Anti-inflammatory compounds produced in hairy roots culture of *Sphaeralcea angustifolia*

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**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 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. After 2 years in culture, *S. angustifolia* hairy roots are producers of scopoletin and sphaeralcic acid and they 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.

**Keywords** *Agrobacterium rhizogenes* · Malvaceae · Scopoletin · Tomentin · Sphaeralcic acid

**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| 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 |
| PGR          | Plant growth regulators |
| pTDT         | Plasmid threonine deaminase gene of tomato |
| TNF-α        | Tumor necrosis factor-alpha |
| WP           | Woody plant medium |
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 tissues of this plant had demonstrated anti-inflammatory activity, with scopoletin identified as the main active compound (Meckes et al. 2004; Juárez-Ciriaco et al. 2008; García-Rodríguez et al. 2012). Topical administration of a gel formulation prepared with 1% of *S. angustifolia* dichloromethane extract standardized in 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 important pharmacological activities had been reported, such as anti-inflammatory (Moon et al. 2007; Ding et al. 2008), antioxidant (Shaw et al. 2003; Gwak et al. 2011), inhibition of nuclear transcription factor (NF–κp) and inflammatory cytokines production, as well as pro-inflammatory mediators (Kim et al. 2004; Deng et al. 2007), anti-angiogenic in arthritis mouse model (Pan et al. 2009), anti-proliferative (Thani et al. 2010), anti-thyroid, anti-hypertensive (Aldi et al. 2015), anti-hyperuricemic (Zeng et al. 2020), and anti-diabetic (Jang et al. 2020), among others.

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

Plant cell culture is considered a potential option for active compounds production, however, only a few large-scale commercial systems have been established due to the low yield of secondary metabolites or due to lose the ability to accumulate these compounds partially or totally (Caili and Meizhe 2021). Then, it is important to explore other biotechnological process for the production of compounds with important antiarthritic activity identified in cell suspension cultures of *S. angustifolia*: scopoletin, tomentin and sphaeralcic acid.

Pharmaceutical compounds from plants can be obtained by direct extraction from barks, stems, leaves, flowers or fruits of plants usually in low yields; moreover, these compounds generally have very complicated structures and some of them cannot be synthesized using an organic method. Hairy roots of many medicinal plants have been established for active compounds production and the generation of novel active compounds not identified in the wild plants. Furthermore, the production of some metabolites synthesized only in specific tissues was achieved in hairy roots cultures, for example, vinblastine and vincristine produced exclusively in the aerial parts of *Catharanthus roseus* plants, and catherantine accumulated in all organs (Schweizer et al. 2018).

The genetically transformed root cultures are considered an important biotechnological tool for secondary metabolites production with biological activity due to they have genetic and biochemical stability, can grow in media lacking of plant growth regulators, and have short duplication times, attributes that give them advantages over cell suspension cultures (Rajashekar et al. 2016; Gutierrez-Valdés et al. 2020).

For active compounds production in hairy root cultures some factors must be considered, such as the stability of compounds and their toxicity to the hairy roots culture due to the large amounts produced, in addition to the cost involved in their production (Shi et al. 2021). Another factors to consider is the hairy roots stably for large quantities of active compounds production, and to produce similar amounts of compounds in each subculture; these issues were achieved in the hairy roots cultures from *Hyoscyamus muticus* (16 years) and *Catharanthus roseus* (11 years) after many years of continuous subculture (Häkkinen et al. 2016; Sün et al. 2017). Finally, for large scale cultivation of hairy root culture a suitable bioreactor could be another limiting factor, the hairy root cultures of *Panax ginseng* hairy roots were successfully grown in an airlift bioreactor of 19 L, and of *Cichorium intybus* L. were scaled up in the acoustic mist bioreactor to produce esculin, a coumarin with anti-inflammatory activity (Bais et al. 2002; Stiles et al. 2013). The accumulation of secondary metabolites can be also increase by means different biotic and abiotic elicitors (Caili and Meizhe 2021).

It is important refer that some pharmaceutical companies (Mibelle, Sederma, and Rootec) use this technology to produce compounds for health; Rootec company produces atropine (anti-cholinergic drug) from hairy roots of *Atropa belladonna*, nicotine (nicotine replacement therapy) from *Nicotiana glauca*, gingsenosides (antioxidant, anticancer, cardiovascular diseases) from *Panax ginseng*, and camptothecin (anticancer and antiviral) from *Camptotheca acuminata* (Ochoa-Villarreal et al. 2016).

It is important to explore the hairy roots cultures for the production of compounds with important antiarthritic...
activity identified in cell suspension cultures of *S. angustifolia*: scopoletin, tomentin and sphaeralcic acid. Considering that there are no reports in the literature about the genetic transformation of *S. angustifolia*, the reports of some species of the Malvaceae family transformed by *A. rhizogenes* ATCC 15,834 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 the base for an industrially competitive biotechnological system in the future.

### 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/cm² 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 (cucumisine type; OD₅₅₀ = 0.5), A4/pTDT or ATCC 15,834/pTDT (agropin type; OD₅₅₀ = 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 15,834/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 absence of plant growth regulators (PGR); 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 PGR, and (3) emission of red fluorescence under an epifluorescence microscope (Carl Zeiss V8).

Each hairy root line selected (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 (16 h)/darkness (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 (Theko 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 of each hairy root line and total DNA of roots from untransformed S. angustifolia plantlets (2.5 months after in vitro germination) were isolated. Each sample (100 mg) was finely cut and placed in a lysis tube and 750 µL of lysis solution were added; then, each sample was agitated for 10 min in a vortex at maximum speed in order to disrupt the tissue. The lysis tube was then centrifuged at 10,000×g for 1 min; after centrifugation, 400 µL of supernatant was transferred to a filter tube connected to a collector tube and centrifuged once again at 7000×g for 1 min. The DNA of lysis solution was 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:

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

Chemical analysis

Extract preparation

The dry biomass of three flasks from each line of hairy roots at 2 and 3 weeks of batch culture was extracted three times by maceration (24 h for each procedure) at room temperature with a mixture of reactive grade solvents (CH2Cl2:CH3OH 9:1; Merck) in a ratio of 1:50 (w/v). The extracts were filtered, pooled, and concentrated to dryness under reduced pressure. Independently, the culture medium of each flask was partitioned (v/v) three times with dichloromethane; extracts from each medium were pooled and concentrated to dryness under reduced pressure. The extracts from hairy roots and media obtained were dissolved in high purity methanol (Merck) for their high performance liquid chromatography (HPLC) analysis. The content of sphaeralcic acid and scopoletin in the CH2Cl2:CH3OH extracts were determined by HPLC (Pérez-Hernández et al. 2014, 2019a; Nicasio-Torres et al. 2016, 2017).

After 2 year in culture, the dry biomasses of hair roots lines were extracted by maceration as was above described and analyzed by HPLC.

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) H2O with TFA (0.5%, Sigma Aldrich) and (B) CH3CN high purity (Merck). The compounds were detected by monitoring scopoletin and tomentin absorbance λ = 344 nm and sphaeralcic acid λ = 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 \( y = 165,407 \times + 16,720, r^2 = 0.9993 \), and for sphaeralcic acid it was \( y = 7381.9 \times + 1362.2, r^2 = 0.9998 \), 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 \( S. \) angustifolia explants infected with the strains ATCC 15,834/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 15,834/pTDT strain; leaf explant also showed callus formation. The highest transformation frequency was obtained in nodal explants infected with the 15,834/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 15,834 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 this plant species.

Selection of hairy root lines

All individualized and actively growing roots in a semi-solid MS medium in the absence of PGR were observed under an epifluorescence microscope, and none of them showed red fluorescence. Obtaining hairy roots cultures showing red fluorescence, implies events of double transfer of T-DNA to the plant genome, one coming from the wild type Ri plasmid and the other from the binary vector pTDT carried by the same strain of \( A. \) rhizogenes. Absence of red fluorescence in these hairy roots could be due to 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, similar results were obtained by Moreno-Azurases et al. (2017) in \( Lopezia \) racemose Cav transformed with \( A. \) rhizogenes strain ATCC 15,834.

Seven putatively transformed root lines obtained from infection with ATCC 15,834/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 higher 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 pigment. 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 hairy roots growth in batch was evaluated after eight subcultures (6 months) in MS liquid medium. The SaTR N5.1, SaTR N12.2, and SaTR N12.4 hairy root lines grown in liquid MS medium presented the highest growth index after 3 weeks of culture (Table 2). These results (5–7 times) are similar to the studies carried out with hairy root cultures of *Pueraria 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 15,834 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).

The genetic transformation of selected hairy root lines was confirmed by an effective and quick procedure used in previous reports for demonstration of genetic transformation of hairy roots, which starts amplifying a fragment of the *rolC* gene by PCR and a fragment of the VirD2 gene; this gene is not transferred from the *Agrobacterium* strain to the plant genome, and it has been used to confirm that hairy root cultures were free of *A. rhizogenes* (Fig. 2; Häkkinen et al. 2016; Moreno-Anzúrez et al. 2017). We observed that the other tested root lines did not amplify the expected fragment of 490 bp, although they show active growth. It is known that with some frequency, the T-DNA of *Agrobacterium* is not completely transferred to the plant genome (Rossi et al. 1996). In this sense, it will be necessary to test the amplification of another *rol* gene.

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

| Strain        | ATCC 15,834/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 ≥ 0.05 and Tukey<sub>0.05</sub> test = 40.676) means with * was different significantly

NR no response

### Table 2 Growth Index of hairy root lines from *Sphaeralcea angustifolia* transformed with *Agrobacterium rhizogenes* 15,834/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<sub>0.05</sub> = 1.59 for 2 weeks; F = 19.26, **p < 0.01, Tukey<sub>0.05</sub> = 2.09 for 3 weeks

### Quantification of anti-inflammatory compounds in dichloromethane-methanol extracts

The presence of scopoletin, tomentin, and sphaeralcic acid in the dichloromethane-methanol (dry hairy roots) and dichlorometane (liquid culture media) extracts from 7 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, 5 lines of the hairy roots had the potential to produce scopoletin and sphaeralcic acid, but tomentin was not detected. The SaTR N12.4 hairy root line only produces sphaeralcic acid and it is accumulated in biomass (Table 3). Scopoletin was identified as the anti-inflammatory compound in the aerial tissues of wild plant and in the cells in suspension culture of *S. angustifolia*; sphaeralcic acid is a de novo compound identified in the cells in suspension cultivated in MS medium under nitrate restriction (García-Rodríguez et al. 2012; Pérez-Hernández et al. 2014). According to the ANOVA and Tukey’s test, the SaTR N7.2 line had the highest scopoletin and sphaeralcic acid contents at 2
and 3 weeks of culture; however, this hairy root 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 440-fold superior to that detected in wild plants. 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 DW) was obtained with nitrate reduction to 0.274 mM (Nicasio-Torres et al. 2016). The scopoletin yield obtained in the cells in suspension was only 2.6-fold higher than that detected in the SaTR N7.2 hairy root line, and the sphaeralcic acid yield was 260-fold superior 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 five-fold lower than that produced in line SaTR N7.2. Hairy root line SaTR N7.2 was identified with the higher scopoletin and sphaeralcic acid production and lower growth index.

The excretion of scopoletin and sphaeralcic acid was similar for the SaTR N7.1, SaTR N7.2 and SaTR N15.1 hairy root lines. The culture media of the hairy root lines SaTR N5.1, SaTR N7.1, SaTR N7.2, and SaTR N15.1 contained sphaeralcic acid; the SaTR N7.2, SaTR N7.1 and SaTR N15.1 lines had the highest content at 2 and/or 3 weeks of culture (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). 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 total nitrates reduced to 2.74 mM and of copper increased to 2 μM, 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 SaTR N7.1 line was the one with the highest scopoletin yield at 3 weeks of culture (0.73 ± 0.44 mg L⁻¹); this content was lower than that reported by Nicasio-Torres et al. (2016) in cell suspension cultures of *S. angustifolia*.

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, but this production negatively affects its growth possibly as a result of its high accumulation. The production of both compounds is similar to the SaTR N5.1, SaTR N7.1, and SaTR N15.1 hairy root lines (Table 5). The SaTR N12.4 hairy root line grows well and only produces sphaeralcic acid and it is accumulated in biomass (Table 3).

The chemical analysis of scopoletin and sphaeralcic acid in the dichloromethane:methanol extracts from SaTR N5.1, SATR N7.1, SATR N7.2 and SATR N15.1 hairy roots showed that after 2 years in culture, the hairy roots preserve the capability to produce scopoletin and sphaeralcic acid at similar levels; scopoletin at similar levels and sphaeralcic acid at high levels to those detected in cell suspension culture of *S. angustifolia* without stimulation (Table 3). The successful production of bioactive compounds by hairy root culture of *S. angustifolia* can be scale up to bioreactors. Stimulating effects of biotic elicitors explored in the *S. angustifolia* cell suspension in the production of scopoletin and sphaeralcic acid, individual or synergetic effects by combination of reduction of total

**Fig. 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 plantlets (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 plantlets (negative control).
nitrate and increase of copper sulfate in MS medium can be included, as well as using elicitors like jasmonic acid or salicylic acid.

Conclusion

In this work, it was possible to obtain four hairy root lines (SaTR N5.1, SaTR N7.1, SaTR N7.2 and SaTR N15.1) of S. angustifolia producers of scopoletin and sphaeralcic acid, both accumulated and excreted into culture media. The SaTR N5.1 line had the best growth, which would allow us to evaluate different strategies to increase the scopoletin

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 years | 2 weeks  | 3 weeks  | 2 years |
| SaTR N5.1 | 0.003 ± 0.001 | 0.004 ± 0.002 | 0.001 ± 0.0003 | 0.64 ± 0.3* | 0.58 ± 0.3 | 1.10 ± 0.33** |
| SaTR N7.1 | 0.001 ± 0.001 | 0.001 ± 0.001 | 0.011 ± 0.002** | 0.18 ± 0.05 | 0.46 ± 0.2 | 1.22 ± 0.17** |
| SaTR N7.2 | 0.151 ± 0.02** | 0.08 ± 0.03** | 0.007 ± 0.002 | 17.6 ± 1.72** | 8.3 ± 2.11** | 1.16 ± 0.08** |
| SaTR N12.2 | ND | ND | ND | ND | ND | ND |
| SaTR N12.4 | ND | ND | ND | 0.02 ± 0.006 | 0.03 ± 0.005 | 0.052 ± 0.01 |
| SaTR N15.1 | 0.003 ± 0.004 | 0.004 ± 0.002 | 0.003 ± 0.0003 | 0.17 ± 0.15 | 0.6 ± 0.26 | 1.57 ± 0.30** |
| SaTR L2.2 | ND | ND | 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; Tukey0.05 = 0.022 for 2 weeks; F = 16.9; p < 0.01; Tukey0.05 = 0.041 for 3 weeks; F = 12.8; p = 0.016; Tukey0.05 = 0.007 for 2 years

Sphaeralcic acid F = 292.9; p < 0.01; Tukey0.05 = 2.11 for 2 weeks; F = 13.75; p < 0.01; Tukey0.05 = 4.46 for 3 weeks; F = 14.1; p = 0.0062; Tukey0.05 = 0.86 for 2 years

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 weeks  | 3 weeks  | 2 weeks  | 3 weeks  |
| 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 ± 2.0 |
| 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). According to ANOVA scopoletin F=1.71, p>0.05 for 2 weeks and F=2.1, p>0.05 for 3 weeks; sphaeralcic acid F=0.88, p>0.05 for 2 weeks and F=0.40, p>0.05 for 3 weeks

nitrate and increase of copper sulfate in MS medium can be included, as well as using elicitors like jasmonic acid or salicylic acid.
and sphaeralcic acid production. Similarly, it is possible to investigate the biosynthesis of sphaeralcic acid using metabolomics analysis and RNA sequencing (RNaseq). The SaTR N7.2 line was highly producer of sphaeralcic acid and scopoletin and negatively affects its growth. 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. 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.

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Author contributions As a PhD student, RRP 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. JAG supervised the establishment of the transformation protocol of Sphaeralcea angustifolia by Agrobacterium rhizogenes, and he was the Thesis Co-Director of the PhD student. PNT participated in establishing hairy roots culture, extraction, and analytical methods for compounds quantification; she was the Thesis Co-Director of the PhD student. FMM participated in the confirmation of the transformation of hairy root lines. MHR and IPA were part of the tutorial committee of the PhD student, and they participated in the design of the project and manuscript revision.

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