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RESEARCH NOTE

# Optimization of active compounds production by interaction between nitrate and copper in *Sphaeralcea angustifolia* cell suspension using Response Surface Methodology

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## Abstract

Cells in suspension from *Sphaeralcea angustifolia* produce compounds with anti-arthritic activity (scopoletin, tomentin, and sphaeralcic acid) in Murashige and Skoog (MS) medium with 2.74 mM of total nitrate content. The effect of nitrate and copper contents in the MS medium on the growth of cell suspension and the production of active compounds were tested by means of $2^k$ factorial design (FD) and Central Composite Design (CCD). The growth rate and the duplication time were statically similar and the maximal biomass (9–11 days) was improved by increases of the nitrate concentration. Highest contents of coumarins (4137.00 µg $L^{-1}$) and sphaeralcic acid (1441.00 µg $L^{-1}$) were obtained in the culture media by the interaction of 2.74 mM of nitrates and 2 µM of copper at 2 and 4 days; sphaeralcic acid contents were similar to those detected intracellularly after 9 and 11 days of culture. According to the CCD, highest contents of coumarins (4008.00 µg $L^{-1}$) and sphaeralcic-acid (6107.00 µg $L^{-1}$) could be obtained with 2.35 µM of copper with 2.42 mM of total nitrate, this condition did not affect the cell growth. The coumarin content is 40-fold and that of the sphaeralcic-acid is 30-fold higher than those detected in biomass. The *S. angustifolia* cell suspension could be cultured in a stirred tank bioreactor in a batch or in a continuous manner for the production of scopoletin, tomentin, and sphaeralcic-acid.

**Keywords** Anti-inflammatories · Inmunomodulators · Scopoletin · Sphaeralcic acid · Tomentin

## Abbreviations

| | |
|---|---|
| CFA | Complete Freud's adjuvant |
| CCD | Central composite design |
| FD | Factorial design |
| $\mu_{max}$ | Maximal growth rate |
| MS | Murashige and Skoog medium |
| RSM | Response surface methodology |
| dt | Duplication time |
| TNF-α | Tumor necrosis factor alpha |

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## Introduction

A gel prepared with the dichloromethane extract of *Sphaeralcea angustifolia* (Cav.) G. Don (Malvaceae) aerial tissues standardized in scopoletin demonstrated therapeutic effectiveness in treating patients with osteoarthritis (Romero-Cerecero et al. 2013). In view of that collection of *S. angustifolia* plants from its natural habitat has been restricted by the Mexican Ministry of the Environment and Natural Resources (SEMARNAT, NOM059-ECOL-1994), the cell suspension culture of this species was established as a potential for the production of active compounds to be used as a reliable source of plant pharmaceuticals (Smetanska 2008; Pérez-Hernández et al. 2014; Nicasio-Torres et al. 2016). The *S. angustifolia* cell suspension developed in Murashige and Skoog (MS) medium with 2.74 mM of total nitrates produced scopoletin, and its concentration (0.04%) was 60-fold higher than that detected in the wild plant (0.00067%); in

addition, tomentin (0.096%), and sphaeralcic-acid (0.0036%) were produced (Pérez-Hernández et al. 2014; Nicasio-Torres et al. 2016). Scopoletin, tomentin and sphaeralcic-acid compounds were active in acute and chronic inflammatory models (Pan et al. 2010; García-Rodríguez et al. 2012; Pérez-Hernández et al. 2014; Nicasio-Torres et al. 2017). The dichloromethane extract from cell suspension of *S. angustifolia* and standardized in scopoletin, tomentin, and sphaeralcic-acid could be an alternative to prepare a gel for its clinical application.

In this study, the effect of total nitrates in combination with copper contents in the MS medium on the cell growth and the production of anti-inflammatory compounds in the cell-suspension cultures of *S. angustifolia* was evaluated using factorial and central composite design.

## Materials and methods

### Cell suspension cultures

The *S. angustifolia* cell suspension in batch cultures was cultivated in MS medium and growing conditions previously reported (Pérez-Hernández et al. 2014; Nicasio-Torres et al. 2016).

### Experimental design

A $2^K$ Factorial design (FD) using total nitrates and copper contents as independent variables X1 and X2 (Table 1), respectively, were coded at two levels (+1, −1) determined from the values of total nitrates (2.74 mM) and copper (0.1 µM) in which scopoletin, tomentin, and sphaeralcic acid were produced (Pérez-Hernández et al. 2014; Nicasio-Torres et al. 2016). In each experimental condition, batch cell suspensions were developed over a period of 23 days in culture, taking three flasks to analyze the cell-growth response (Y1), from which growth rate ($\mu$), duplication time (dt), and maximal biomass were calculated; whereas analyses of a mixture of coumarins as tomentin and scopoletin (Y2), and sphaeralcic-acid (Y3) contents were performed from the extracts obtained from the media and the cellular biomass. The results of the dependent variables were compared through an analysis of variance (ANOVA), from which a linear mathematical model was obtained:

$$Y = B_0 + B_1X_1 + B_2X_2 + B_{1,2}X_1X_2$$

where Y is the predicted responses according to the model (growth rate, duplication time, maximal biomass, coumarins or sphaeralcic-acid), $B_0$ is the intercept term, $B_1$ is the co-efficient indicative of linear effect of total nitrates on the response, $B_2$ is the co-efficient indicative of linear effect of copper on the response, $B_{1,2}$ is the co-efficient of interaction effect of total nitrates and copper on the response.

With this model, it was possible to detect the experimental levels of the stimulators to establish a Central composite design (CCD) for the two independent variables with five (− 1.4, − 1, 0, 1, and 1.41) codified levels (Ryswyk and Van Hecke 1991; Palasota and Deming 1992) that would define the region of optimal response on the growth (Y1), and for the intra-cellular and extracellular production of scopoletin and tomentin (Y2), and sphaeralcic-acid (Y3) at days 2 and 4 of culture. The response variables (Y2 and Y3) were analyzed through an ANOVA whose results can be expressed according to the following quadratic equation

$$Y = B_0 + B_1X_1 + B_2X_2 + B_{12}X_{12} + B_{11}X_1^2 + B_{22}X_2^2$$

**Table 1** Coefficients of the linear model of growth parameters of *Sphaeralcea angustifolia* cell suspension in batch culture grown in Murashige and Skoog (MS) medium supplemented with different levels of nitrate and copper in the $2^K$ factorial design

| X1 ($NO_3^-$) | X2 ($Cu^{2+}$) | $NO_3^-$ (mM) | $Cu^{2+}$ (µM) | $\mu_{max}$ (days $^{-1}$) | dt (days) | Maximal biomass (g $L^{-1}$) |
|---|---|---|---|---|---|---|
| +1 | −1 | 2.74 | 0.1 | 0.389 | 1.78 | 14.25 |
| −1 | −1 | 0.685 | 0.1 | 0.317 | 2.18 | 7.54 |
| +1 | +1 | 2.74 | 2 | 0.339 | 2.04 | 12.79 |
| −1 | +1 | 0.685 | 2 | 0.351 | 1.97 | 8.41 |
| 0 | 0 | 1.37 | 1 | 0.364 | 1.90 | 11.08 |

| Coefficients (linear model) | µ | dt | Maximal biomass |
|---|---|---|---|
| β0 | 0.376 | 1.708 | 9.580 |
| β1($NO_3^-$) | 0.012 | 0.017 | 2.601** |
| β2 ($Cu^{2+}$) | −0.007 | 0.081 | −0.421** |
| β1,2 ($NO_3^-$)($Cu^{2+}$) | 0.008 | −0.072 | 0.346** |

Coefficient values were significantly different when these were followed by $**(p<0.01)$

where Y is coumarins (Y2), and the sphaeralcic-acid (Y3) contents intra- and extra-cellular, $B_0$ is the intercept term, $B_1$ is the linear effect of total nitrate on response, $B_2$ is the linear effect of copper on response, $B_{11}$ is the quadratic effect of total nitrate on response, $B_{22}$ is the quadratic effect of copper on response, $B_{12}$ is the co-efficient of the effect of the interaction between variables.

From the above model, the response surface was plotted for each independent variable to describe the behavior of every response concerning to the nitrate and copper concentrations contained in the culture medium.

## Analytical determinations

### Cell growth

The cell suspension from each flask was vacuum- filtered in a Buchner funnel (Whatman No. 1, 5.5-cm diameter) and the biomass retained; subsequently, the biomass was dried at room temperature and dry weight (DW) was determined (g $L^{-1}$). Maximal biomass was obtained after analyzing cellular growth under each nutrient condition; the maximal growth rate ($\mu_{max}$) was calculated by a semi-log calculation of exponential phase and time (graph not shown), and duplication time was ascertained from the equation $dt = \ln 2/\mu_{max}$ (Quintero 1981).

### Tomentin, scopoletin, and sphaeralcic-acid analyses

Dry biomasses were extracted at room temperature at a 1:20 (w/v) proportion with dichloromethane:methanol (9:1) three times by maceration (24 h for each procedure). The extracts obtained for each sample were filtered through Whatman No. 1 filter paper, pooled, and concentrated to dryness. The culture media were partitioned three times with dichloromethane; extracts from each medium were pooled and concentrated to dryness (Pérez-Hernández et al. 2014; Nicasio-Torres et al. 2016).

Analyses of HPLC of media and biomass extracts were carried out in a Waters system (2695 Separation Module) coupled to a diode array detector (2996) with a 190–600 nm detection range and operated by the Manager Millennium software system (Empower ver. 1; Waters Corp., Boston, MA, USA). Separations were performed in a Spherisorb® RP-18 column (250 × 4.6 mm, 5 µm; Waters) employing a constant temperature of 25 °C during analyses. Samples (20-µL) were eluted at a 1.2 mL $min^{-1}$ flow rate with (A) high-purity $H_2O$ ($H_3PO_4$-1.0%) and (B) high-purity $CH_3CN$-gradient mobile phases (Merck), and were detected by monitoring absorbance at λ = 343 nm and λ = 357 nm, as previously described (Pérez-Hernández et al. 2014; Nicasio-Torres et al. 2016). Analyses of a mixture of scopoletin (99%, Sigma-Aldrich, México) and tomentin (88%), and sphaeralcic acid (95%) were performed by comparing their retention times (tomentin, 10.96 min, scopoletin, 11.2 min, and sphaeralcic acid, 17.3 min) and the absorbance spectra, and their quantification using calibration curves.

## Results and discussion

### Cell growth

The growth curves obtained in the cell suspensions grown in MS medium under the experimental conditions determined by the $2^k$ FD showed a similar sigmoid-like curve (Fig. 1). The ANOVA showed that maximal growth rate ($\mu_{max}$) and duplication time (dt) did not vary by effect of nitrate and copper levels in the MS medium (Table 1); nevertheless, the maximal biomass achieved varied by the combined action of nitrate and copper ($p < 0.05$). The positive effect of nitrates means that for increasing maximal biomass, nitrates concentration in culture media must be higher, and the negative effect of the copper means that its concentration must be lower. Copper levels of 1–10 µM reduced the growth of carrot embryo cultures (Górecka et al. 2007) and 45 mg $L^{-1}$ reduced the growth of *Bacopa monnieri* shoot culture (Sharma et al. 2015), while 100–200 µM of copper were toxic for cells in the suspension of *Nicotiana tabacum* (Szafranska et al. 2011). The largest maximal biomass (14.25 g $L^{-1}$) acquired was similar to that obtained before for cell cultures (13.37 g $L^{-1}$) grown with 2.74 mM of total nitrates in combination with 0.1 µM of copper (Nicasio-Torres et al. 2016).

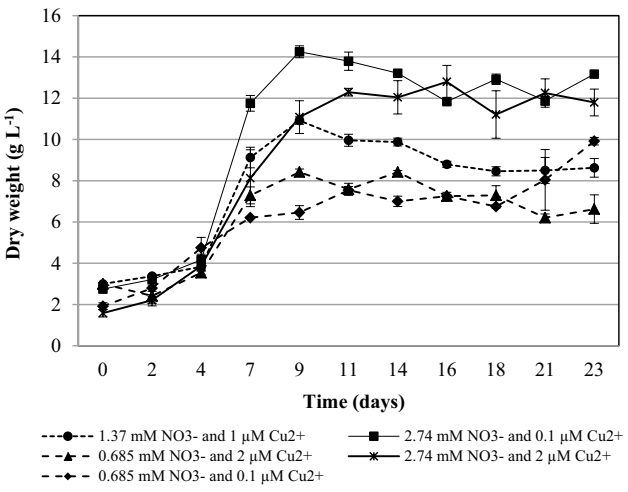


**Fig. 1** Growth curves of *Sphaeralcea angustifolia* cell suspensions in batch cultures by variation of total nitrate and copper concentrations in the MS medium by the $2^K$ factorial design. Mean ± SEM (n = 3)

## Production of scopoletin, tomentin, and sphaeralcic acid

The intra-cellular production of coumarins (scopoletin and tomentin) and sphaeralcic-acid is associated with growth (Fig. 2a, b), and highest concentrations were obtained during the maintenance phase; at days 9 and 11, the concentrations are similar in the suspensions cultured with 2.74 mM–0.1 µM, 2.74 mM–2 µM, and 1.37 mM–1.0 µM of nitrate and copper, respectively. Sphaeralcic-acid contents were 10-fold higher than those previously determined (112.04 µg $L^{-1}$) after 11 days of culture (Nicasio-Torres et al. 2016). These compounds are also excreted into the culture medium (Fig. 2c, d) at the beginning (2 and 4 days) of logarithmic growth phase (Pérez-Hernández et al. 2014; Nicasio-Torres et al. 2016); subsequently, the content of these compounds decreased considerably. Highest contents of coumarins (4137.00 µg $L^{-1}$) and sphaeralcic-acid (1441.00 µg $L^{-1}$) at days 2 and 4 of culture, respectively, were obtained in the MS medium by the interaction of 2.74 mM of nitrates and 2 µM of copper; in addition, the growth of the cell suspension was not modified (Fig. 1). The excretion of coumarins is higher than that reported for scopoletin (999.00 µg $L^{-1}$) on the same days of culture, and sphaeralcic-acid had not been detected under these same culture conditions (Nicasio-Torres et al. 2016). Sphaeralcic-acid contents in the culture media at day 4 were similar to those detected in biomass after 9 and 11 days of culture (Fig. 2b, d). This behavior could be an advantage for biotechnological production of these compounds, as they could be removed from culture media after 4 days of culture letting the cells continue growing for producing them intracellularly.

In order to optimize extra-celular productions obtained during the first 4 days of culture, a CCD was developed. The model coefficients indicate that nitrate and copper concentrations should increase ($\beta1$, $\beta2 = 423.97$; $p < 0.05$) in order to favor coumarin (scopoletin and tomentin) excretion at day 2; for sphaeralcic-acid the significant effect [$\beta2^2$ ($Cu^{2+}$) = 1301.94; $p < 0.05$] indicated that copper concentrations should increase at day 4. Instead of favoring the coumarin accumulation, the nitrate concentration should be reduced and that of copper should be increased ($\beta1$, $\beta2 = 39.45$; $p < 0.05$). The maximal levels of extra- (4136.96 ± 188.17 µg $L^{-1}$) and intra-cellular

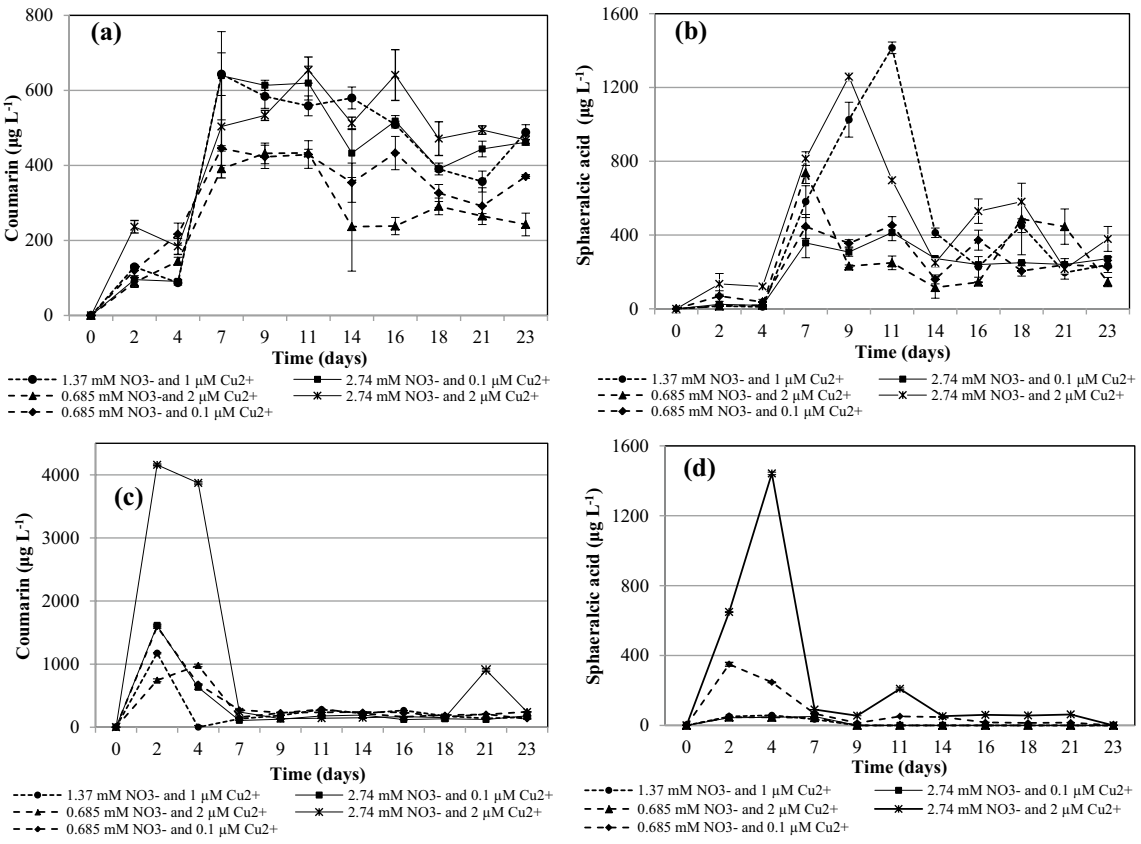


**Fig. 2** Production kinetics of coumarins biomass (**a**) and in culture medium (**c**), and sphaeralcic-acid biomass (**b**) and in culture medium (**d**) in *Sphaeralcea angustifolia* cell suspension, by varying the nitrate and copper concentrations in the MS medium by the $2^K$ factorial design. Mean ± SEM (n = 3)

($236.45 \pm 16.59$ µg $L^{-1}$) coumarins were obtained with 2.74 mM of nitrate and 2 µM of copper after 2 days of culture. The best condition for sphaeralcic-acid production was obtained with 1.37 mM nitrate without copper for its excretion ($5258.12 \pm 156.34$ µg $L^{-1}$), and with 2.41 µM copper for its accumulation ($511.18 \pm 150.39$ µg $L^{-1}$) after 4 days. According to the response surface, the highest intracellular contents of coumarins could be obtained in combination with nitrate and copper reduction (268.00 µg $L^{-1}$) or high copper levels (2 µM) with 2.42 mM of nitrate (297.00 µg $L^{-1}$) at day 2 of culture (Fig. 3a); this latter condition of cultivation does not affect growth. In addition, coumarin excretion was improved at the highest copper concentrations (4008.00 µg $L^{-1}$): this content is 40-fold higher than that detected in biomass (Fig. 3a, b). In the cell cultures of *Angelica archangelica*, the accumulation and excretion of scopoletin were induced with a dose-dependent effect utilizing concentrations between 5 and 50 µM of copper sulfate in cultures exposed to light (Siatka et al. 2017).

The intra-cellular production of sphaeralcic acid was high with the lowest copper concentration during the growth phase, but increased in the maintenance phase when high copper concentrations (2.35 µM) were used in combination with the nitrate reduction after 4 days of culture (Fig. 3c). In the response surface is possible to observe that higher contents of sphaeralcic-acid (Fig. 3d) can be obtained by reducing the nitrate content and/or increasing the copper content (0.32 mM of nitrates and 2.35 µM of copper). The sphaeralcic-acid extra-cellular content is 30-fold higher than that detected in biomass.

The effects of macronutrients ($NO_3^-$, $NH_4^+$, and $PO_4^{3-}$) on cell growth and triterpenoid saponins production (centellosides, madecassoside, and asiaticoside and their aglycones, madecassic and asiatic acids) in *Centella asiatica* cell-suspension cultures were analyzed using the Box-Behnken response-surface-model experimental design. The optimal values predicted from the RSM were 5.05 mM $NH_4^+$, 15.00 mM $NO_3^-$, and 2.60 mM $PO_4^{3-}$, yielding 16.00 g $L^{-1}$ cell DW. However, production of triterpenoids was lower than 4.00 mg $g^{-1}$ cell DW (Rozita et al. 2005). Further, asiaticoside production in shoot cultures of *C. asiatica* increased from 5.30 to 8.90 and 8.37 mg $g^{-1}$ DW when the total nitrogen concentration of 60 mM was reduced to 50 and 40 mM, respectively.

The study of nitrate and copper as inducers of the production of phenolic compounds has been not evaluated, to our knowledge, in combination. Nitrate-deficient in *Arabidopsis*

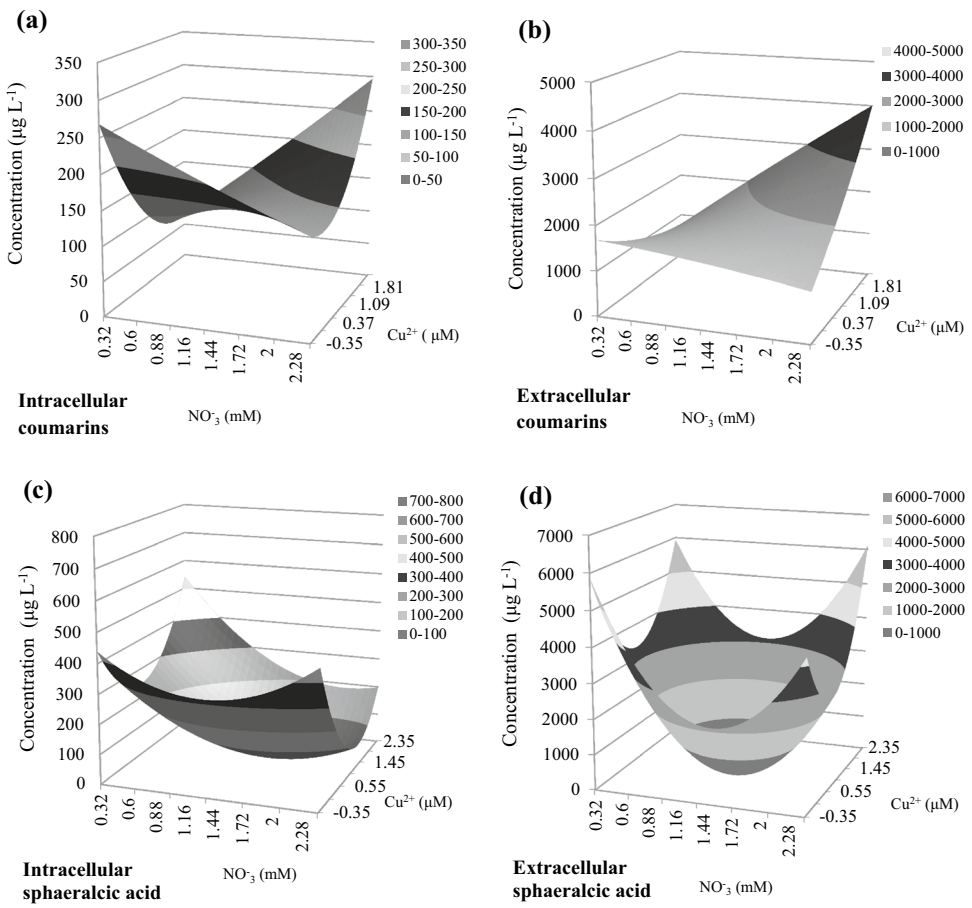


**Fig. 3** Coumarin (**a, b**) and sphaeralcic-acid (**c, d**) production at 2 and 4 days in culture, respectively, of *Sphaeralcea angustifolia* cell suspensions

*thaliana* wild-type plants accumulate high levels of a range of phenylpropanoids including chlorogenic acid and rutin, stimulated by the reduction of nitrate and mediated by the induction of enzymes established in the early steps of the phenylpropanoid biosynthetic pathway (Fritz et al. 2006). Additionally, in *A. thaliana* plants grown in soil, on agar plates, and by means of hydroponics, anthocyanin and flavonol accumulation was induced by diminishing nitrogen in the growth medium (Lea et al. 2007). In *Matricaria chamomilla* plant cultures, nitrate reduction increased the levels of chlorogenic acid and the coumarins herniarine and umbelliferone (Kováčik and Klejdus 2013). In *in-vitro Bacopa monnieri*, shoot cultures treated with 45.00 mg $L^{-1}$ of $CuSO_4$, shown highest bacoside content i.e., ~ 1.42-fold higher than in control cultures (Sharma et al. 2015). Contrary to the results obtained in cell-suspension cultures of *S. angustifolia*, in *C. asiatica* the medium devoid of copper also favored higher asiaticoside accumulation (7.05 mg $g^{-1}$ DW) compared with shoots (4.40 mg $g^{-1}$ DW) cultivated with 0.1 µM copper (Prasad et al. 2012). In the cultivation of *Hypericum perforatum* plants, it was possible to increase (two fold) the concentration of hypericin by decreasing the nitrate concentration from 4.42 to 0.2 mM and increasing irradiance from 100 to 400 µmol $m^{-2}$ $s^{-1}$ (Briski and Gawienowski 2001).

After analyzing the effects of nitrates and copper on metabolites production, the CCD showed that the *S. angustifolia* cell suspension could be cultivated in a stirrer tank bioreactor for producing high concentrations of scopoletin, tomentin, and sphaeralcic-acid in MS medium with 2.42 mM of total nitrate in combination with a high copper concentration (2.35 µM) at relatively short culture times.

The main contribution of the results obtained in the present work is that they offer the bases to design a bioprocess that allows producing coumarins and sphaeralcic-acid at the bioreactor level. Thus, during the first four days of culture, high excretions of the metabolites would occur into the broth; which would facilitate its removal and subsequent recovery, and then the concentrations of nitrates and copper in the culture medium could be modified to favor the production of these metabolites during the maintenance phase. This would allow maximum use of the ability of the cells to produce coumarins and sphaeralcic acid.

**Author Contributions** JP-H participated in the collection, analysis and interpretation of data, and the writing of the manuscript. AM-T participated in the design of the study, interpretation of data, and writing of manuscript. PN-T participated in the conception and design of the study, as well as the writing of manuscript, and approved the final version to be submitted.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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