Effect of Sucrose on Agrobacterium rhizogenes C58C1-Induced Hairy Root Growth in Tobacco (Postprint)

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Abstract

Robust growth of hairy roots is crucial for establishing hairy root-AM fungus dual culture systems. To optimize hairy root culture medium components, determine the appropriate sucrose concentration for hairy root growth, and improve the growth status of tobacco hairy roots, this study used Agrobacterium rhizogenes strain C58C1 to induce hairy root formation from leaves of two tobacco varieties, NC82 and Va116. Following PCR verification, the hairy roots were cultured in 1/2MS medium containing different sucrose concentrations under both solid and liquid culture conditions. The effects of sucrose on hairy root growth in the two tobacco varieties were investigated by measuring branch number, fresh weight (FW), and dry weight (DW). The results demonstrated that C58C1 could induce hairy root formation from leaves of both tobacco varieties, but with different induction rates. NC82 exhibited a higher induction rate (87.3%), which was 2.26 times that of Va116 (38.6%). Medium sucrose concentration significantly affected hairy root growth, with effects varying according to tobacco variety and initial branch number. For solid culture optimization of hairy roots induced from NC82 and Va116, the sucrose concentrations that inhibited branch number increase were 25 g · L-1 and 15 g · L-1, respectively. For liquid culture optimization, maximum FW(DW) was achieved at 25 g · L-1 and 15 g · L-1, respectively, reaching 0.541 g (0.055 g) and 0.474 g (0.050 g). Considering branch number, FW(DW), and hairy root growth vigor comprehensively, the optimal medium sucrose concentration for C58C1-induced hairy roots of NC82 was 25 g · L-1, while that for Va116 was 15 g · L-1. This study optimized the appropriate sucrose concentration and culture method for tobacco hairy root medium composition, laying a foundation for subsequent large-scale propagation of hairy roots and addressing the critical issue of poor host growth in establishing hairy root-AM fungus dual culture systems.

Full Text

Effects of Sucrose on the Growth of Tobacco Hairy Roots Induced by *Agrobacterium rhizogenes* C58C1

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Abstract

Healthy hairy root growth is critical for establishing hairy root-AM fungus dual culture systems. To optimize medium composition and determine the optimal sucrose concentration for hairy root growth, this study investigated the effects of sucrose on hairy root development in two tobacco varieties. Hairy roots were induced from leaves of *Nicotiana tabacum* varieties NC82 and Va116 using *Agrobacterium rhizogenes* strain C58C1 and verified by PCR. The roots were then cultured on 1/2MS medium containing various sucrose concentrations under both solid and liquid conditions. Branch number, fresh weight (FW), and dry weight (DW) were measured to assess growth.

The results showed that C58C1 successfully induced hairy roots from both tobacco varieties, though induction rates differed significantly. NC82 exhibited a higher induction rate (87.3%) than Va116 (38.6%), representing a 2.26-fold difference. Sucrose concentration significantly affected hairy root growth in a variety- and initial branch number-dependent manner. For solid culture, the inhibitory sucrose concentrations for branch number increase were 25 $g \cdot L^{-1}$ for NC82 and 15 $g \cdot L^{-1}$ for Va116. In liquid culture, maximum FW and DW were achieved at 25 $g \cdot L^{-1}$ for NC82 (0.541 g FW, 0.055 g DW) and 15 $g \cdot L^{-1}$ for Va116 (0.474 g FW, 0.050 g DW). Considering branch number, biomass accumulation, and overall growth vigor, the optimal sucrose concentrations were determined to be 25 $g \cdot L^{-1}$ for NC82 hairy roots and 15 $g \cdot L^{-1}$ for Va116 hairy roots. This study optimizes the sucrose concentration for tobacco hairy root culture, providing a foundation for large-scale propagation and addressing the critical challenge of poor host growth in hairy root-AM fungus dual culture systems.

Keywords: hairy root, sucrose, medium optimization, branch number, fresh weight, dry weight

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Introduction

Arbuscular mycorrhizal (AM) fungi form mutualistic symbioses with over 80% of terrestrial vascular plants, enhancing mineral nutrient uptake, improving stress and disease resistance, modifying soil structure, and facilitating remediation of heavy metal contamination. These fungi hold tremendous potential as natural biofertilizers and biocontrol agents in agricultural and forestry production. However, AM fungi are obligate symbionts that require host plants to complete their life cycle, which has prevented their axenic cultivation and limited large-scale inoculum production.

Hairy root-AM fungus dual culture systems, which utilize Ri T-DNA transformed roots (hairy roots) as hosts for AM fungal spores, represent an effective solution for AM fungus cultivation and mass production. Previous research demonstrates that hairy root growth significantly influences AM fungal infection, development, and sporulation, making robust hairy root culture a critical prerequisite for successful dual culture systems.

Agrobacterium rhizogenes infection of plant tissues induces hairy root formation through integration and expression of T-DNA from the Ri plasmid, yielding genetically stable, transgenic root clones. Our preliminary studies showed that *A. rhizogenes* strain C58C1 can induce hairy roots from tobacco leaves, but growth performance is affected by medium composition, plant growth regulators, and culture conditions. Among carbon sources used in hairy root culture (sucrose, fructose, and glucose), sucrose provides more carbon atoms and energy, is more readily perceived by plant cells, and supports superior growth compared to glucose or fructose. Establishing an optimal tobacco hairy root culture system is essential for developing a tobacco hairy root-AM fungus dual culture platform, and sucrose concentration warrants careful investigation as a key medium component.

This study induced hairy roots from two tobacco varieties (NC82 and Va116) using *A. rhizogenes* C58C1. After PCR verification, the roots were cultured on 1/2MS medium containing various sucrose concentrations under solid and liquid conditions. By measuring branch number, fresh weight, and dry weight, we determined the optimal sucrose concentration for hairy root growth, providing fundamental conditions for establishing a tobacco hairy root-AM fungus dual culture system and offering reference data for other plant species.

1.1 Materials

Bacterial strain: *Agrobacterium rhizogenes* C58C1, provided by the Biochemistry Laboratory, College of Life Sciences, Guizhou University.
Tobacco varieties: NC82 and Va116, provided by the Plant Physiology Laboratory, College of Life Sciences, Guizhou University.

1.2.1 Hairy Root Induction

Sterile tobacco seedlings: Established following the method of Lu (2020) using 75% ethanol and 10% sodium hypochlorite for surface sterilization.

Bacterial suspension preparation: C58C1 cultures were activated following Lu (2020) and suspended in MS liquid medium containing 100 $\text{mol} \cdot \text{L}^{-1}$ acetosyringone to prepare the inoculum.

Induction procedure: Following Lu (2020), tobacco leaf explants (2 cm × 2 cm) were pre-cultured for 2–3 days, immersed in bacterial suspension for 8–10 minutes, then co-cultivated on MS medium (30 $\text{g} \cdot \text{L}^{-1}$ sucrose) in darkness for 2–3 days. Explants were transferred to antibiotic medium (500 $\text{mg} \cdot \text{L}^{-1}$ cefotaxime, 100 $\text{mg} \cdot \text{L}^{-1}$ timentin) to induce hairy roots. Fast-growing, vigorously branching roots were selected and propagated on 1/2MS medium at 25 °C in darkness for use in optimization experiments.

1.2.2 Optimization and Growth Measurement

PCR verification: Conducted following Hu et al. (2015) to confirm hairy root identity for both varieties.

Solid culture optimization: Hairy roots were cultured on 1/2MS solid medium with three factors: tobacco variety (NC82, Va116), initial branch number (0 or 1–2 branches), and sucrose concentration (0, 5, 10, 15, 20, 25, 30, 35, 40 $\text{g} \cdot \text{L}^{-1}$), yielding 36 treatments with three replicates of three plates each (90 mm diameter). Medium pH was adjusted to 5.8–6.0, and cultures were maintained at 25 °C in darkness. Branch numbers were recorded every 2 days for 20 days.

Liquid culture optimization: Based on solid culture results, 1/2MS liquid medium was prepared with sucrose concentrations of 15, 20, 25, and 30 $\text{g} \cdot \text{L}^{-1}$ (four treatments, three replicates). Eighty milliliters of medium were dispensed into 150 mL flasks, sterilized, and inoculated with vigorous hairy roots. Cultures were incubated at 25 °C in darkness with shaking at 110 rpm. After 30 days, roots were blotted dry, weighed for fresh weight, then oven-dried at 102–105 °C for 10 minutes, followed by drying at 70–80 °C to constant weight for dry weight measurement.

Induction rate (%) = (Number of explants producing hairy roots / Total number of explants) × 100.

Data were analyzed using Microsoft Excel 2010 and SPSS 22.0.

Results

2.1 Induction and PCR Verification

A. rhizogenes C58C1 successfully induced hairy roots from both NC82 and Va116 tobacco leaves. Hairy roots emerged at wound sites approximately one week post-infection. Induction rates at 12 days were 54.4% for NC82 and 20.5% for Va116, increasing to 87.3% and 38.6% respectively by 19 days. Induced hairy roots exhibited extensive branching, rapid growth, and normal development on hormone-free medium [Figure 1: see original paper].

PCR analysis confirmed that roots induced from both varieties were true hairy roots. Amplified DNA from both tobacco hairy roots showed specific products. Comparison with the C58C1 positive control and DNA molecular weight marker (M) confirmed the presence of the rolB gene fragment (~741 bp) [Figure 2: see original paper], indicating successful integration of the Ri plasmid rolB gene into the NC82 and Va116 genomes.

2.2.1 Effects of Sucrose Concentration on Branch Number in Solid Culture

Branch numbers in both NC82 and Va116 hairy roots increased over time, with the magnitude of increase varying by sucrose concentration [FIGURE:3, FIGURE:4]. At 0 and 5 $g \cdot L^{-1}$ sucrose, branch increase was minimal for all treatments, plateauing at approximately 8 branches after 4 days. At 10 $g \cdot L^{-1}$, only NC82 roots with initial 1–2 branches continued increasing to 18 branches by day 14, while other treatments ceased growth. At 15–40 $g \cdot L^{-1}$, branch numbers increased continuously throughout the 20-day culture period, reaching maximum values. For NC82, roots starting with 0 branches achieved 18, 27, 26, 23, 13, and 15 branches at 15, 20, 25, 30, 35, and 40 $g \cdot L^{-1}$ sucrose, respectively; those starting with 1–2 branches reached 32, 29, 71, 54, 24, and 31 branches. For Va116, roots starting with 0 branches reached 29, 12, 25, 8, 8, and 16 branches, while those starting with 1–2 branches reached 75, 30, 40, 25, 24, and 25 branches.

Sucrose concentration significantly affected branch number in a variety- and initial branch-dependent manner [FIGURE:5, FIGURE:6]. Across 0–40 $g \cdot L^{-1}$, all treatments showed an initial increase followed by decrease in branch number, with characteristic inhibitory concentrations. For NC82, roots starting with 0 branches showed increasing branch numbers up to 20 $g \cdot L^{-1}$ (27 branches at day 20), which represented the inhibitory concentration; beyond this, branch numbers declined. For NC82 starting with 1–2 branches, the inhibitory concentration was 25 $g \cdot L^{-1}$. For Va116, the inhibitory concentration was 15 $g \cdot L^{-1}$ for both initial branch categories.

Therefore, optimal sucrose concentrations for solid culture were 20–30 $g \cdot L^{-1}$ for NC82 hairy roots and 15–25 $g \cdot L^{-1}$ for Va116 hairy roots.

2.2.2 Effects of Sucrose Concentration on Hairy Root Biomass in Liquid Culture

Sucrose concentration significantly affected biomass accumulation and growth status in a variety-dependent manner [TABLE:1, FIGURE:7]. For NC82, FW and DW increased then decreased across 15–30 $g \cdot L^{-1}$, peaking at 25 $g \cdot L^{-1}$ (0.541 g FW, 0.055 g DW). For Va116, maximum FW and DW occurred at 15 $g \cdot L^{-1}$ (0.474 g FW, 0.050 g DW), declining with higher concentrations. After 30 days, roots at 15 $g \cdot L^{-1}$ showed extensive branching, slender morphology, and minimal browning, while those at 30 $g \cdot L^{-1}$ exhibited reduced branching and pronounced browning, with browned roots becoming thick, hardened, and brittle [Figure 7: see original paper].

TABLE:1 Fresh and dry weights of two hairy root types under different sucrose concentrations (mean ± SD, n=3)

Sucrose ($g \cdot L^{-1}$)	NC82 FW (g)	NC82 DW (g)	Va116 FW (g)	Va116 DW (g)
15	0.371$±0.020*b*|0.038±0.005*AB*|0.474±0.026*a*|0.050±0.004*A*||20|0.317±0.059*bc*|0.034±0.006 C			

Different letters indicate significant differences between treatments (P<0.05).

Integrating data from solid culture (branch number) and liquid culture (biomass), the optimal sucrose concentrations for *A. rhizogenes* C58C1-induced hairy roots were determined to be 25 $g \cdot L^{-1}$ for NC82 and 15 $g \cdot L^{-1}$ for Va116.

Discussion and Conclusion

The obligate symbiotic nature of AM fungi limits their agricultural application. Hairy root-AM fungus dual culture systems offer an effective solution for axenic cultivation and mass production. Our previous research demonstrated that *Scutellospora* spp. could establish symbiosis with tobacco hairy roots induced by *A. rhizogenes* A4 and produce new spores, while *Claroideoglomus etunicatum* could form symbiotic associations with tobacco hairy roots induced by both C58C1 and A4 strains. We also observed that hairy root growth status, medium composition, exogenous substances, pH, and temperature affect AM fungal spore development in dual culture systems.

As the host, hairy root growth critically impacts large-scale AM fungus production; poor hairy root performance restricts AM fungal development. Among factors influencing hairy root growth (medium, pH, light, temperature), sucrose provides essential energy and carbon, representing a decisive factor and key medium component in dual culture systems. Sucrose concentration significantly affects both hairy root growth and spore development.

In plant tissue culture, carbon sources provide energy substrates and regulate osmotic potential. Sucrose is the most commonly used carbon source and standard for plant cell culture, with most synthetic media employing sucrose as the sole carbon source, though concentration optimization is crucial. Previous studies report optimal sucrose concentrations of 20–40 $g \cdot L^{-1}$ for plant tissue culture, with specific examples including 30 $g \cdot L^{-1}$ for *Dendrobium*, *Pueraria phaseoloides*, and *Psammosilene tunicoides* hairy roots. However, dual culture studies often use lower concentrations: 10 $g \cdot L^{-1}$ sucrose in M medium for carrot hairy roots with *Gigaspora margarita* and *Rhizophagus intraradices*; 10 $g \cdot L^{-1}$ in 1/10MS medium for citrus hairy roots with *Gi. margarita* and *Glomus mosseae*; and 10 $g \cdot L^{-1}$ in MSR medium for various other systems. Interestingly, Srinivasan et al. (2014) observed higher sporulation in *Rhizophagus irregularis* after 70 days, possibly due to reduced sucrose, while D' Souza et al. (2013) found sucrose-free medium promoted spore germination. Mohan et al. (2017) reported that 10 $g \cdot L^{-1}$ sucrose optimized root colonization, though 20–30 $g \cdot L^{-1}$ produced more new spores.

Most AM fungus pure culture studies have used carrot, with few reports on tobacco, and no previous investigations of optimal sucrose concentrations for tobacco hairy root-AM fungus dual culture systems. Our results demonstrate that 30 $g \cdot L^{-1}$ sucrose effectively induces hairy roots from both tobacco varieties, though induction rates differ (NC82: 87.3%; Va116: 38.6%), confirming that genotype affects transformation efficiency. Branch number increased over time in a sucrose concentration-dependent manner, with inhibitory concentrations of 25 $g \cdot L^{-1}$ for NC82 and 15 $g \cdot L^{-1}$ for Va116. Biomass accumulation peaked at 25 $g \cdot L^{-1}$ for NC82 and 15 $g \cdot L^{-1}$ for Va116. Considering all growth parameters, the optimal sucrose concentrations are 25 $g \cdot L^{-1}$ for NC82 and 15 $g \cdot L^{-1}$ for Va116.

An optimized hairy root culture system is fundamental for successful dual culture establishment. This study determined optimal sucrose concentrations and culture methods for two tobacco varieties, laying the groundwork for large-scale hairy root propagation and tobacco hairy root-AM fungus dual culture systems, while providing valuable reference data for other plant species.

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