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OPTIMIZATION OF *Agrobacterium rhizogenes* - MEDIATED HAIRY ROOT INDUCTION IN *Salvia officinalis*

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Abstract

Sage (Salvia sp.) is a key medicinal plant from the Lamiaceae family. Recently, the mass production of hairy roots has become important for producing plant secondary metabolites, offering fast growth and stable bioactive compound production, making them ideal for transgenic research. In this study, the optimization of hairy root induction conditions by Agrobacterium rhizogenes in Salvia officinalis, has been evaluated. For this purpose, the effect of 3 different times of bacterial co-culture (24, 48, 72 h) with explants (whole seedling, leaf, stem and petiole) was investigated. The 48h co-culturing interval was considered the most suitable time. The highest rate of success in hairy root production (88.4 %) belonged to the leaf explants. In order to evaluate the stability and quantity of hairy roots, liquid 0.5X MS medium with 3 treatments of IBA (0, 0.5, 1 mg/L) were used. The highest percentage of growth was observed in liquid 0.5X MS and at the hormone level of 1 mg/L. Transgenic hairy roots produced were confirmed by PCR.

Key words*: hairy root, secondary metabolites, Salvia officinalis, Agrobacterium rhizogenes, PCR.*

INTRODUCTION

The history of treating diseases with medicinal plants is as old as the history of human life on Earth. Humans have always treated themselves with the help of medicinal plants, based on experience, science, and according to their needs. Medicinal plants are a crucial resource for both traditional medicine and the pharmaceutical industry. In modern times, the interest in using herbal remedies for disease treatment and prevention has grown significantly. As a result, the incorporation of medicinal plants into the production of pharmaceutical, food, cosmetic, and health products is expanding rapidly, raising their value and significance in the global market (Cunningham, 1993; Angelova-Teneva & Beshkova, 2015).

Some bioactive compounds, essential in the pharmaceutical industry, cannot be synthesized in the lab and can only be extracted from plants. These compounds either have unknown chemical structures or possess such complex structures that their synthetic production is challenging and costly, which is why pharmaceutical companies show great interest in plant-based medicines (Omid Beygi, 2010).

Salvia officinalis L. is one of the best-known species of the mint family (Lamiaceae), native to the Mediterranean regions but distributed throughout the world (Hojati, 2024; Rahmanifar et al., 2023). It has a long-standing history in traditional medicine for treating conditions such as fever, rheumatism, bronchitis, and various mental disorders. Numerous studies have highlighted its beneficial effects, including anti-diabetic (Christensen et al., 2010), anti-inflammatory (Rodrigues et al., 2011), antimicrobial (Garcia et al., 2012), antioxidant (Generalić et al., 2012; Mihai et al., 2021), neurodegenerative disease prevention (Takano et al., 2011), and anti-tumor properties (Al Barzanji et al., 2013).

To enhance the production of biochemical substances, various biotechnological strategies have been developed (Asghari, 2006; Zayova et al., 2016). Hairy root cultures, generated by the soil bacterium *Agrobacterium rhizogenes*, are an effective and efficient method for mass-producing secondary metabolites. These cultures grow rapidly, are easy to maintain, and

can synthesize a diverse array of beneficial compounds (Shanks & Morgan, 1999). The bacterium induces the development of hairy roots by transferring a T-DNA fragment from its root-inducing plasmid (Ri) into the plant's genome, leading to the expression of stable *rol* genes (*rol A*, *rol B*, *rol C*, and *rol D*) in the plant cells (Chaudhuri et al., 2005). The efficiency of this transformation process depends on factors such as plant species, tissue age, physiological state, bacterial strain, bacterial concentration, and co-culture duration (Kumar et al., 2006). Additionally, the culture medium's composition, especially its hormonal components like auxins, plays a significant role in the growth rate and secondary metabolite production (Washida et al., 2004; Pakdin Parizi et al., 2014).

Although hairy root induction using *A. rhizogenes* is a well-known process, there have been few studies focused on inducing hairy roots in sage. This research seeks to optimize of *A. rhizogenes*-mediated hairy root induction in *S. officinalis* and investigate the factors that influence this process.

MATERIALS AND METHODS

Preparation of sterile seedlings

Sage seeds were obtained from the National Agricultural Research and Development Institute. To prepare sterile seedlings, seeds were surface disinfected with 75% ethanol for 30 seconds, followed by a 10-minute treatment with a 5% sodium hypochlorite solution. Seeds were abundantly rinsed with distilled water and then cultured in hormone-free MS medium supplemented with 3% sucrose (Murashige & Skoog, 1962) (Table 1).

The pH of the medium was adjusted to 5.6-5.8. The cultures were maintained under a 16-hour light/8-hour dark photoperiod at 25°C.

Bacterial strain and growth conditions

The reference strain *A. rhizogenes* ATCC 15834 was used for hairy root transformation in *S. officinale*.

The bacterial strain was reactivated from lyophilized stock on Nutrient Agar medium (purchased VWR International BVBA, Leuven, Belgium). The refreshed, pure strain was then grown in Nutrient Broth (purchased VWR International BVBA, Leuven, Belgium) for 3 days at 26°C and 150 rpm shaking. Cells were then harvested by centrifugation at 6000 rpm, for 10 min. The obtained pellet was washed with sterile 0.5X MS medium and resuspended in the same nutritional solution, at 10^8 CFU/ml.

Table 1. MS culture medium substances

Category of substances	Substance	Concentration (mg/L)
Macroelements	NH_4NO_3	1650.0
	KNO_3	1900.0
	$CaCl_2.2H_2O$	440.0
	KH_2PO_4	170.0
	$MgSO_4.7H_2O$	370.0
Microelements	$ZnSO_4.7H_2O$	8.6
	$MnSO_4.4H_2O$	22.3
	$CuSO_4.5H_2O$	0.025
	H_3BO_3	6.2
	KI	0.83
	$Na_2MoO_4.2H_2O$	0.25
	$CoCl_2.6H_2O$	0.025
Chelater iron	$FeSO_4.7H_20$	27.8
	Na_2EDTA	37.3
Organic supplements	Nicotinic acid	0.5
	Pyridoxine (B_6)	0.5
	Thiamine (B_1)	0.1
	Glycine	2.0
	Myoinositol	100.0

Inoculation of explants

To inoculate the explants with bacteria, leaves, stems, petioles and whole seedlings were used. The explants were divided into 2 cm pieces and shaken with bacterial suspension for 20 minutes, to allow the transfer of the rooting genes within the vegetal tissue. The explants were then placed on 0.5X MS-NH_4 solid culture medium without hormones and transferred to a growth room at 25°C under dark conditions. After 24, 48 and 72 hours of inoculation, the explants were washed and transferred to 0.5X MS-NH4 medium supplemented with 500 ppm of Cefotaxime. This process was then repeated weekly, each time decreasing the antibiotic amount with a 100 ppm. After complete removal of the bacteria, the explants were placed in antibiotic-free medium. Then, daily observations were made to check the appearance of hairy roots in the explants inoculated with bacteria. After one month of hairy root growth, various characteristics such as T-DNA transfer success

and the rate of hairy root production were evaluated.

Development and maintenance of hairy roots

Hairy roots longer than 2 cm were isolated and transferred into Erlenmeyer flasks containing 0.5X MS liquid culture medium. A shaker was used at 120 rpm in the dark at 25°C to maintain the hairy roots and they were subcultured every two weeks. To investigate the stability and growth rate of the hairy roots, three concentrations of IBA hormone (0, 0.5 and 1) were used in 0.5X MS liquid culture medium, and finally, after 60 days, their wet and dry weights were measured.

Molecular analysis

Plant DNA was extracted using a classical cetyltrimethylammonium bromide (CTAB) isolation protocol (Ciucă et al., 2020). Briefly, fresh roots from untreated plants and bacterial inoculated explants were aseptically mortared. Then 100 mg of root paste was treated with 1 ml CTAB lysis buffer, containing 2% CTAB, 1.42M NaCl, 200 mM EDTA, 100 mM Tris, 1% sodium thiosulphate, and 2% β-mercaptoethanol in sterile deionized water. After 1h of water bath incubation at 65°C, with periodic stirring, the samples were centrifuged at 10,000 ×g for 10 min. The supernatant was treated with an equal volume of chloroform: isoamyl alcohol 24:1 (v/v), and the mixture was centrifuged at 10,000 ×g for 10 min. The aqueous phase was then collected and treated with 1.5 ml RN-ase solution (10 mg/ml) for 45 min at 37°C. Samples were then treated with 60 µl 3M sodium acetate (pH 5.5), 60 µl 10 mM ammonium acetate, and 0.7 volumes of 2-propanol, and incubated overnight at -20°C. Samples were then centrifuged for 6 min at 12,000 × g, and the translucent pellet was hydrated in TE (1M Tris, 0.5M EDTA, pH 8) and stored at -20°C.

Bacterial DNA was extracted from fresh cultures obtained in NB. The DNA was isolated using the ZR Fungal/Bacterial DNA MiniPrep™ (Zymo Research, SUA), according to the manufacturer recommendations.

The PCR analysis was conducted to confirm the sage transformation by the *A. rhizogenes.* The targeted genes were *rolB* and *rolC* (Li et al., 2015). The oligonucleotide primer pairs used to amplify these genes were 5′- GCT CTT GCA GTG CTA GAT TT -3′ and 5′- GAA GGT GCA AGC TAC CTC TC -3′ for *rolB*, 5′- CTC CTG ACA TCA AAC TCG TC -3′ and 5′- TGC TTC GAG TTA TGG GTA CA -3′ for *rolC* (Grzegorczyk et al., 2006).

Each reaction was performed with 2 µl of template DNA, 1X Dream Taq buffer, 10 mM dNTPs, 10 µM primers, 0.5U Dream Taq and up to 25 µl of MilliQ water. The PCR was performed with Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany) and began with a 3 min initial denaturation step at 94°C, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 1 min, extension at 72°C for 1 min, and finally 7 min at 72°C. The PCR products migrated to 1.2% agarose gel, in 0.5X TBE, stained with ethidium bromide. The expected amplification products should have 383 bp for *rolB* and 585 bp for *rolC* (Grzegorczyk et al., 2006).

Statistical analysis

Statistical calculations, analysis of variance, and graph drawing were performed using SPSS software ver. 22, and comparison of means was performed using Duncan's test at a confidence level of 95%.

RESULTS AND DISCUSSIONS

After 2-3 weeks after *A. rhizogenes* inoculation of leaf, stem, petiole and seedlings explants, hairy roots appeared (Figure 1).

Figure 1. *Salvia* plant grown *in vitro* condition (a); Hairy roots grown after inoculation (b)

The highest percentage of hairy root induction and the largest number of hairy roots were observed at the wounded areas of *A. rhizogenes* treated leaf explants, with a success rate of

88.4%. In contrast, stem explants showed the lowest success in hairy root formations (10.4%). Following stem explants, petiole explants had the second-lowest induction rate (12.4%), while whole seedlings demonstrated an average ability of 32.4% to form hairy roots. Additionally, all control explants lacked hairy roots. These suggest that the type of explant plays a significant influence in the succeeding rate of *A. rhizogenes* transformation and hairy roots formation. Differences in the rate of hairy root production due to the use of different types of explants have been noted in other previous studies. Several research groups also reported leaf explants to have the highest efficiency in producing hairy roots (Mirzaie Delbari et al., 2022; Zare et al., 2022; Kardoost & Mahmoodnia, 2023). In only a few cases, leaf explants were the only ones capable of developing hairy roots, while to the rest of the explants the transformation was ineffective. When studying *A. rhizogenes* transformation in sage, Norouzi et al. (2017) were able to observe hairy roots formation only on bacterial treated leaf explants, while on petiole and stem explants no hairy roots were developed (Norouzi et al., 2017). However, there are also cases in which leaf explants produced fewer hairy roots than other treated explants (Lee at al., 2007; Brijwal & Tamta, 2015). Differences in physiological status, DNA synthesis, and cell division of various tissues may account for their competence to produce hairy roots (Pirian et al., 2012).

On the other hand, in the present study, petiole and stem explants were very sensitive to displacement and quickly turned brown. There losses caused by necrosis occurred during the subcultures in which the cefotaxime concentration was reduced. And although sage stem and petiole explants formed callus and hairy roots, they eventually died due to the browning and necrosis (Figure 2).

Infection of plant tissues by different strains of *A. rhizogenes* in many cases causes necrosis and browning of plant tissues. One of the reasons for the browning of explants after infection by *A. rhizogenes* is the presence of phenolic compounds and their oxidation by the enzyme polyphenol oxidase. This enzyme is responsible for their browning in plant tissue and plays an important role in activating programmed cell death (Shakeran & Keyhanfar, 2017). Hypersensitivity reactions are considered one of the plant defense responses, which are generally characterized by features such as localized and rapid cell death and the accumulation of antimicrobial agents *in situ* (Richter & Ronald, 2000). This is a series of events that cause necrosis and cell destruction. This can occur in the cell layer where the T-DNA was introduced or it may prevent the regeneration of transgenic cells isolated from such necrotic tissues. Thus, the yield of clones with transgenic cells is reduced (Shakeran & Keyhanfar, 2017).

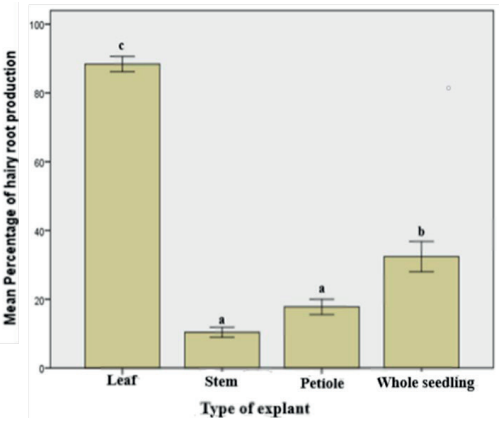

Figure 2. The hairy roots production on different explants. The data are presented as average values, while bars reflect the standard deviation. Different letters of columns indicate significant differences based on Duncan's test ($p < 0.05$)

In several studies, different types of explants from various plant species exhibit differing percentages of browning after being infected with *A. rhizogenes*, highlighting the influence of both plant genotype and bacterial strain on the rate of browning (Putalun et al., 2007; Zolala et al., 2007; Rahnama et al., 2008; Shakeran & Keyhanfar, 2017). In addition, the current study shows that in most cases, before hairy roots formation on the explants, callus formed at the site of cuts and wounds, and then hairy roots appeared. In some other studies, callus formation in explants has been reported after induction with *A. rhizogenus* and during the process of hairy root formation (Li et al., 2018; Sohrabinejad et al., 2018).

In this study, the rate of hairy root induction in a bacterial co-culture system was evaluated at

three different time points: 24, 48, and 72 h. The results revealed a significant variation in induction efficiency across the time intervals. The lowest percentage of hairy root induction occurred within explants from the 24 h treatment, with only 12.4% successful hairy root formation. In contrast, the highest induction rate was observed at 48 h, with a substantial 80% of the cultures successfully forming hairy roots. However, after the 72 h treatment, excessive explants colonization with *A. rhizogenes* made difficult the removal of the bacteria, and despite the antibiotic treatment, a complete bacterial elimination was not always achieved, leading to explant destruction. As a result, the transformation rate dropped significantly to 43.4% (Figure 3).

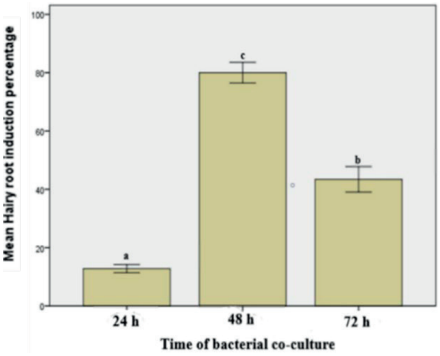

Figure 3. The rate of hairy roots induction after different times of co-culturing the explants with the bacteria. The data are presented as average values, while bars reflect the standard deviation. Different letters of columns indicate significant differences based on Duncan's test ($p < 0.05$)

Similar studies have also identified 48 hours as the ideal duration for bacterial co-culture and T-DNA transfer, supporting the findings of this research (Shakeran & Keyhanfar, 2017; Mirzaie Delbari et al., 2022; Kardoost Parizi & Mahmoodnia Meimand, 2023).

To confirm the transgenic nature of the hairy root lines and verify the presence of the *rolB* and *rolC* genes, polymerase chain reaction (PCR) was conducted using specific primers for these genes. The PCR results confirmed the presence of T-DNA, validating that the hairy roots had been successfully transformed by *A. rhizogenes* (Figure 4).

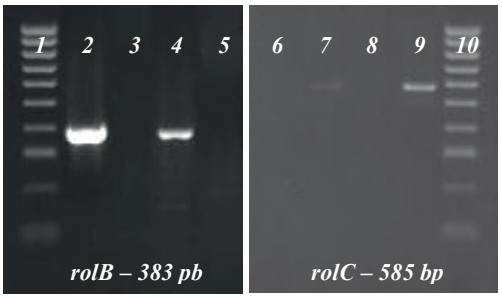

Figure 4. PCR amplification of *rol B* and *rolC* oncogenes in *A. rhizogenes* transformed roots and *A. rhizogenes* ATCC 15834 strain. The corresponding lanes are as follows: 1 and 10 = Molecular marker of 100bp, 2 and 9 = transformed sage roots, 3 and 8 = untreated sage roots, 4 and 7 = *A. rhizogenes* ATCC 15834 strain, 5 and 6 = negative control (MilliQ water)

The variance analysis table (Table 2) in this study indicated that the application of the auxin hormone IBA significantly affected the growth characteristics of the hairy roots, including both fresh and dry biomass. However, a treatment of 0.5 mg/l of IBA had no significant effect on hairy root growth. The highest growth rate was observed at a concentration of 1 mg of IBA hormone, where sage roots had 10.65 g fresh weight, and 0.36 g dry weight (Figure 5).

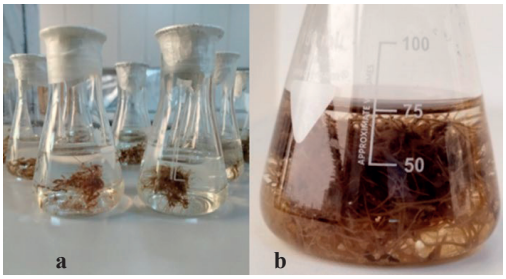

Figure 5. Sage hairy roots:
a. Hairy roots on the first day of hormone treatment;
b. Hairy roots after 2 months of treatment with 1 mg IBA/L

These results are consistent with the findings of Sohrabinejad et al. (2018) about production of hairy root in *Calendula officinalis*. Their study showed that among treatments with IAA concentrations of 0, 0.5, and 1 mg/l, the best root growth occurred at the 1 mg/l concentration in 0.5X B5 medium (Sohrabinejad et al., 2018).

Table 2. Results of analysis of variance, the effect of hormone concentration on hairy root growth

Tests of Between-Subjects Effects						
Source	Dependent variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected model	Wet weight (g)	112.963[a]	2	56.482	76.642	0.000
	Dry weight (g)	0.065[b]	2	0.033	12.933	0.001
Intercept	Wet weight (g)	499.278	1	499.278	677.493	0.000
	Dry weight (g)	0.541	1	0.541	214.032	0.000
IBA	Wet weight (g)	112.963	2	56.482	76.642	0.000
	Dry weight (g)	0.065	2	0.033	12.933	0.001
Error	Wet weight (g)	8.843	12	0.737		
	Dry weight (g)	0.030	12	0.003		
Total	Wet weight (g)	621.085	15			
	Dry weight (g)	0.637	15			
Corrected Total	Wet weight (g)	121.807	14			
	Dry weight (g)	0.096	14			

[a]R Squared = 0.927 (Adjusted R Squared = 0.915)
[b]R Squared = 0.683 (Adjusted R Squared = 0.630)

Furthermore, studies on secondary metabolite production in *Cichorium intybus* L. through hairy root induction, in the presence of four concentrations of naphthyl acetic acid (0, 0.5, 1, and 1.5 mg NAA/l), showed that combination of 1.5 mg NAA/l with 3% and 4% sucrose produced higher increases in both fresh and dry weight, as well as other growth parameters (Fathi et al., 2019).

CONCLUSIONS

Hairy roots induction on medicinal plants to produce high-value therapeutic compounds has become increasingly important in recent years. This raised interest is driven by the substantial demand for plant-derived metabolites, which are often found in low quantities in natural plant sources. As a result, there is a growing emphasis on exploring methods that can enhance the production of such bioactive compounds.

Hairy roots are known to generate plants secondary metabolites more effectively, rapidly, and consistently than other plant tissues such as aerial parts or callus cultures. Therefore, advancing *in vitro* plant propagation techniques, particularly for generating and producing hairy roots, is a key research objective. This study focuses on *Salvia officinalis*, a plant of significant medicinal value, and examines the production of hairy roots within this species.

Different plant explants exhibited varying capacities for hairy root formation, and factors such as the duration of bacterial co-culture and the concentration of plant hormones were found to significantly influence both the induction and growth of the hairy roots. Co-inoculation on sage leaves for 48 h in *A. rhizogenes* ATCC15834 suspension, as well as 1 mg of IBA hormone treatment revealed to be the most promising way for increasing the biomass production of hairy roots in this medicinal plant species. Although research on hairy root production in sage species has been limited, the results of this study suggest that inducing hairy roots in this plant holds great potential for producing valuable medicinal compounds.

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