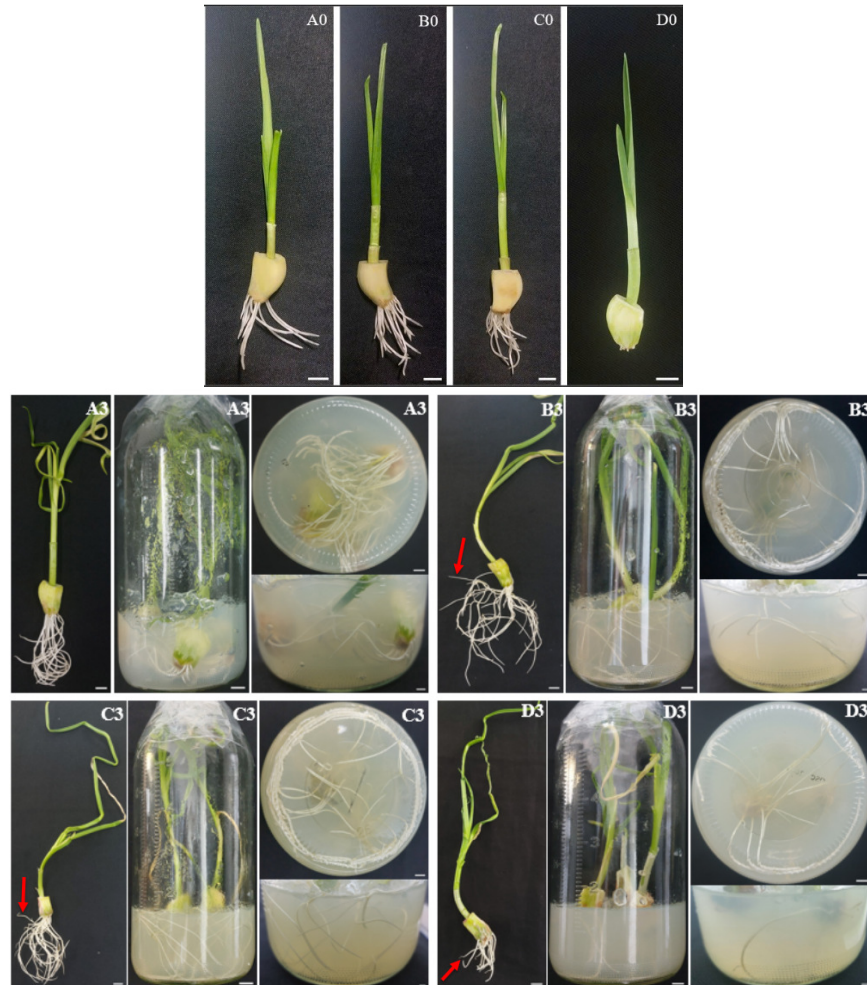


Figure 6. Hairy root induction in *A. sativum* under different inoculation and wounding conditions

Note: (A) Non-inoculated control. (B) Inoculated with *A. rhizogenes* while retaining the entire root system. (C) Inoculated with *A. rhizogenes*, with the root system retained and local wounding applied at the basal stem and root junction. (D) Inoculated with *A. rhizogenes*, with the entire root system removed prior to inoculation. “0”: Day 0 (prior to inoculation); “3”: Three weeks after inoculation. Photographs include whole plants, *in vitro* culture bottles, and root morphology on agar plates. Red arrows: the non-geotropic growth characteristic of hairy roots. Scale bar = 1 cm

Root morphology varied markedly among treatments. Control plants developed normal roots with typical geotropic growth and minimal lateral branching. In contrast, roots induced by *A. rhizogenes* displayed characteristics typical of hairy roots, including multidirectional growth, extensive lateral branching, and emergence of roots above the agar surface (Figure 6). Among the treated groups, the most vigorous and well-developed hairy root development was observed in plants with intact root systems during inoculation (treatment B). In comparison, physical wounding (treatment C) or complete root removal (treatment D) appeared to reduce both root biomass and the typical hairy root architecture, likely due to wound-induced physiological stress.

Although all infected treatments showed evidence of transformation, significant differences were observed in both root formation rate and fresh root biomass (Table 4). Treatments retaining the root system - with or without localized wounding - achieved a 100% root formation rate. However, the fresh biomass significantly differed between the two: the unwounded group produced the highest biomass (1.00 ± 0.02 g), while the wounded group showed reduced biomass (0.71 ± 0.16 g). In contrast, removing the root system prior

to inoculation resulted in a lower formation rate (63.33%) and the lowest root biomass (0.27 ± 0.02 g). One-way ANOVA followed by Duncan's test confirmed significant differences ($p < 0.05$) among treatments.

Table 4. Fresh root biomass of *A. sativum* seedlings after 3 weeks of hairy root induction

Treatment	Root formation rate (%)	Fresh root biomass per sample (g)
Control (intact roots, not inoculated with <i>A. rhizogenes</i>)	0.00 ^c ± 0.00	1.00 ^a ± 0.13
Root system retained (1)	100.00 ^a ± 0.00	1.00 ^a ± 0.02
Root system retained with localized wounding (2)	100.00 ^a ± 0.00	0.71 ^b ± 0.16
Root system completely removed (3)	63.33 ^b ± 6.35	0.27 ^c ± 0.02

Different superscript letters within a column indicate significant differences between treatments at $p < 0.05$ (Duncan's multiple range test)

PCR analysis confirmed that *A. rhizogenes* successfully transferred T-DNA in all three treatments involving bacterial exposure, including the one without mechanical wounding. This raises an important question: can *A. rhizogenes* induce hairy root formation without physical injury to plant tissues? Morphological observations (Figure 6), root formation rates, and fresh root biomass (Table 4) revealed clear differences among treatments.

Treatments 1 (root system retained, no wounding) and 2 (root system retained with localized wounding) both achieved 100% root formation rates. However, their biomass differed significantly - Treatment 1 produced the highest biomass (1.00 ± 0.02 g), while Treatment 2 yielded only 0.71 ± 0.16 g. Treatment 3, which combined complete root removal and wounding at the stem disc (similar to Treatment 2), showed the lowest transformation efficiency (63.33%) and minimal biomass (0.27 ± 0.02 g). These results suggest that while wounding may not prevent T-DNA transfer, it compromises post-transformation growth, likely due to stress responses or damage to receptive tissues.

A reasonable explanation is in the presence of acetosyringone in all infection treatments. This phenolic compound strongly induces *vir* gene activation on the Ri plasmid and can trigger T-DNA transfer even in the absence of mechanical damage when supplied exogenously (Gelvin, 2003; Kumar *et al.*, 2006). Several studies confirm that transformation can occur in intact tissues, as evidenced by *rol* gene expression and opine production (Brencic *et al.*, 2005; Chen and Otten, 2017).

The garlic stem disc, rich in parenchyma and intercellular spaces, likely facilitates bacterial access through natural openings, reducing the need for injury. Maintaining tissue integrity also preserves endogenous auxin balance, which plays a central role in hairy root induction (Spanò *et al.*, 1981; Gelvin, 1990). Conversely, wounding may trigger oxidative stress or lignification, which impairs root development (Tepfer, 1984; Rana *et al.*, 2017).

This phenomenon of injury-associated inhibition has also been observed in tomato (*Solanum lycopersicum*) and potato (*S. tuberosum*), where excessive wounding leads to browning, necrosis, or poor root growth (Christey, 2001; Ahsan *et al.*, 2007). Notably, garlic appears particularly sensitive, showing a pronounced decline in root biomass even when root induction efficiency remains high.

Altogether, these findings indicate that *A. rhizogenes* can induce hairy roots in garlic without mechanical wounding, provided acetosyringone is present and physiological conditions are favorable. Retaining the original root system and minimizing tissue damage enhances both transformation success and post-infection root development. This offers a practical and effective strategy for hairy root induction in *A. sativum* under *in vitro* conditions.

Conclusions

Hairy root induction in garlic - a monocotyledonous species traditionally considered recalcitrant to transformation - presents a significant technical challenge. This study demonstrates that the basal plate of stem disc is the most suitable tissue region for inducing hairy roots, particularly when using 10-day-old garlic seedlings with intact clove tissue and root system, without wounding, and in the presence of acetosyringone. Under these conditions, both the induction efficiency and the stability of hairy root growth were markedly improved. Importantly, these findings are supported by both morphological observations and molecular confirmation via PCR analysis of *rol* genes. These results not only contribute to optimizing the hairy root induction protocol in garlic but also open new possibilities for applying hairy root culture techniques to other monocot species, for which reports on hairy root culture remain very limited. Further studies could explore the scalability of this protocol or its applicability to other economically and medicinally important monocot crops.

Authors' Contributions

Conceptualization: QNDP, VTBP, TQT; Data curation: VTBP, NDK; Formal analysis: VTBP; Funding acquisition: QNDP, VTBP; Investigation: VTBP, NDK, TH; Methodology: QNDP, VTBP; Project administration: QNDP, VTBP; Resources: VTBP; Software: VTBP; Supervision: QNDP, VTBP; Validation: VTBP; Visualization: VTBP; Roles/Writing - original draft: VTBP, TQT; and Writing - review & editing: QNDP, VTBP, TQT, CMD.

All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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