Anti-inflammatory Compounds Produced in Hairy Roots Culture of *Sphaeralcea Angustifolia*

Rogelio Reyes-Pérez
Universidad Autonoma del Estado de Morelos Centro de Investigacion en Biotecnologia
Maribel Herrera-Ruiz
Centro de Investigación Biomédica del Sur: Centro de Investigacion Biomedica del Sur
Irene Perea-Arango
Universidad Autonoma del Estado de Morelos Centro de Investigacion en Biotecnologia
Fernando Martínez-Morales
Universidad Autonoma del Estado de Morelos Centro de Investigacion en Biotecnologia
José De Jesús Arellano-García
Universidad Autonoma del Estado de Morelos Centro de Investigacion en Biotecnologia
Maria del Pilar Nicasio Torres (pisaliva@yahoo.com.mx)
Instituto Mexicano del Seguro Social https://orcid.org/0000-0003-2045-6665

Research Article

Keywords: Agrobacterium rhizogenes, Malvaceae, scopoletin, tomentin, sphaeralcic acid

Posted Date: April 30th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-473254/v1

Version of Record: A version of this preprint was published at Plant Cell, Tissue and Organ Culture (PCTOC) on September 8th, 2021. See the published version at https://doi.org/10.1007/s11240-021-02162-8.

Abstract

Sphaeralcea angustifolia (Cav) G. Don is used in traditional Mexican medicine to treat inflammations and gastric disease. Its anti-inflammatory and immunomodulatory activities in mice and rats acute and chronic models have been attributed mainly to scopoletin. Scopoletin reduced joint inflammation, the number of new vessels, production of endogenous angiogenic inducers, and reversed the histopathological alterations in rat adjuvant-induced arthritis. Tomentin and sphaeralcic acid from *S. angustifolia* cells in suspension proved anti-inflammatory and immunomodulatory activities in mice kaolin/λ-carrageenan-induced arthritis. Transformed roots of *S. angustifolia* have been proposed as active compounds producers. A high transformation frequency mediated by *Agrobacterium rhizogenes* ATCC15834/pTDT was obtained from nodal segments (59.5 ± 10.5%, 145 hairy root lines) and leaves (40.0 ± 25, 52 hairy root lines) of 2-month-old plantlets. Among seven lines selected according to their phenotypic characteristics and growth index, the SaTR N7.2 line presented the highest sphaeralcic acid production (17.6 ± 1.72 mg/g DW); this production was 440-fold superior to that reported in *S. angustifolia* wild plants, and in comparison to cells in the suspension of *S. angustifolia* in MS medium with nitrate restriction this was 263-fold higher when cultured in flasks and 5-fold higher in a stirred-tank type bioreactor. The SaTR N7.2, SaTR N5.1, SaTR N7.1, and SaTR N15.1 lines excreted sphaeralcic acid into the culture medium at similar levels. Genetic transformation of hairy roots was confirmed by amplifying a 490 bp fragment of the *rolC* gene. *S. angustifolia* hairy root cultures producers of scopoletin and sphaeralcic acid can be stressed by nitrate reduction and/or copper increased to stimulate scopoletin and sphaeralcic acid production.

Key Message

Hairy roots lines of *Sphaeralcea angustifolia*, producers of scopoletin and sphaeralcic acid anti-inflammatory compounds, were established by first time through plant transformation mediated by *Agrobacterium rhizogenes* ATCC15834/pTDT.

Introduction

Sphaeralcea angustifolia (Cav) G. Don (Malvaceae) is known in Mexico as “Vara de San José” or “Hierba del negro” (Aguilar et al., 1994). This plant is used in Mexican traditional medicine to treat blows, fractures, inflammation, and gastric problems (Aguilar et al., 1994; Argueta et al., 1994). Extracts from aerial parts of this plant had demonstrated anti-inflammatory activity, with scopoletin identified as the main active compound (García-Rodríguez et al., 2012; Juárez-Ciriaco et al., 2008; Meckes et al., 2004). Topical administration of a gel formulation made with 1% of *S. angustifolia* dichloromethane extract standardized in the scopoletin content showed therapeutic effectiveness and tolerability in patients with osteoarthritis of hands, capable of reducing the associated symptoms: pain, inflammation, and joint stiffness (Romero-Cerecero et al., 2013).

Scopoletin has been isolated from many plants (Jain et al., 2002), and several biological activities had been reported, such as anti-inflammatory (Ding et al., 2008; Moon et al., 2007), antioxidant (Gwak et al., 2011; Shaw et al., 2003), anti-proliferative (Thani et al., 2010), anti-angiogenic (Pan et al., 2009), anti-thyroid, anti-hypertensive (Aldi et al., 2015), anti-hyperuricemic (Zeng et al., 2020), and anti-diabetic (Jang et al., 2020), among others.

In a previous report, a cell suspension culture of *S. angustifolia* was employed to improve scopoletin production. In such a report, tomentin and sphaeralcic acid were found in the dichloromethane-methanol extract of the cell suspension culture, and they were isolated and identified as potent anti-inflammatories in acute and chronic mice models (Pérez-Hernández et al., 2014; Nicasio-Torres et al., 2016). Both compounds were able to modulate the production of pro- and anti-inflammatory cytokines (Serrano-Román *et al.*, 2015; Nicasio-Torres et al., 2017). Production of scopoletin, tomentin, and sphaeralcic acid in cell suspension culture was scale up to bioreactor of stirred tank (Pérez-Hernández et al., 2019a). Antioxidant activity of tomentin isolated from the stem bark of *Jatropha podagrica* was also reported (Minh et al., 2019).

Hairy root cultures are generated by the insertion of the transferred DNA (T-DNA) fragment of the root-inducing (Ri) plasmid after the infection of plant tissues with *Agrobacterium rhizogenes*. These cultures are considered an important biotechnological tool for secondary metabolites production with biological activity (Gutierrez-Valdes et al., 2020). Hairy roots have genetic and biochemical stability, can grow in media lacking plant growth regulators, and have short duplication times, attributes that give them advantages over cell suspension cultures (Rajashekar et al., 2016). Hairy root cultures of *Cichorium intybus* L. have been scaled up to acoustic mist bioreactor to produce esculin, a coumarin with anti-inflammatory activity (Bais et al., 2002). Some pharmaceutical companies (Mibelle, Sederma, and Rootec) use this technology to produce compounds for health; Rootec company produces camptothecin (anticancer and antiviral) from *Camptotheca acuminata* (Ochoa-Villarreal *et al.*, 2016).

There are no reports in the literature about the genetic transformation of *S. angustifolia*, so studies of species of the Malvaceae family transformed by *A. rhizogenes* ATCC 15834 strains were used as a starting point. Among such species, we found *Gossypium hirsutum* and *Gossypium barbadense* (Triplett et al., 2008); *Abutilon indicum* L. (Indian mallow; Sajjalaguddam and Paladugu, 2016); *Althaea officinalis* L. (marshmallow; Tavassoli and Safipour Afshar, 2018); and *Urena lobata* L (Phuong et al., 2018). Based on the above, we decided to establish a transformation protocol for *S. angustifolia* using *A. rhizogenes* strains and select hairy root lines with a good growth rate and a high production of scopoletin, tomentin, and sphaeralcic acid, as an industrially competitive biotechnological system.

Materials And Methods

Plant material

Sphaeralcea angustifolia seeds were collected at Huichapan, Hidalgo State, Mexico, and taxonomically identified by Santiago Xolalpa Molina, M.Sc., responsible for the medicinal plants Herbarium of the

Mexican Institute of Social Security from Mexico City (Herbarium, IMSSM) with voucher number 16,412.

Aseptic culture

Seeds of *S. angustifolia* were disinfected with 70% ethanol for 3 min, then with 1.2% sodium hypochlorite solution for 10 min, and finally washed four times with sterile distilled water for 10 min. The sterile seeds were sprouted in glass bottles with 50% nutrients of Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), supplemented with 30 g/L sucrose, pH 5.7 and 3.0 g/L phytagel. Before use, the culture medium was autoclaved at 121°C and 1.2 kg/cm^2 pressure for 18 min. The glass containers with the seeds were incubated for their germination in a culture room at 25 ± 2°C with a light/dark (16 h:8 h) photoperiod under 50 μM m^{-2} s^{-1} of warm white-fluorescent light intensity.

Transformation procedure

Leaves and nodal segments were obtained from 2-month-old seedlings. The explants were dipped in a suspension of the bacterial strains *A. rhizogenes* K599/pTDT (cucumopine type; OD_{550} = 0.5), A4/pTDT or ATCC 15834/pTDT (agropin type; OD_{550} = 0.5) for 10 min. The binary vector pTDT contains the gene that encodes the tomato threonine deaminase enzyme, which gives a red fluorescence to the transformed plant tissue. Subsequently, each explant was dried on sterile filter paper to remove excess of bacterial suspension and transferred to a glass bottle with co-culture medium based on MS medium, pH 5.7 and 3.0 g/L phytagel, and incubated at previously described conditions for 48 h. At the end of this time, the bacteria were eliminated with three washes with sterile distilled water containing the antibiotics ceftriaxone and cefotaxime (300 mg/L each) for 10 min each. Consecutively, the explants were dried on a piece of sterile filter paper and then transferred to a glass bottle with MS medium supplemented with 10 g/L of sucrose and the above-mentioned antibiotics at the same concentrations and incubation conditions described.

Selection of putatively transformed roots

The number of nodal segments and leaf explants infected with the *A. rhizogenes* strain ATCC 15834/pTDT, A4/pTDT, and K599/pTDT generated hairy root were recorded.

Each hairy root sprouting from any explant infected with *A. rhizogenes* strain was individualized when reaching a length of approximately 1.0 cm and considered a putatively transformed root line. Root and explant numbers were used for classification and identification. The roots were cultured individually in new glass bottles containing semi-solid MS medium supplemented with 10 g/L sucrose, 3.0 g/L phytagel, pH 5.7, and 300 mg/L cefotaxime and ceftriaxone; in the subsequent subculture, the antibiotic concentration was reduced to 100 mg/L. Finally, hairy roots free of *A. rhizogenes* were transferred to glass bottles with basal MS medium added with 30 g/L, pH 5.7, 2.5 g/L of phytagel without antibiotics and incubated under the conditions previously described.

The selection criteria for hairy roots were: 1) physical characteristics (plagiotropic roots and increased branching), 2) growth capacity in the absence of growth regulators, and 3) emission of red fluorescence under an epifluorescence microscope (Carl Zeiss V8).

Each hairy root line (2 g) was cultivated into 250 ml Erlenmeyer flasks with 80 mL of liquid MS medium added with 30 g/L sucrose and incubated at 25 ± 2°C with light/darkness (16 h:8 h) photoperiod under 50 µM m^{-2} s^{-1} intensity of warm white-fluorescent light in an orbital shaker at 110 rpm (New Brunswick Scientific Co., Inc.). Every 4 weeks, hairy root cultures were vacuum filtered using a Buchner funnel (Whatman filter paper No. 1, 9-cm diameter), and the retained roots weighted and transferred (2 g) to flasks with fresh medium.

Growth of hairy root lines

From selected hairy root lines, the growth was evaluated after 2 and 3 weeks in culture; three flasks were taken at the beginning of the culture and at each time of culture. Each flask was vacuum filtered using a Buchner funnel (Whatman filter paper No. 1, 9-cm diameter) and the retained roots washed with sterile distilled water; the fresh weight (FW) was determined, and the roots dried in an oven (Thelco 160 DM) at 65°C for 48 h. The growth index (GI) at 2 and 3 weeks of growth was determined in dry weight (DW) considering the following formula:

$$GI = \frac{(final\ dry\ weight\ -\ initial\ dry\ weight\ )}{initial\ dry\ weight}$$

Transformation confirmation

Total DNA samples of each hairy root line were isolated and used as a template for PCR analysis to determine the presence of the *rolC* gene (Bonhomme *et al*., 2000) and the absence of *virD2* gene (Haas et al., 1995) in transformed hairy roots using specific primers: the forward primer 5' TGTGACAAGCAGCGATGAGC 3' and reverse 5' GATTGCAAACTTGCACTCGC 3' was used to amplify a 490 bp fragment of the *rol*C gene, and the second one forward 5' ATGCCCGATCGAGCTCAAGT 3' and reverse 5' ACCGTCGGCTCTACAAACCCAGTCC 3' to amplify a 338 bp fragment of the *virD2* gene, this gene was used as a control since it is not transferred to the plant cell during the transformation process. A DNA amplification kit from Vivantis was used following the manufacturer directions. Each sample was prepared in 200 µL PCR tubes on ice to obtain a total reaction volume of 50 µL, comprising 1 µL DNA template, 5 µL 10X Taq DNA polymerase reaction buffer, 2 µL 50 mM $MgCl_2$, 1 µL of each primer (10 µM), 2 µL dNTPs mixture (2 mM), 0.5 µL of recombinant Taq DNA polymerase (5 U/µL) and 37.5 µL of nuclease free water. PCR amplification for both genes was carried out in an Mastercycler Gradient device (Eppendorf, Hamburg, Germany) under the following conditions: 1 cycle of 5 min at 95°C, 35 cycles of 1

min denaturing at 95°C, 1 min annealing at 50°C, and 1 min extension at 72°C; as a final point, one cycle of 5 min final extension at 72°C (Moreno-Anzúrez *et al.*, 2017).

The PCR products were subjected to electrophoresis in 1% agarose gel at 100 volts for 60 min and visualized on a UV transilluminator (BioDoc-It™ Imaging System, Upland, CA, USA) using ethidium bromide staining for visualization and documentation.

Chemical analysis

Extract preparation

The dry biomass of three flasks from each line of hair roots was extracted three times by maceration (24 h for each procedure) at room temperature with a mixture of reactive grade solvents (CH_2Cl_2:CH_3OH 9:1; Merck) in a ratio of 1:50 (w/v). The extracts were filtered, pooled, and concentrated to dryness under reduced pressure. The content of sphaeralcic acid and scopoletin in the CH_2Cl_2:CH_3OH extract was determined by high-performance liquid chromatography (HPLC, Pérez-Hernández *et al.*, 2014; Nicasio-Torres *et al.*, 2016; Nicasio-Torres *et al.*, 2017; Pérez-Hernández *et al.*, 2019a).

HPLC conditions

HPLC analyses were performed using a Waters system (2695 Separation Module) coupled to a diode array detector (2996) with a 190–600-nm detection range and operated through the Manager Millennium software system (Empower 1; Waters Corp., Boston, MA, USA). Separations were performed on a Spherisorb RP-18 column (250 × 4.6 mm, 5.0 µm; Waters Corporation) using a constant temperature of 25 °C during analyses. Samples (20 µL) were eluted at a flow rate of 1.0 mL/min with a mobile phase gradient of high purity: (A) H_2O with TFA (0.5%, Sigma Aldrich) and (B) CH_3CN high purity (Merck). The compounds were detected by monitoring scopoletin and tomentin absorbance l= 344 nm and sphaeralcic acid l= 357 nm. The identification of scopoletin (99% purity; Sigma-Aldrich Chemical, Mexico), tomentin (93%), and sphaeralcic acid (95%) was carried out by comparing their absorption spectra and their retention times (scopoletin 10.3 min, tomentin 10.2 min, and sphaeralcic acid 22.8 min). The concentration ratio for scopoletin quantification ranges from 1.25 to 20 µg/mL and sphaeralcic acid from 2.5 to 40 µg/mL. The regression equation for scopoletin was (y) = 165407 (x) +16720, r^2 = 0.9993, and for sphaeralcic acid it was (y) = 7381.9 (x) + 1362.2, r^2 = 0.9998, with r^2> 0.99 (Nicasio-Torres *et al.*, 2016; Serrano-Román *et al.*, 2020).

Results And Discussion

Hairy roots induction

Many factors influence the transformation of plants, such as the type of explant, the culture conditions, bacterial strain, and the *Agrobacterium*-host interactions, among others (Colling et al., 2010). Hairy roots from explants infected with the strains ATCC 15834/pTDT and A4/pTDT appeared about 7 days post co-

culture; from the K599/pTDT strain, roots emerged after 20 days; in the nodal segments appearing first a whitish callus at the site of infection (Fig. 1). Both types of explants showed susceptibility to the *A. rhizogenes* ATCC 15834/pTDT strain; also, leaf explant showed callus formation. The highest transformation frequency was obtained in nodal explants infected with the 15834/pTDT strain, being 59.50 ± 10.5 (Table 1). These results are inferior to those reported for other species of the Malvaceae family transformed with *A. rhizogenes* ATCC 15834 strains. In 15-day-old seedlings shoot explants of *Althaea officinalis*, a transformation frequency of 83 ± 5.2% was reported after 5 days of co-cultivation, and in 15-days-old seedlings leaf explants of *Urena lobate* L. the transformation frequency was 97.33% and 86.33% for stems explants (Phuong et al., 2018; Tavassoli and Afshar, 2018). These relatively low frequencies could be due to the age of *S. angustifolia* explants, co-cultivation time, and the competence for transforming the plant species.

Table 1
Response of leaf explants and nodal segments of 2-month-old *Sphaeralcea angustifolia* seedlings to infection with *Agrobacterium rhizogenes* strains

Strain	**ATCC 15834/pTDT**		**A4/pTDT**		**K599/pTDT**	
Explant	Nodal segment	Leaf	Nodal segment	Leaf	Nodal segment	Leaf
Transformation frequency (%)	59.50 ± 10.5*	40.0 ± 25	33.1 ± 10.5	24.0 ± 4	1.65 ± 1.65	NR
Root number	145	52	43	31	2	NR
Hairy root line number	6	4	NR	NR	NR	NR
The values of transformation frequency are the mean of 2 experiments ± standard error of the mean (n = 60). According to ANOVA of transformation frequency (F = 17.7, $p \geq 0.05$ and $Tukey_{0.05}$ test = 40.676) means with * was different significantly.						
NR = No Response						

Selection Of Hairy Root Lines

All individualized roots grown in a semi-solid MS medium were observed under an epifluorescence microscope, and none of them showed red fluorescence. This may be because there was no insertion of the T-DNA from the binary vector pTDT into the plant's genome, or the site where the T-DNA was inserted does not allow the expression of this gene. Seven putatively transformed root lines obtained from infection with ATCC 15834/pTDT strain presented variability in their morphology and growth; these differences could be due to each root clone was resulting from an independent transformation event (Batra et al., 2004). The selected hairy root lines were those that showed active growth in a semi-solid medium without plant growth regulators, presenting greater lateral branching and plagiotropic growth. When the hairy roots were transferred to the liquid culture medium, the SaTR N5.1, SaTR N12.2, and SaTR N12.4 lines showed the best growth index; the SaTR N7.2 line showed small non-friable callus; and the

SaTR N5.1, SaTR N7.1, SaTR N7.2, and SaTR N15.1 lines turned the culture medium to a reddish colour. This variation could be due to genetic alterations provoked by the expression of T-DNA genes of *A. rhizogenes* and to their integration site or the number of copies and the insertion orientation in the plant genome (Chandra, 2012).

The SaTR N5.1, SaTR N12.2, and SaTR N12.4 hairy root lines grown in liquid MS medium presented the highest growth index at 3 weeks of culture (Table 2). These results are similar to the studies carried out with hairy root cultures of *P. candollei* var. Candollei grown in flasks with an inoculum of 1% (W/V) in B5 culture medium at 25°C, with a growth index 9-times higher at 25 days of culture than its initial weight (Danphitsanuparn et al., 2012). The hairy root cultures of *Salvia officinalis* achieved with *A. rhizogenes* strain ATCC 15834 and cultivated in Wooden Plants liquid medium under light and dark conditions showed similar physical characteristics; the growth index was 11-times greater at 4 weeks of culture than the initial dry weight (Grzegorczyk et al., 2006).

Table 2
Growth Index of hairy root lines from *Sphaeralcea angustifolia* transformed with *Agrobacterium rhizogenes* 15834/pTDT

Root line	Initial dry weight (g)	Growth Index	
		2 weeks	3 weeks
SaTR N5.1	0.153 ± 0.02	5.02± 0.47**	6.84 ± 0.84**
SaTR N7.1	0.206 ± 0.04	1.17 ± 0.77	3.33 ± 1.46
SaTR N7.2	0.175 ± 0.03	1.0 ± 0.15	1.47 ± 0.30
SaTR N12.2	0.121 ± 0.01	2.33 ± 0.86	5.82 ± 0.54*
SaTR N12.4	0.184 ± 0.01	2.51 ± 0.11	5.95 ± 0.45*
SaTR N15.1	0.186 ± 0.03	1.15 ± 0.37	3.20 ± 0.68
SaTR L2.2	0.155 ± 0.20	2.65 ± 0.76	4.86 ± 0.22
The values are the mean ± standard deviation (n = 3). According to ANOVA and Tukey test means with * and ** were different significantly.			
F = 18.04, $^{**}p < 0.01$, $Tukey_{0.05}$= 1.59 for 2 weeks; F = 19.26, $^{**}p < 0.01$, $Tukey_{0.05}$= 2.09 for 3 weeks.			

The genetic transformation of all hairy root lines was confirmed by amplifying a fragment of the *rolC* gene by PCR. A VirD2 gene fragment that is not transferred to the plant genome was also amplified to confirm that hairy root cultures were free of *A. rhizogenes* (Fig. 2).

Quantification of anti-inflammatory compounds in dichloromethane-methanol extracts

The presence of scopoletin, tomentin, and sphaeralcic acid in the dichloromethane-methanol extracts of dry hairy roots and the liquid culture media from different hairy roots lines of *S. angustifolia* was confirmed through comparison of their retention times and absorption spectra as previously described

(Fig. 3). Except for the SaTR N12.2 and SaTR L2.2 lines, all of the hairy roots lines produced scopoletin and sphaeralcic acid, but tomentin was not detected. According to the ANOVA and Tukey's test, the SaTR N7.2 line had the highest scopoletin content at 2 weeks of culture. However, this line had a lower growth index. The scopoletin yield was 75-fold higher than that detected in the wild plant and 39-fold higher than that reported for cell suspension cultures of *S. angustifolia* (0.038 mg g^{-1} DW) grown in complete MS medium added with 1.0 mg L^{-1} of naphthalene-acetic acid and 0.1 mg L^{-1} of kinetin (Nicasio-Torres et al., 2016). Sphaeralcic acid yield was also significantly higher in line SaTR N7.2 at the second week of culture, and this yield was 440-fold superior to that detected in wild plants.

Table 3
Accumulation of scopoletin and sphaeralcic acid in hairy root cultures of *Sphaeralcea angustifolia*

Root line	Scopoletin (mg/g DW)		Sphaeralcic acid (mg/g DW)	
	2 weeks	3 Weeks	2 weeks	3 Weeks
SaTR N5.1	0.003 ± 0.001	0.004 ± 0.002	0.64 ± 0.3*	0.58 ± 0.3
SaTR N7.1	0.001 ± 0.001	0.001 ± 0.001	0.18 ± 0.05	0.46 ± 0.2
SaTR N7.2	0.1512 ± 0.02**	0.08 ± 0.03**	17.6 ± 1.72**	8.3 ± 2.11**
SaTR N12.2	ND	ND	ND	ND
SaTR N12.4	ND	ND	0.02 ± 0.006	0.03 ± 0.005
SaTR N15.1	0.003 ± 0.004	0.004 ± 0.002	0.17 ± 0.15	0.6 ± 0.26
SaTR H2.2	ND	ND	ND	ND
Wild plant	0.002 ± 0.02		0.04 ± 0.001	
The values are the mean ± standard deviation (n = 3). According to ANOVA and Tukey test means with * and ** were different significantly.				
Scopoletin F = 224.2; $p < 0.01$; $Tukey_{0.05}$= 0.022 for 2 weeks; F = 16.9; $p < 0.01$; $Tukey_{0.05}$= 0.041 for 3 weeks				
Sphaeralcic acid F = 292.9; $p < 0.01$; $Tukey_{0.05}$= 2.11 for 2 weeks; F = 13.75; $p < 0.01$; $Tukey_{0.05}$= 4.46 for 3 weeks.				

Research in cell suspension cultures of *S. angustifolia* reported an increase in the production of sphaeralcic acid and scopoletin by reducing the concentration of total nitrates in the MS culture medium. In a previous report, the highest yield for sphaeralcic acid (0.0672 mg g^{-1} DW) was obtained with nitrate reduction to 0.274 mM and for scopoletin was (0.4 mg g^{-1} DW) with the same nitrate restriction medium (Nicasio-Torres et al., 2016); this scopoletin yield was 2.6-fold higher than that detected in the SaTR N7.2 hairy root line.

In another study, cell suspensions cultures of *S. angustifolia* cultivated in a stirred tank-type bioreactor with MS medium and nitrate restriction (2.74 mM) produced mainly sphaeralcic acid (Pérez-Hernández et al., 2014 Nicasio-Torres, et al., 2016); sphaeralcic acid yield (3.47 mg/g DW) was 5-fold lower than that produced in line SaTR N7.2.

Similarly, scopoletin and tomentin production in a mixture (4.137 mg L^{-1}) in cell suspension cultures of *S. angustifolia* stimulated by the interaction of reduced 2.74 mM total nitrates and 2 µM of copper, was superior to that accumulated in the SaTR N7.2 hairy root line. However, coumarins levels (4.008 mg L^{-1}) and sphaeralcic acid (6.107 mg L^{-1}) excreted to the culture medium was 3-fold lower than the excreted by SaTR N7.2 hairy root line (Pérez-Hernández, et al., 2019b).

The culture media of the hairy root lines SaTR N5.1, SaTR N7.1, SaTR N7.2, and SaTR N15.1 were extracted and analyzed by HPLC to determine the sphaeralcic acid and scopoletin content excreted to the culture medium. All these culture media contained sphaeralcic acid; the SaTR N7.2 and SaTR N7.1 lines had the highest content at 2 and 3 weeks of culture, respectively (Table 4). These yields are higher than those reported in cell suspension cultures (0.244 mg L^{-1}) with 0.274 mM nitrate restriction (Nicasio-Torres et al., 2016). The SaRT N7.1 line was the one with the highest scopoletin yield at 3 weeks of culture (0.73 ± 0.44); this content was lower than that reported by Nicasio-Torres et al. (2016) in cell suspension cultures of *Sphaeralcea angustifolia*.

Table 4
Secretion of scopoletin and sphaeralcic acid to the medium in hairy root cultures of *Sphaeralcea angustifolia*

Root line transformed	Scopoletin (mg L^{-1})		Sphaeralcic acid (mg L^{-1})	
	2 Week	3 Week	2 Week	3 Week
SaTR N5.1	0.16 ± 0.08	0.04 ± 0.03	11.99 ± 3	11.14 ± 6.4
SaTR N7.1	0.27 ± 0.1	0.73 ± 0.44	8.9 ± 2.5	18.25 ± 2.8
SaTR N7.2	0.43 ± 0.06	0.21 ± 0.03	15.5 ± 2.2	15.50 ± 2
SaTR N15.1	0.35 ± 0.1	0.06 ± 0.04	9.06 ± 4.8	17.11 ± 6.6
The values are the mean ± standard deviation (n = 3).				

The relationship of growth and active compounds production indicated that SaTR N7.2 is the best hairy root line for scopoletin and sphaeralcic acid production, and more experimental work could be needed to improve its growth index. The production of both compounds is similar to the SaTR N5.1, SaTR N7.1, and SaTR 15.1 hairy root lines (Table 5). The excretion of scopoletin and sphaeralcic acid was similar for the SaTR N7.1 and SaTR N7.2 hairy root lines. The SaTR N12.4 hairy root line grows well and only produces sphaeralcic acid (Table 3).

Table 5
Production of scopoletin and sphaeralcic acid by total biomass in culture of hairy roots of *Sphaeralcea angustifolia*

Root line	Scopoletin (mg/total biomass)		Sphaeralcic acid (mg/total biomass)	
	2 weeks	3 Weeks	2 weeks	3 Weeks
SaTR N5.1	0.003 ± 0.001	0.005 ± 0.002	0.572 ± 0.25	0.612 ± 0.33
SaTR N7.1	0.0006 ± 0.001	0.001 ± 0.001	0.125 ± 0.03	0.416 ± 0.21
SaTR N7.2	0.052 ± 0.01**	0.033 ± 0.013**	6.140 ± 1.352**	3.6 ± 1.63**
SaTR N15.1	0.002 ± 0.002	0.004 ± 0.002	0.125 ± 0.1	0.645 ± 0.43
The values are the mean ± standard deviation (n = 3). According to ANOVA and Tukey test means with ** were different significantly.				
Scopoletin F = 13.90; $p < 0.01$; $Tukey_{0.05}$= 0.0181 for 2 weeks; F = 92.83; $p < 0.01$; $Tukey_{0.05}$= 0.0119 for 3 weeks				
Sphaeralcic acid F = 52.81; $p < 0.01$; $Tukey_{0.05}$= 1.82 for 2 weeks; F = 9.26; $p < 0.01$; $Tukey_{0.05}$= 2.27 for 3 weeks.				

Conclusion

In this work, it was possible to obtain three hairy root lines (SaTR N5.1, SaTR N7.1, and SaTR N15.1) of *S. angustifolia* producers of scopoletin and sphaeralcic acid. The SaTR N5.1 line had the best growth, which would allow us to evaluate different strategies to increase the scopoletin and sphaeralcic acid production. The SaTR N7.2 line had the lowest growth index. However, this line was highly producer of sphaeralcic acid and scopoletin. Also, this line secretes sphaeralcic acid to the culture medium, and this condition can facilitate its purification and implement a system of continuous production of this compound. Also, it is possible to investigate the biosynthesis of sphaeralcic acid using metabolomics analysis. On the other hand, phytochemical studies can be carried out for the chemical analysis of hairy SaTR N12.2 and SaTR N12.4, which may be root lines producers of other secondary metabolites not yet identified in *S. angustifolia* species.

Abbreviations

DW Dry Weight

ERK Extracellular signal-regulated kinases

FW Fresh weight

FCA Freund´s complete adjuvant

GI Growth Index

IL Interleukin

MS Murashige and Skoog medium

OD Optical density

Ri Root Inducing

pTDT Plasmid threonine deaminase gene of tomato

TNF-α Tumor necrosis factor-alpha

WP Woody plant medium

Declarations

Author contributions

As a PhD student, Rogelio Reyes-Pérez participated in all the experimental work under the advice of the co-authors in the collection, analysis, and interpretation of data and the writing of the manuscript. Jesús Arellano-García supervised the establishment of the transformation protocol of *Sphaeralcea angustifolia* by *Agrobacterium rhizogenes*, and he was the Thesis Co-Director of the PhD student. Pilar Nicasio-Torres participated in establishing hairy roots culture, extraction, and analytical methods for compounds quantification; she was the Thesis Co-Director of the PhD student. Fernando Martínez-Morales participated in the confirmation of the transformation of hairy root lines. Maribel Herrera-Ruiz and Irene Perea-Arango were part of the tutorial committee of the PhD student, and they participated in the design of the project and manuscript revision.

Acknowledgements

This work was supported by Basic Grant 622122 from Consejo Nacional de Ciencia y Tecnología, México (CONACyT-México) for the Doctoral studies of Rogelio Reyes-Pérez at the Natural Science Doctoral Program of Centro de investigación en Biotecnología (CeIB), Universidad Autónoma del Estado de Morelos, Mexico, and by Complementary Grant 99188274 from the Instituto Mexicano del Seguro Social (IMSS, México). This research had a registration number R-2018-1702-016.

References

1. Aguilar A et al (1994) Herbario Medicinal del Instituto Mexicano del Seguro Social. Publicación del IMSS. México D.F. 140–251

2. Aldi Y, Yuliandra Y, Nasrul E, Yanwirasti, Handayani D, Bakhtiar A (2015) Decreased interleukin-4 level of type i hypersensitive mice using scopoloetin isolated from noni fruit (Morinda citrifolia L.). Research Journal of Pharmaceutical Biological Chemical Sciences 6(4):1823–1829
3. Argueta V, Cano A, Rodarte AL (1994) M. L. Atlas de las plantas de la medicina tradicional mexicana. Instituto Nacional Indigenista, México, pp 44–52
4. Bais HP, Suresh B, Raghavarao KSMS, Ravishankar GA (2002) Performance of hairy root cultures of Cichorium intybus L. in bioreactors of different configurations. In Vitro Cellular Developmental Biology - Plant 38(6):573–580. https://doi.org/10.1079/IVP2002334
5. Batra J, Dutta A, Singh D, Kumar S, Sen J (2004) Growth and terpenoid indole alkaloid production in Catharanthus roseus hairy root clones in relation to left- and right-termini-linked Ri T-DNA gene integration. Plant Cell Rep 23(3):148–154. https://doi.org/10.1007/s00299-004-0815-x
6. Bonhomme V, Laurain-Mattar D, Lacoux J, Fliniaux MA, Jacquin-Dubreuil A (2000) Tropane alkaloid production by hairy roots of Atropa belladonna obtained after transformation with Agrobacterium rhizogenes 15834 and Agrobacterium tumefaciens containing rol A, B, C genes only. J Biotechnol 81(2–3):151–158. https://doi.org/10.1016/S0168-1656(00)00287-X
7. Chandra S (2012) Natural plant genetic engineer Agrobacterium rhizogenes: Role of T-DNA in plant secondary metabolism. Biotech Lett 34(3):407–415. https://doi.org/10.1007/s10529-011-0785-3
8. Colling J, Groenewald JH, Makunga NP (2010) Genetic alterations for increased coumarin production lead to metabolic changes in the medicinally important Pelargonium sidoides DC (Geraniaceae). Metab Eng 12(6):561–572. https://doi.org/10.1016/j.ymben.2010.08.001
9. Danphitsanuparn P, Boonsnongcheep P, Boriboonkaset T, Chintapakorn Y, Prathanturarug S (2012) Effects of Agrobacterium rhizogenes strains and other parameters on production of isoflavonoids in hairy roots of Pueraria candollei Grah. ex Benth. var. candollei. Plant Cell Tissue Organ Cult 111(3):315–322. https://doi.org/10.1007/s11240-012-0196-8
10. Ding Z, Dai Y, Hao H, Pan R, Yao X, Wang Z (2008) Anti-inflammatory effects of scopoletin and underlying mechanisms. Pharm Biol 46(12):854–860. https://doi.org/10.1080/13880200802367155
11. García-Rodríguez RV, Cevallos C, Siordia G, Jiménez-Arellanes G, Chávez-Soto MA, M. A., & Meckes-Fischer M (2012) Sphaeralcea angustifolia (Cav.) G. Don extract, a potential phytomedicine to treat chronic inflammation. Boletin Latinoamericano y Del Caribe de Plantas Medicinales y Aromaticas 11(5):454–463
12. Grzegorczyk I, Królicka A, Wysokińska H (2006) Establishment of Salvia officinalis L. hairy root cultures for the production of rosmarinic acid. Zeitschrift Fur Naturforschung - Section C Journal of Biosciences 61(5–6):351–356. https://doi.org/10.1515/znc-2006-5-609
13. Gutierrez-Valdes N, Häkkinen ST, Lemasson C, Guillet M, Oksman-Caldentey KM, Ritala A, Cardon F (2020) Hairy Root Cultures—A Versatile Tool With Multiple Applications. Front Plant Sci 11(March):1–11. https://doi.org/10.3389/fpls.2020.00033
14. Gwak et al., G et (2011) Extraction Procedures for Free Radical Scavenging Activity from Noni Fruit (Morinda citrifolia). Korean Journal of Medicinal Crop Science 19(1):38–46.

https://doi.org/10.7783/KJMCS.2011.19.1.038

15. Haas JH, Moore LW, Ream W, Manulis S (1995) Universal PCR primers for detection of phytopathogenic Agrobacterium strains. Appl Environ Microbiol 61(8):2879–2884. https://doi.org/10.1128/aem.61.8.2879-2884.1995
16. Jain DC, Pant N, Gupta MM, Bhakuni RS, Verma RK, Tandon S, … Kumar S (2002) *U.S. Patent No. 6,337,095*. Washington, DC: U.S. Patent and Trademark Office
17. Jang JH, Park JE, Han JS (2020) Scopoletin increases glucose uptake through activation of PI3K and AMPK signaling pathway and improves insulin sensitivity in 3T3-L1 cells. Nutr Res 74:52–61. https://doi.org/10.1016/j.nutres.2019.12.003
18. Juárez-Ciriaco M, Román-Ramos R, González-Márquez H, Meckes-Fischer M (2008) Efecto de *Sphaeralcea angustifolia* sobre la expresión de citocinas pro y anti-inflamatorias. LabCiencia con noticias técnicas de laboratorio 2:21–23
19. Meckes M, David-Rivera AD, Nava-Aguilar V, Jimenez A (2004) Activity of some Mexican medicinal plant extracts on carrageenan-induced rat paw edema. Phytomedicine 11(5):446–451. https://doi.org/10.1016/j.phymed.2003.06.002
20. Minh TN, Xuan TD, Tran HD, Van TM, Andriana Y, Khanh TD, Van Quan N, Ahmad A (2019) Isolation and purification of bioactive compounds from the stem bark of Jatropha podagrica. Molecules 24(5):1–15. https://doi.org/10.3390/molecules24050889
21. Moon PD, Lee BH, Jeong HJ, An HJ, Park SJ, Kim HR, Ko SG, Um JY, Hong SH, Kim HM (2007) Use of scopoletin to inhibit the production of inflammatory cytokines through inhibition of the IκB/NF-κB signal cascade in the human mast cell line HMC-1. Eur J Pharmacol 555(2–3):218–225. https://doi.org/10.1016/j.ejphar.2006.10.021
22. Moreno-Anzúrez NE, Marquina S, Alvarez L, Zamilpa A, Castillo-España P, Perea-Arango I, Torres PN, Herrera-Ruiz M, Díaz García ER, García JT, Arellano-García J (2017) A cytotoxic and anti-inflammatory campesterol derivative from genetically transformed hairy roots of Lopezia racemosa Cav. (Onagraceae) *Molecules*, 22(1). https://doi.org/10.3390/molecules22010118
23. Murashige T, Skoog F (1962) Murashige1962Revised.Pdf. Physiol Plant 15:474–497
24. Ochoa-Villarreal, Howat S, Hong SM, Jang MO, Jin YW, Lee EK, Loake GJ (2016) Plant cell culture strategies for the production of natural products. BMB Reports 49(3):149–158. https://doi.org/10.5483/BMBRep.2016.49.3.264
25. Nicasio-Torres M, Pérez-Hernández J, González-Cortazar M, Meckes-Fischer M, Tortoriello J, Cruz-Sosa F (2016) Production of potential anti-inflammatory compounds in cell suspension cultures of Sphaeralcea angustifolia (Cav.) G. Don. Acta Physiol Plant, 38(8). https://doi.org/10.1007/s11738-016-2211-x
26. Nicasio-Torres M, Serrano-Román J, Pérez-Hernández J, Jiménez-Ferrer E, Herrera-Ruiz M (2017) Effect of Dichloromethane-Methanol Extract and Tomentin Obtained from Sphaeralcea angustifolia Cell Suspensions in a Model of Kaolin/Carrageenan-Induced Arthritis. Planta Medica International Open 4(01):e35–e42. https://doi.org/10.1055/s-0043-108760

27. Pan R, Dai Y, Yang J, Li Y, Yao X, Xia Y (2009) Anti-angiogenic potential of scopoletin is associated with the inhibition of ERK1/2 activation. Drug Dev Res 70(3):214–219. https://doi.org/10.1002/ddr.20297
28. Pérez-Hernández J, González-Cortazar M, Marquina S, Herrera-Ruiz M, Meckes-Fischer M, Tortoriello J, Cruz-Sosa F, Nicasio-Torres MDP (2014) Sphaeralcic acid and tomentin, anti-inflammatory compounds produced in cell suspension cultures of Sphaeralcea angustifolia. Planta Med 80(2–3):209–214. https://doi.org/10.1055/s-0033-1360302
29. Pérez-Hernández J, Nicasio-Torres MdelP, Sarmiento-López LG, Rodríguez-Monroy M (2019a) Production of anti-inflammatory compounds in Sphaeralcea angustifolia cell suspension cultivated in stirred tank bioreactor. Eng Life Sci 19(3):196–205. https://doi.org/10.1002/elsc.201800134
30. Pérez-Hernández J, Martínez-Trujillo A, Nicasio-Torres P (2019b) Optimization of active compounds production by interaction between nitrate and copper in Sphaeralcea angustifolia cell suspension using Response Surface Methodology. Plant Cell Tissue Organ Cult 136(2):407–413. https://doi.org/10.1007/s11240-018-1516-4
31. Phuong VTB, Hong PTA, Phuong QND (2018) Improving hairy root induction of Urena lobata L. by Agrobacterium rhizogenes ATCC 15834 by some factors. Science Technology Development Journal 21(3):90–97. https://doi.org/10.32508/stdj.v21i3.430
32. Rajashekar J, Kumar V, Veerashree V, Poornima DV, Sannabommaji T, Gajula H, Giridhara B (2016) *Protocols for In Vitro Cultures and Secondary Metabolite Analysis of Aromatic and Medicinal Plants, Second Edition*. *1391*(April), 427–443. https://doi.org/10.1007/978-1-4939-3332-7
33. Romero-Cerecero O, Meckes-Fischer M, Zamilpa A, Enrique Jiménez-Ferrer J, Nicasio-Torres P, Pérez-García D, Tortoriello J (2013) Clinical trial for evaluating the effectiveness and tolerability of topical Sphaeralcea angustifolia treatment in hand osteoarthritis. J Ethnopharmacol 147(2):467–473. https://doi.org/10.1016/j.jep.2013.03.040
34. Rossi L, Hohn B, Tinland B (1996) Integration of complete transferred DNA units is dependent on the activity of virulence E2 protein of Agrobacterium tumefaciens. Proc Natl Acad Sci USA 93(1):126–130. https://doi.org/10.1073/pnas.93.1.126
35. Sajjalaguddam RR, Paladugu A (2016) *Quercetin Production From Hairy Root Cultures of Indian Mallow (Abutilon Indicum L.)*. *5*(11), 956–967. https://doi.org/10.20959/wjpps201611-7962
36. Serrano-Román J. Bióloga Jade Leonor Serrano Román, Maestría en Medicina Molecular, Facultad de Medicina, Universidad Autónoma del Estado de Morelos. Caracterización farmacológica de tomentina y ácido sphaeralcico aislados de suspensiones celulares de *Sphaeralcea angustifolia* en un modelo de artritis experimental. Cuernavaca Morelos, 2015, 100 pp
37. Serrano-Román J, Nicasio-Torres P, Hernández-Pérez E, Jiménez-Ferrer E (2020) Elimination pharmacokinetics of sphaeralcic acid, tomentin and scopoletin mixture from a standardized fraction of Sphaeralcea angustifolia (Cav.) G. Don orally administered. *Journal of Pharmaceutical and Biomedical Analysis*, *183*. https://doi.org/10.1016/j.jpba.2020.113143

38. Shaw CY, Chen CH, Hsu CC, Chen CC, Tsai YC (2003) Antioxidant properties of scopoletin isolated from Sinomonium acutum. Phytother Res 17(7):823–825. https://doi.org/10.1002/ptr.1170
39. Tavassoli P, Safipour Afshar A (2018) Influence of different Agrobacterium rhizogenes strains on hairy root induction and analysis of phenolic and flavonoid compounds in marshmallow (Althaea officinalis L.). 3 Biotech 8(8):0. https://doi.org/10.1007/s13205-018-1375-z
40. Thani W, Vallisuta O, Siripong P, Ruangwises N (2010) Anti-proliferative and antioxidative activities of Thai noni/Yor (Morinda citrifolia Linn.) leaf extract. Southeast Asian J Trop Med Public Health 41(2):482–489
41. Triplett BA, Moss SC, Bland JM, Dowd MK (2008) Induction of hairy root cultures from Gossypium hirsutum and Gossypium barbadense to produce gossypol and related compounds. *In* Vitro Cellular Developmental Biology - Plant 44(6):508–517. https://doi.org/10.1007/s11627-008-9141-2
42. Zeng Y, Ma Y, Yang Z, Mao J, Zheng Y (2020) Antihyperuricemic efficacy of Scopoletin-loaded Soluplus micelles in yeast extract/potassium oxonate-induced hyperuricemic mice. Drug Dev Ind Pharm 46(9):1550–1557. https://doi.org/10.1080/03639045.2020.1811302

Figures

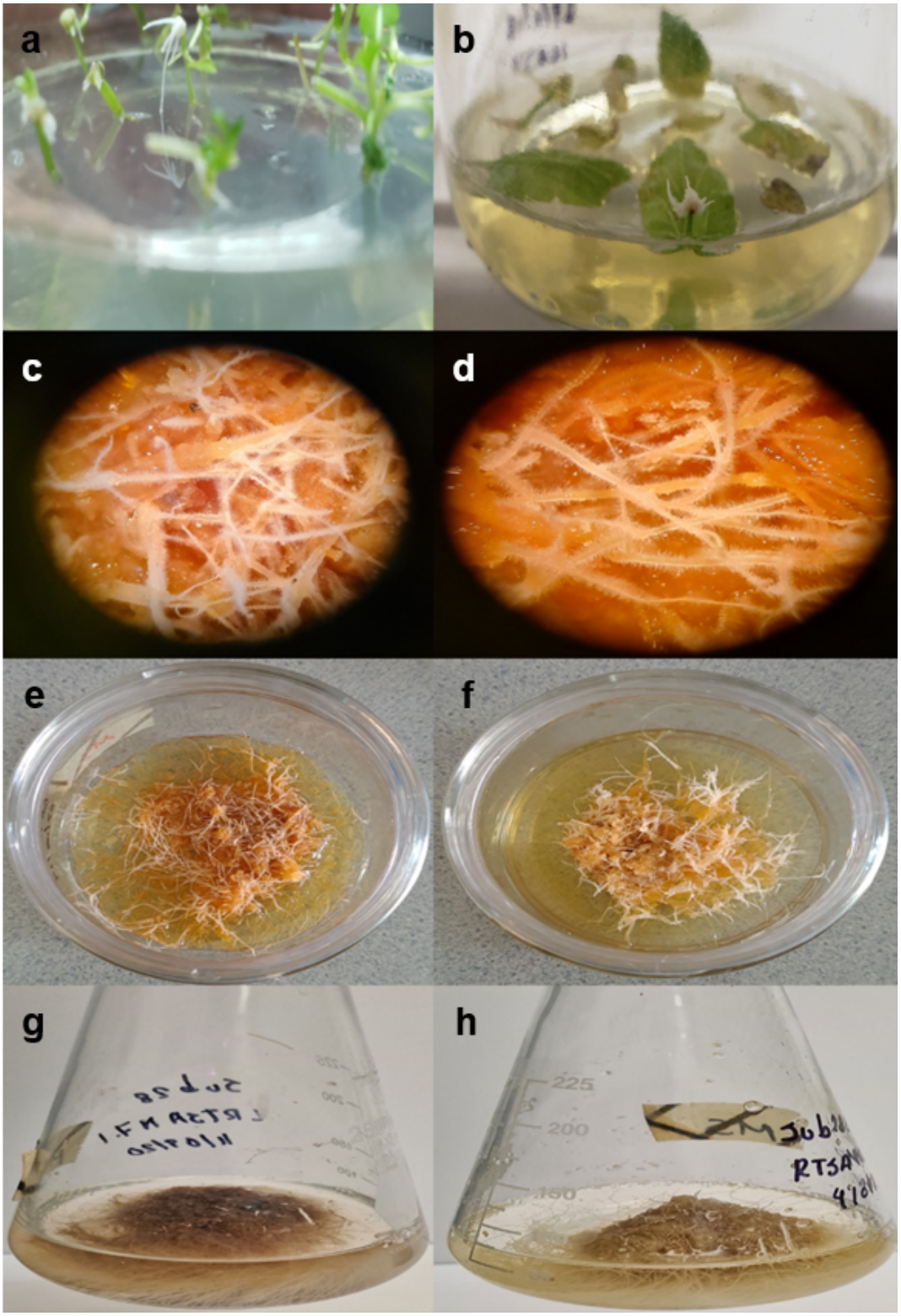

Figure 1

Induction of hairy roots in leaf explants and nodal segments from Sphaeralcea angustifolia plantlets. Emergence of hairy roots in: a) nodal segments and b) leaf explants; c and d) morphological characteristic of hairy root; e and f) roots grown on semi-solid MS medium; g and h) propagation of biomasses in liquid MS medium

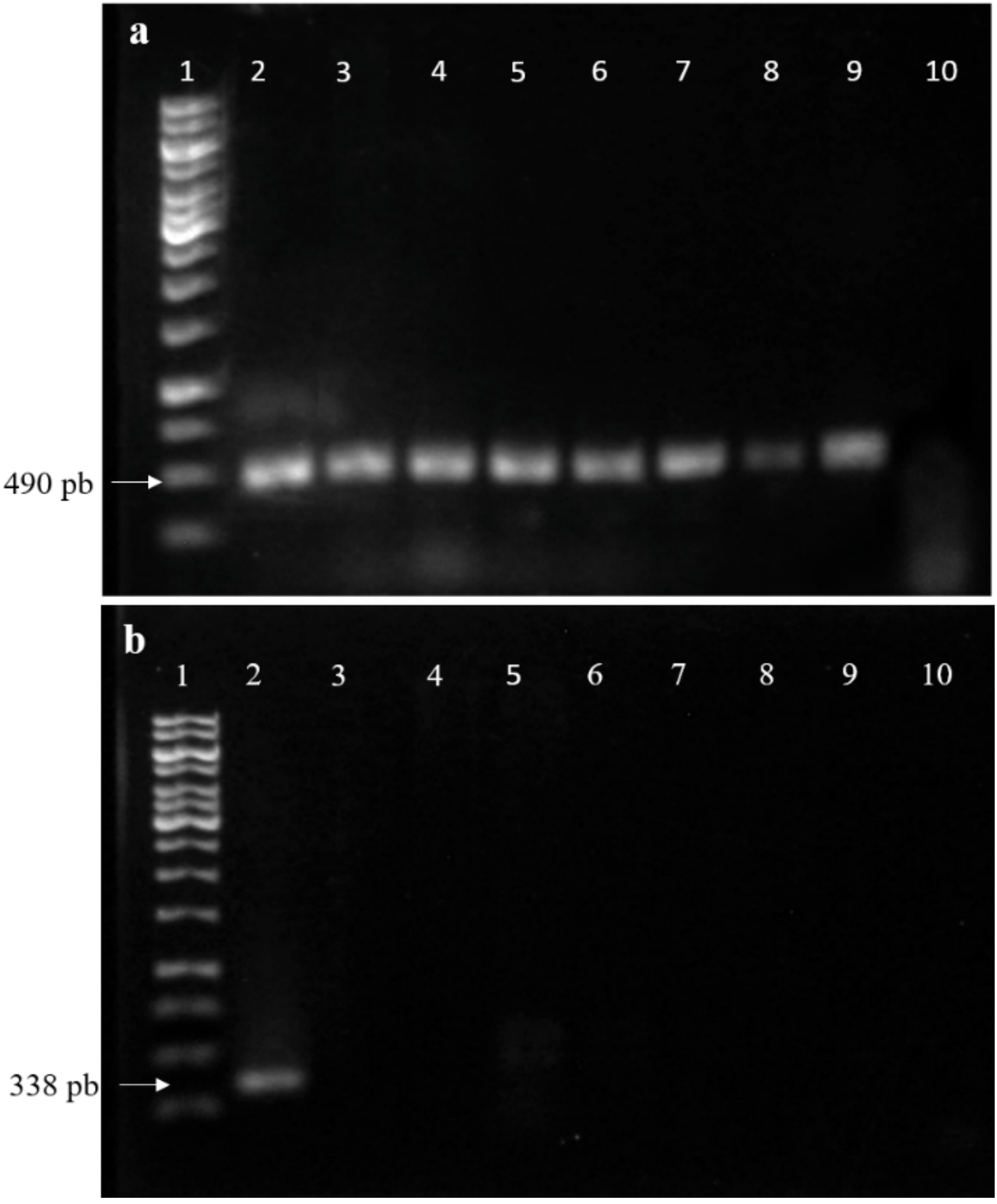

Figure 2

PCR products of hairy root transformed lines of Sphaeralcea angustifolia a) Lane 1: 1 Kb DNA marker, lane: 2 amplified band of rolC from DNA of Agrobacterium rhizogenes (positive control), lane 3 to 9 amplified band of rolC from the DNA of hairy roots (SaTR H2.2, N5.1, N7,1, N7.2, N12.2, N12.4, N15.1, respectively), lane 10 DNA of Sphaeralcea angustifolia wild plant (negative control). b) Lane 1: 1 Kb DNA marker, lane: 2 amplified band of VirD2 from DNA of Agrobacterium rhizogenes (positive control), lane 3

to 9 DNA of hairy roots (SaTR H2.2, N5.1, N7,1, N7.2, N12.2, N12.4, N15.1, respectively), lane 10 DNA of Sphaeralcea angustifolia wild plant (negative control)

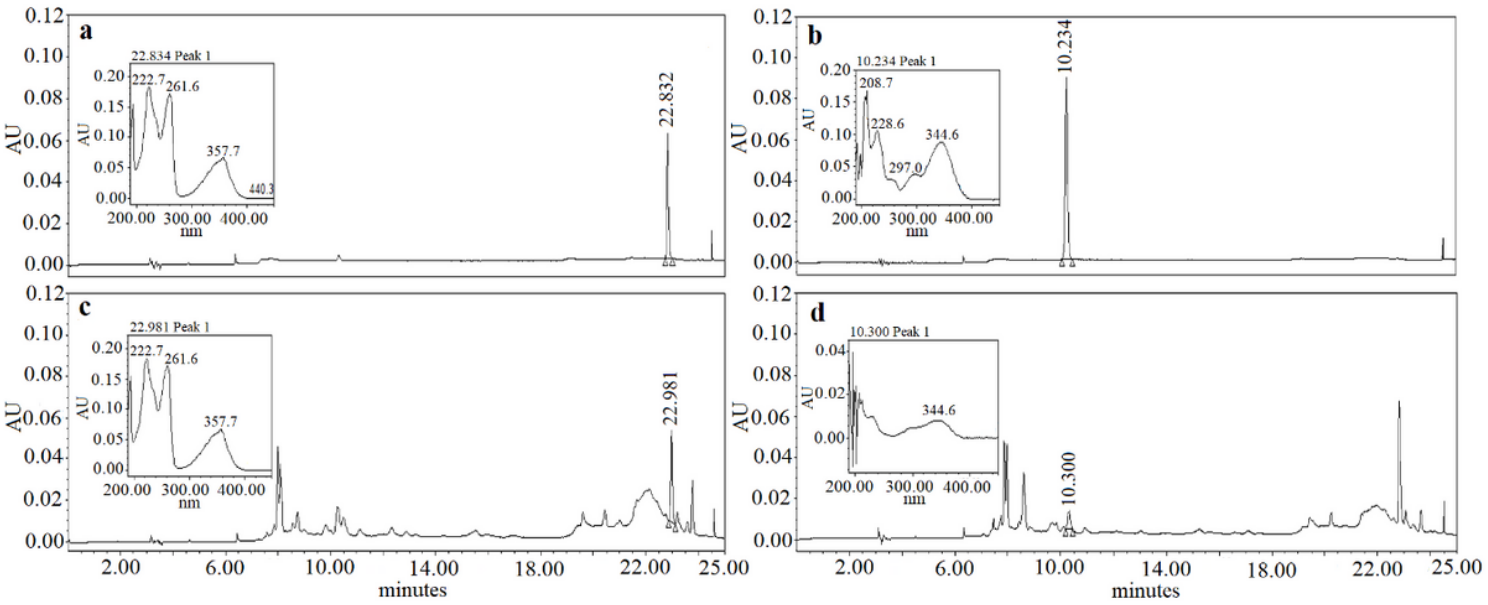

Figure 3

Chromatograms of HPLC and absorption spectra of a) sphaeralcic acid retention time 22.83 min and b) scopoletin retention time 10.27 standards, and sphaeralcic acid (c) and scopoletin (d) detected in the dichloromethane-methanol extract from SaTR N7.2 hairy root line of Sphaeralcea angustifolia