MORPHOGENESIS AND SECONDARY METABOLITES PRODUCTION IN THE MEDICINAL PLANT *Castilleja tenuiflora* Benth. UNDER NITROGEN DEFICIENCY AND STARVATION STRESS IN A TEMPORARY IMMERSION SYSTEM

MORFOGÉNESIS Y PRODUCCIÓN DE METABOLITOS SECUNDARIOS EN LA PLANTA MEDICINAL *Castilleja tenuiflora* Benth. BAJO ESTRÉS POR DEFICIENCIA Y AUSENCIA DE NITRÓGENO EN UN SISTEMA DE INMERSIÓN TEMPORAL

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Abstract

Castilleja tenuiflora Benth, is a medicinal plant that synthesizes secondary metabolites such as phenylethanoid glycosides (PhGs), flavonoids, lignans, and iridoid glycosides with pharmacological properties. Nutrient deficiency stress in *in vitro* culturing of plants has been shown to be a good biotechnological strategy to stimulate secondary metabolism. Our aim was to evaluate morphogenesis (growth and development) and the production of secondary metabolites in *C. tenuiflora* shoots in response to stress by nitrogen (N) deficiency and starvation in temporary immersion bioreactors. Shoots were grown in B5 liquid medium with 25.74 mM (control), 1.32, 0.66 (deficiency) and 0 mM (starvation) N concentration. In all treatments, matter and shoot multiplication rate (SMR) decreased over 40%, chlorophylls content decreased, roots length increased, and leaves showed chlorosis respect to control. Under 0.66 mM N deficiency, the enzyme phenylalanine ammonia lyase (PAL) was more active (twice more than control) and total phenolic compounds were enhanced. At day 21: maximum concentration of PhGs (verbascoside and isoverbascoside) and iridoids (aucubin) was observed in 0.66, 1.32 and 0 mM N treatments (283 ± 0.5, 90 ± 0.3 and 12.5 ± 0.1 mg g^{-1} DM, respectively); these concentrations were 175, 143 and 34% greater than respective controls on the same day. Additionally, presence of anthocyanins was evident in stems of all plants developed under N deficiency and starvation. Despite the fact that, the matter, SMR and chlorophylls decreased because N stress deficiency, the production of secondary metabolites was enhanced, demonstrating that *in vitro* culture in temporary immersion bioreactors under suitable nutrients deficit such as N is an appropriate strategy to stimulate secondary metabolism in *C. tenuiflora*.

Keywords: abiotic stress, Aucubin, nitrogen starvation, phenylethanoid glycosides, RITA® bioreactor.

Resumen

Castilleja tenuiflora Benth., es una planta medicinal que sintetiza metabolitos secundarios como feniletanoides glicosilados (PhGs), flavonoides, lignanos, e iridoides; que presentan actividad farmacológica. El estrés por deficiencia de nutrientes en el cultivo de plantas *in vitro* ha mostrado ser una estrategia biotecnológica apropiada para estimular su metabolismo secundario. En este trabajo se evaluaron la morfogénesis (crecimiento y desarrollo) y la producción de metabolitos secundarios en brotes de *C. tenuiflora* en biorreactores de inmersión temporal expuestos a estrés por deficiencia y ausencia de nitrógeno (N). El estrés se aplicó a un cultivo de los brotes cultivados en medio líquido B5 con 25.74 mM (control), 1.32, 0.66 (deficiencia) y 0 mM (ausencia) de N. En comparación con el control, todos los tratamientos mostraron una disminución de más del 40% en la biomasa y el índice de multiplicación de brotes, el contenido de clorofila disminuyó, la longitud de las raíces aumentó y las hojas mostraron clorosis. En deficiencia de N (0.66 mM), la enzima fenilalanina amonio liasa (PAL) mostró la mayor actividad (dos veces más que el control) y el contenido de compuestos fenólicos se incrementó. En el día 21 se encontraron las concentraciones máximas de PhGs (verbascósido e isoverbascósido) e iridoides (aucubina) (283 ± 0.5, 90 ± 0.3 y 12.5 ± 0.1 mg g^{-1} BS, en los tratamientos con 0.66, 1.32 and 0 mM de N, respectivamente); estas concentraciones fueron 175, 143 y 34% más altas en comparación con el control. Además, la presencia de antocianinas en los tallos de todas las plantas desarrolladas bajo deficiencia y ausencia de N fue

evidente. A pesar de que la biomasa, la multiplicación de brotes y las clorofilas disminuyeron debido al estrés por deficiencia y ausencia de N, la producción de metabolitos secundarios se incrementó significativamente, lo que demuestra que esta estrategia es apropiada para estimular el metabolismo secundario de *C. tenuiflora*.

Palabras clave: estrés abiótico, Aucubina, ausencia de nitrógeno, feniletanoides glicosilados, bioreactores RITA®.

1 Introduction

Plant cell, tissue, and organ cultures have been used to increase plant matter and produce a wide range of phytochemicals of pharmacological interest (Nieto-Trujillo *et al*. 2017; Murthy *et al*. 2015). There are different systems that can be used for these purposes, one of them being temporary immersion bioreactors, which offer among other advantages the reduction of labour cost, reduction of vitrification compared with permanent immersion, complete renewal of atmosphere at each immersion and secondary metabolite enhancement (Ashraf *et al*. 2013; Valdez-Tapia *et al*. 2014). Secondary metabolites biosynthesis in plants varies depending on environmental stress, and the biosynthesis of these compounds can be stimulated by biotic and abiotic factors (Ramakrishna and Ravishankar 2011). In fact, by induction of a particular stress response, plants can be stimulated to produce desired phytochemicals for industrial purposes (Trivellini *et al*. 2016).

Nitrogen (N) is a major limiting macronutrient for growth, development and productivity in most agricultural systems (Yang *et al*. 2015). It is mainly absorbed by the root and is an essential component of molecules such as amino acids, proteins, nucleic acids and chlorophyll (Ková čik and Bačkor 2007; Kraiser *et al*. 2011). Because more than half of nitrogen is used in processes involved in photosynthesis, insufficient N supply results in a stress condition for plants and other photosynthetic organisms as microalgae (Navarro-Peraza *et al*. 2017), leading to reduced growth, changes in root architecture, and chlorophyll degradation (Lea and Azevedo 2007; Giorgi *et al*. 2009). A shift from biosynthesis of secondary metabolites based on N towards carbon (C)-based compounds occurs, mainly to phenolic compounds such as hydroxycinnamic acids, flavonoids and anthocyanins (Lovdal *et al*. 2010; Ncube *et al*. 2012; Rubio-Wilhelmi *et al*. 2012). Under nitrogen-deficiency conditions, the accumulation of reactive oxygen species is induced (Kovacik *et al*. 2009; Giorgi *et al*. 2009; Kovacik *et al*. 2014). To counteract this oxidative stress, plants increase both enzymatic and non-enzymatic systems to protect against reactive oxygen species; part of non-enzymatic system consists of the biosynthesis of secondary metabolites such as phenolic compounds. The biosynthesis of these metabolites is correlated with an increment of PAL activity (Wang *et al*. 2014), which is a key enzyme in the biosynthesis of phenolic compounds.

Castilleja tenuiflora Benth. (Orobanchaceae) is a wild plant used in traditional medicine to relieve gastrointestinal diseases, and "treat" nerves and tumors (Graham *et al*. 2000; Alonso-Castro *et al*. 2011). This species synthesizes secondary metabolites such as iridoid glycosides (aucubin, bartsioside, mussaenosidic acid, geniposidic acid, methyl 8-*epi*-loganin, geniposide, carioptoside, shanzhiside) (Jiménez *et al*. 1995, Herrera-Ruiz *et al*. 2015), phenylethanoid glycosides (verbascoside, isoverbascoside) (Gómez-Aguirre *et al*. 2012), flavonoids (apigenin, quercetin glycosides, luteolin-5-methyl ether) (López -Laredo *et al*. 2012; Herrera-Ruiz *et al*. 2015) and lignans (tenuifloroside) (Herrera-Ruiz *et al*. 2015). These compounds have shown valuable pharmacological activities such as cytotoxic, antioxidant, anti-inflammatory, antiulcer and anti-depressant effects (Moreno-Escobar *et al*. 2011; Sanchez *et al*. 2013; Herrera-Ruiz *et al*. 2015).

Our group developed *in vitro* cultures of *C. tenuiflora* that synthetizes both phenolics and iridoid glycosides, and we have grown them in liquid medium in flasks (permanent immersion) and in temporary immersion bioreactors. Only whole plants have the ability to synthetize iridoids (Martínez-Bonfil *et al*. 2011; Sanchez *et al*. 2013); root and shoot cultures synthetize only PhGs (Gómez-Aguirre *et al*. 2012; Medina-Perez *et al*. 2015). In some of these cultures, we have evaluated oxidative stress conditions affecting secondary metabolites synthesis. For example, we induced biotic stress with cell-wall oligosaccharides from *Fusarium oxysporum* f. sp. *Lycopersicum* race 3, which stimulated PhGs biosynthesis in shoots of *C. tenuiflora* grown in shake flasks (Cardenas-Sandoval *et al*. 2015). We previously showed that abiotic stress by nitrogen deficiency (1.32 mM N) in shoots of *C. tenuiflora* grown in a temporary immersion system stimulated PhGs production with induction of anthocyanin biosynthesis (Medina-Perez *et al*. 2015). In that work, initial plant material was developed in

B5 liquid medium without plant growth regulators so iridoid glycosides were not synthetized and only one N-stress condition was tested. Therefore, *in vitro* culture of *C. tenuiflora* in temporary immersion bioreactors with suitable N deficit in the culture medium is a good alternative to obtain these two kinds of secondary metabolites. Here we report the effect of N stress (deficiency and starvation) on the morphogenesis (growth and development) and secondary metabolism of *C. tenuiflora* plants culturing in temporary immersion bioreactors.

2 Materials and methods

2.1 Plant material and treatments

C. tenuiflora plants were cultured *in vitro* in MS semisolid medium (0.8% agar w/v) (Murashige and Skoog 1962) with sucrose (0.09 M) and supplemented with the plant growth regulators indole butyric acid (0.5 μM) and 6-benzylaminopurine (0.55 μM). For experiments, forty explants (21-day-old) of 2.5 cm size were transferred into a temporary immersion bioreactor system (RITA®) with 200 mL of B5 liquid medium (Gamborg *et al*. 1968) and sucrose (0.09 M) without plant growth regulators. In each bioreactor the flow rate in the inlet air was 1 L min^{-1} and the immersion cycle was 5 min every 24 h. (Valdez-Tapia *et al*. 2014). Nitrogen stress was induced by modifying the KNO$_3$ and (NH$_4$)$_2$SO$_4$ basal concentration in B5 medium without altering the nitrate:ammonium ratio (24:1). The treatments were: the N basal concentration of B5 medium as the control (25.74 mM N), N-deficiency (1.32 and 0.66 mM N) and N-starvation (0 mM N). For each treatment, three bioreactors were run simultaneously; and the experiment was repeated twice. All cultures used in this study were maintained in a growth chamber under controlled conditions: 25 ± 2°C with a 16-h light/8-h dark photoperiod and an irradiance of 77 μmol m^{-2} s^{-1}. Total chlorophyll content, PAL activity, total phenolic compounds, phenylethanoids and aucubin concentrations were measured on days 0, 4, 9 and 21 d of culture growth. For the morphogenesis variables, at the start (day 0) and at the end of the experiment (day 21), all of the plants were counted and the shoot multiplication rate was calculated as follows: (number of shoots and buds at the end of culture period)/(number of shoots inoculated). We also evaluated plant and root length (mm); and the water

content, fresh and dry matter through gravimetric measurements.

2.2 Total chlorophyll and nitrogen content

The total chlorophyll concentration was determined as the sum of chlorophyll *a* and *b* concentration, according to methods described by Lichtenthaler and Wellburn (1987), with minor modifications. Fresh tissue (15 mg of whole plant) was frozen with liquid N and grounded to a fine powder. This was extracted with acetone (80%) for 30 min and then centrifuged at 16,060 × g for 10 min at 10 °C. The absorbance of chlorophyll *a* and *b* was measured at 663.2 and 646.8 nm, respectively (UV-A 160, Shimadzu, Japan). To determinate total N content, matter from three bioreactors of each N stress treatment was collected, frozen in liquid nitrogen and lyophilized. The samples were analyzed by Kjeldahl method (Allen, 1989).

2.3 Phenylalanine ammonia-lyase activity

PAL enzyme activity was determined by measuring cinnamic acid production from phenylalanine using the method proposed by Yan *et al*. (2006). Fresh tissue (200 mg of whole plant) was frozen in liquid nitrogen, pulverized in a cold mortar with 40 mg of polyvinylpolypyrrolidone (PVPP) and homogenized with an extraction solution (3 mL at 4 °C) containing 100 mM sodium phosphate buffer (pH 6.0), 2 mM EDTA and 4 mM dithiothreitol (DTT). The mixture was centrifuged at 16,060 × g for 15 min at 4 °C (Biofuge fresco, Heraeus®, United Kingdom), and the extract was used for enzymatic reaction. The reaction mixture consisted of 550 μL of 50 mM Tris-HCl (pH 8.8), 250 μL of L-phenylalanine 20 mM (pH 8.8) and 200 μL of enzyme extract (2-16 μg protein). After incubation (60 min shaking at 600 rpm at 40 °C), the reaction was stopped with 50 μL of 5N HCl and the absorbance was measured at 290 nm. To avoid interference by endogenous L-phenylalanine, a blank without L-phenylalanine was used. PAL activity was expressed as nmol cinnamic acid h^{-1} mg^{-1} protein (nmol CA h^{-1} mg^{-1} protein). The content of soluble protein was determined by the Bradford assay (1976).

2.4 Quantification of total phenolics content

Total phenolic compounds were measured by colorimetric method as described before by López-Laredo *et al*. (2009). Fresh samples (200 mg of whole

plant) were frozen in liquid nitrogen and grounded to a fine powder in a cold mortar. For extraction, the powder was suspended in 1 mL of HPLC-grade methanol, stirred vigorously and allowed to stand for 30 min. After centrifugation (16,060 x g for 15 min at 4 °C), 100 μL of supernatant was mixed with 2.5 mL of deionized water and 0.1 mL of Folin-Ciocalteu reagent, followed by gentle shaking. The solution was allowed to stand for 6 min. Then, 0.5 mL of sodium carbonate (20% w/v) was added and mixing vigorously. After 30 min, absorbance at λ=760 nm was measured and concentration of phenolics was calculated by comparison with a calibration curve prepared with gallic acid (0-25 μg mL^{-1}). All samples were analyzed in triplicate. Results are reported as mg of gallic acid equivalents per g of dry matter (mg GAE g^{-1} DM).

2.5 Quantification of phenylethanoid glycosides and aucubin by HPLC

Plant material of each treatment was collected from bioreactors, frozen, lyophilized and grounded in a mortar. Extraction was carried out by maceration with methanol (10 mg:1 mL) for 24 h with stirring. The extracts were filtered under vacuum using a Whatman paper No. 1, and the solvent was evaporated using a rotary evaporator (V-250, Buchi, Switzerland) at 210 mbar, 40 °C at 50 rpm.

HPLC analysis was carried out using an HPLC system (Delta Prep 4000, Waters, Milford, MA, USA) equipped with a Waters 2695 separation module, photodiode array detector (Waters 996) and Pro Empower software (Waters), using a reverse phase Lichrospher 100 RP-18 column (250 mm x 4 mm, 5 μm) (Merck, Darmstadt, Germany) connected to a guard column. Commercial aucubin (Fluka Analytical®, 55561) and from *C. tenuiflora* isolated verbascoside (Gómez-Aguirre *et al.*, 2012) were used as reference standards. The mobile phase consisted of a 0.5% v/v acetic acid aqueous solution (solvent A) and acetonitrile (solvent B) for analysis of phenylethanoids. The gradient system was as follows: 0-2 min, 100-0% B; 3-10 min, 77-23% B; 11-13 min, 70-30% B; 14-15 min, 0-100% B; 16-17 min, 100-0% B. Fingerprints of both previously isolated verbascoside standard and samples of *C. tenuiflora* treatments were analyzed at 330 nm. For aucubin quantification, the method established by Sanchez *et al.* (2013) was used. The mobile phase consisted of a mixture of water:acetonitrile (97:3

v/v, solvent A) and acetonitrile (solvent B) with a gradient system as follows: 0-8 min, 100-0% B; 9-10 min, 80-20% B; 11-15 min, 70-30% B; 16-18 min, 40-60% B; 19-22 min, 0-100% B; 23-26 min, 100-0% B. The sample injection volume was 10 μL and the flow rate was maintained at 1 mL min^{-1}. Fingerprints of commercial aucubin standard and samples of *C. tenuiflora* treatments were recorded at 205 nm. The retention time peaks, area under curve peaks and absorption spectrum of reference compounds were recorded. Phenylethanoids (verbascoside and isoverbascoside) and aucubin concentrations were estimated based on a calibration curve for phenylethanoids (y=5035.3x + 4274.3, R^2=0.99) and aucubin (y=5720.5x+2409.9, R^2=0.99), respectively. In both cases, specific concentration is reported as mg per g of dry matter (mg g^{-1} DM) and total yield in mg per bioreactor.

2.6 Statistical analysis

Data were analyzed using one-way ANOVA. Dunnett´s test with a significance level of 5% was performed to determine whether nitrogen deficiency or starvation showed significant influence on the parameters assessed respect to control. All tests were performed using SAS® System for Windows version 9.0 (SAS Institute, Inc., Cary, NC, USA).

3 Results

3.1 Effect of nitrogen stress on growth and development

In all treatments, the stress induced by N-deficiency and N-starvation appeared as reductions in shoots matter, shoots and roots length, and inhibition of shoot multiplication (Table 1). The stress conditions had a negative effect on the SMR of plants developed between 2-3 shoots per explant compared to 7 shoots per explant in control conditions. In all evaluated N stress treatments, plant matter (fresh and dry) was strongly decreased over 40% respect to the control, and shoot length was N dependent: plants with an N supply of 1.32 mM showed 20% lower, with a lower or without N supply resulted in a shoot length reduction of 36 and 49%, respectively. Although, shoots developed roots under all treatments, N stress strongly influenced root length; it was observed that

Table 1 Growth and development parameters of *C. tenuiflora* plants grown in temporary immersion system for 21 days under control (25.74 mM N) and nitrogen stress conditions

	Control	N-deficient		N-starved
	25.74 mM	1.32 mM	0.66 mM	0 mM
FM (g per RITA system)	3.4 ± 0.4	2.3 ± 0.1***	2.2 ± 0.2***	2.0 ± 0.2***
DM (g per RITA system)	0.3 ± 0.06	0.2 ± 0.02***	0.2 ± 0.01***	0.2 ± 0.01***
WC (%)	90.1	90	91.2	89.8
SMR	7.0 ± 1.6	2.0 ± 1.9***	3.0 ± 1.2***	2.1 ± 1.1***
Shoots length (cm)	3.9 ± 0.7	3.1 ± 0.7***	2.5 ± 0.6***	2.0 ± 0.8***
Roots length (cm)	2.9 ± 0.9	4.0 ± 0.7***	3.9 ± 0.9***	3.8 ± 1.1***
Total chlorophyll (μg mg^{-1} FM)	0.73 ± 0.1	0.72 ± 0.1	0.52 ± 0.1***	0.52 ± 0.1***
Total nitrogen (mg g^{-1} DM)	6.6 ± 0.8	2.6 ± 0.4***	1.8 ± 0.1***	1.5 ± 0.5***
Soluble proteins (mg g^{-1} DM)	2.2 ± 0.002	2.2 ± 0.006	1.8 ± 0.002***	1.6 ± 0.001***

FM = Fresh matter; DM = Dry matter; WC = Water content; SMR = Shoot multiplication rate. Data are means ± SD (n=3). (***) indicate significant difference between the control and each treatment in each of the assessment variables, according to the comparison of Dunnett's test ($P < 0.05$).

the roots of plants grown under nitrogen stress were 30% longer than the control plants. Tissue water content showed no significant change (Table 1).

N deficiency and starvation caused a decrease of total chlorophyll content. On day 0, all treatments showed a chlorophylls content average of 1.02 ± 0.1 μg g^{-1} FM. Similar to control, this variable in 1.32 mM N deficiency group dropped to 0.7 ± 0.1 μg g^{-1} FM at day 4 and then remained constant without significant differences until day 21. Decrease of total chlorophyll was significant with the lowest N supply (0.66 mM N) and the N starvation by 9 days, when it decreased to 0.52 ± 0.1 μg g^{-1} FM (29% less than control) and then remained constant until day 21 (Table 1). Total nitrogen content declined as N deficiency was worsened, compared with control plants (6.6 ± 0.8 mg g^{-1} DM), reaching the lowest value (1.5 ± 0.5 mg g^{-1} DM) in the N starvation group. Similarly, soluble protein content decreased in the 0.66 mM N deficiency group and the N starvation group (1.8 ± 0.002 and 1.6 ± 0.001 mg g^{-1} DM, respectively) compared with control (Table 1).

The stress also caused a strong chlorosis in all treatments denoted by a yellowish color of the oldest leaves (Fig 1b-d), a slight purple coloration in the stems (Fig 1e-g), and root architecture modification (Fig 1i-k). These changes were most evident after 21 days of culture compared to plants from control (Fig 1a, h).

3.2 PAL activity and total phenolic compounds

The stress by N deficiency and starvation positively stimulated the PAL enzyme activity and total phenolic compounds were enhanced. Among the groups undergoing N-stress treatment, PAL activity (140 ± 8 nmol CA h^{-1} mg^{-1} protein) was significantly higher in the 0.66 mM N group (twice that compared to every other treatment) by day 9. However, at day 21 the highest activity was recorded in the starvation treatment. PAL enzyme activity in control plants showed an increase from the start of culture to 9 day (58 ± 7 nmol CA h^{-1} mg^{-1} protein) and displayed similar levels until the end of experiment (Fig 2a).

The amount of total phenolic compounds showed a significant increase from day 4 in the 1.32 and 0.66 mM N-deficiency groups, reaching the highest concentrations of these metabolites (29.5 ± 0.3 and 27.6 ± 0.05 mg GAE g^{-1} DM, respectively) at day 21. In contrast, the plants under N starvation showed lower or similar content of phenolic compounds compared to control in all evaluated days of culture (Fig 2b).

3.3 PhGs and aucubin concentration

PhGs and aucubin were identified and its concentration was differentially enhanced because N stress deficiency and starvation. The HPLC profile recorded at $\lambda_{max} = 330$ nm showed the presence of two major peaks. By comparison of the reference standard and UV/VIS spectrum, these peaks corresponded to the verbascoside and isoverbascoside.
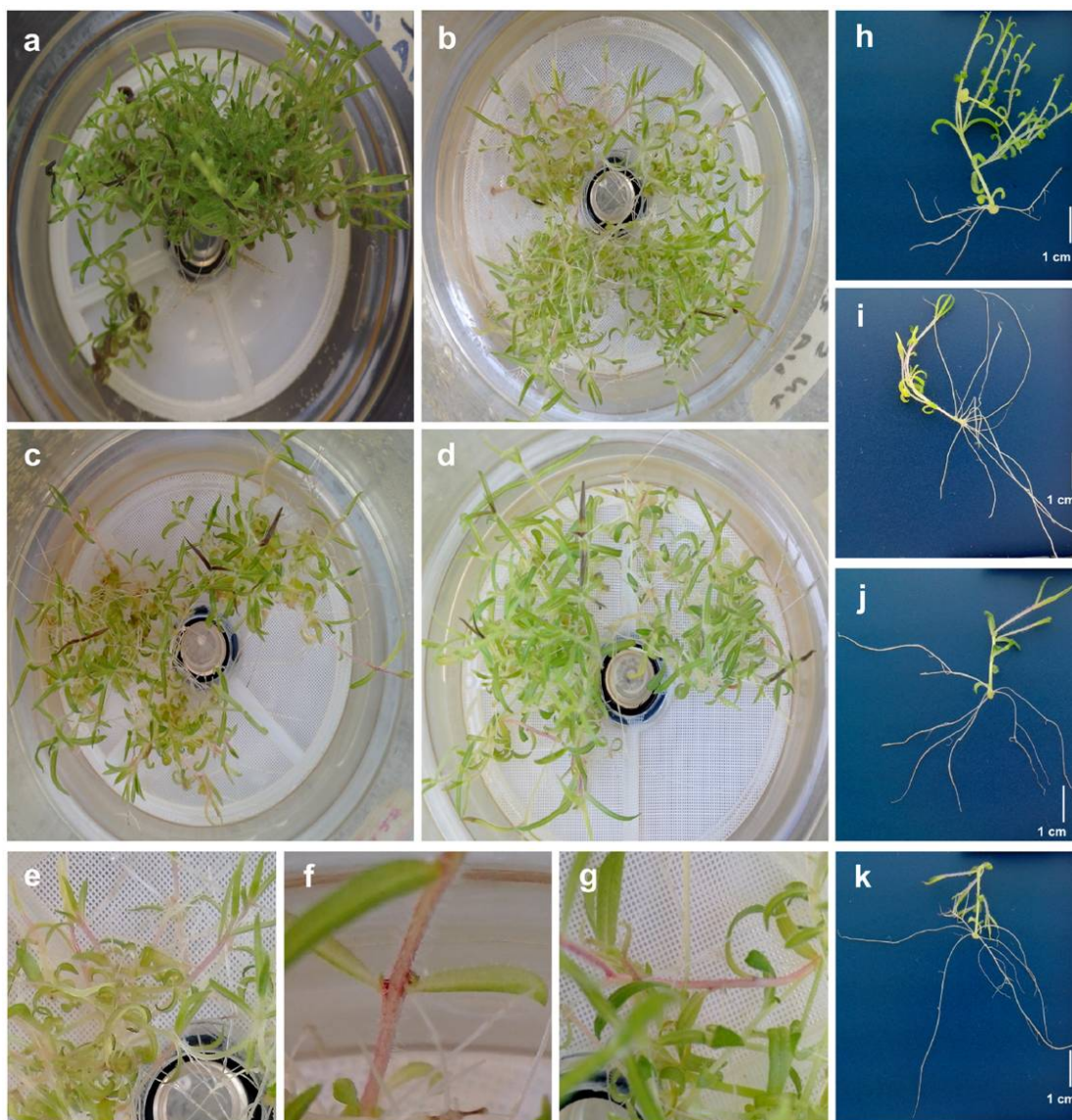
Fig. 1 *Castilleja tenuiflora* plants grown in temporary immersion system after 21 days of culture under Nitrogen deficiency and starvation stress. a Plants from control treatment (25.74 mM N); b Plants from N deficiency treatment (1.32 mM N); c Plants from N deficiency treatment (0.66 mM N); d Plants from N starvation treatment (0 mM N); e Plants with slight purple-pigment stems under N deficiency treatment (1.32 mM N); f Plants with slight purple-pigment stems under N deficiency treatment (0.66 mM N); g Plants with slight purple-pigment stems under N starvation treatment (0 mM N); h Plants with roots from control treatment (25.74 mM N);i Plants with roots from N deficiency treatment (1.32 mM N); j Plants with roots from N deficiency treatment (0.66 mM N); k Plants with roots from N starvation treatment (0 mM N). Scale bar: 1 cm (h, i, j and k).

Figure 3 shows specific and total concentration of these metabolites and it can be seen that, the specific concentration increases as culture time passes in all treatments evaluated. However, under N deficiency the maximum specific concentration of verbascoside (Fig 3a) and isoverbascoside (Fig 3c) was 283 ± 0.5 mg g^{-1} DM and 90 ± 0.3 mg g^{-1} DM, respectively (day 21); these concentrations were 175 and 143% greater than respective controls on the same day (108 ± 0.4 and 37 ± 0.01 mg g^{-1} DM, respectively). Meanwhile, in plants under N-starvation the highest value of verbascoside was observed at day 9 ($276 \pm$
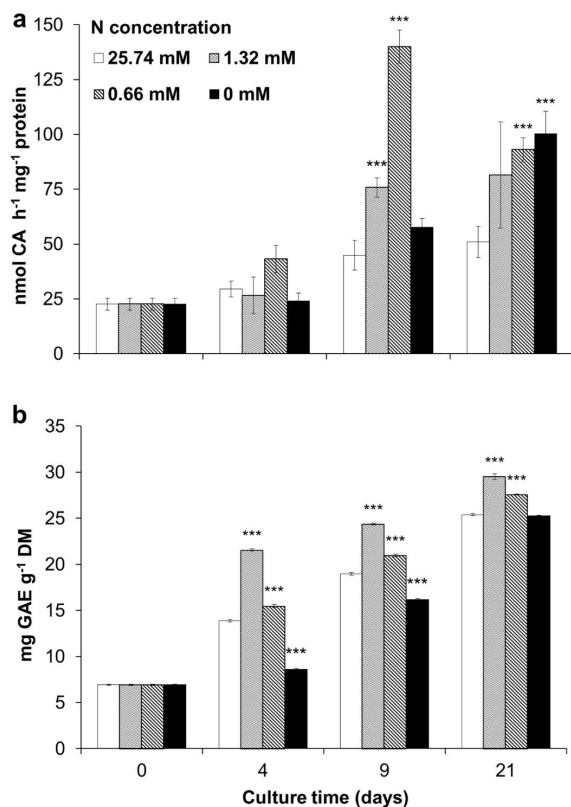
Fig. 2 PAL activity (a) and total phenolic compounds content (b) in *C. tenuiflora* plantlets grown in temporary immersion system for 21 days under control (25.74 mM N) and nitrogen stress conditions. Data are means ± SD (n = 3). (***) Indicate significant difference between the control and each treatment in each of the assessment times, according to the comparison of Dunnett´s test ($P < 0.05$).

0.7 mg g^{-1} DM) with an increment of 64% respect to control, for isoverbascoside was observed at day 21 (64 ± 0.6 mg g^{-1} DM, 78% higher than control). According to figures 3b and 3d, total concentration of PhGs per bioreactor was also higher under N-deficiency (between 47 to 70%) and N-starvation treatment (22%) compared to control.

According to comparison of the reference standard and the UV/VIS spectrum, the HPLC profile recorded at λ_{max} = 205 nm showed the presence of the iridoid aucubin. The specific concentration of this metabolite showed an increase according to culture time passes in all treatments assessed and was higher than control under all N stress treatments (Fig 4a). However, the greatest concentration of aucubin was 12.5 ± 0.3 mg g^{-1} DM, and was observed at day 21 in plants grown under N-starvation with an increase of 34% respect to
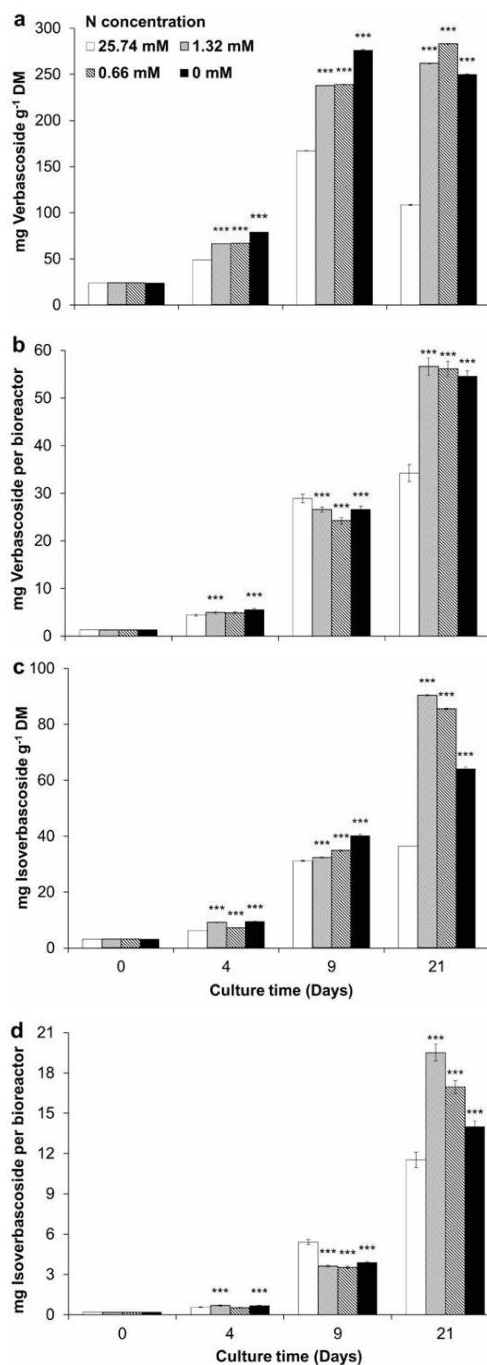
Fig. 3 Specific and total concentration of verbascoside (a, b) and isoverbascoside (c, d) in C. tenuiflora plants grown in temporary immersion system for 21 days under control (25.74 mM N) and nitrogen stress conditions. Data are means ± SD (*n* = 3). (***) Indicate significant difference between the control and each treatment in each of the assessment times, according to the comparison of Dunnett's test ($P < 0.05$).
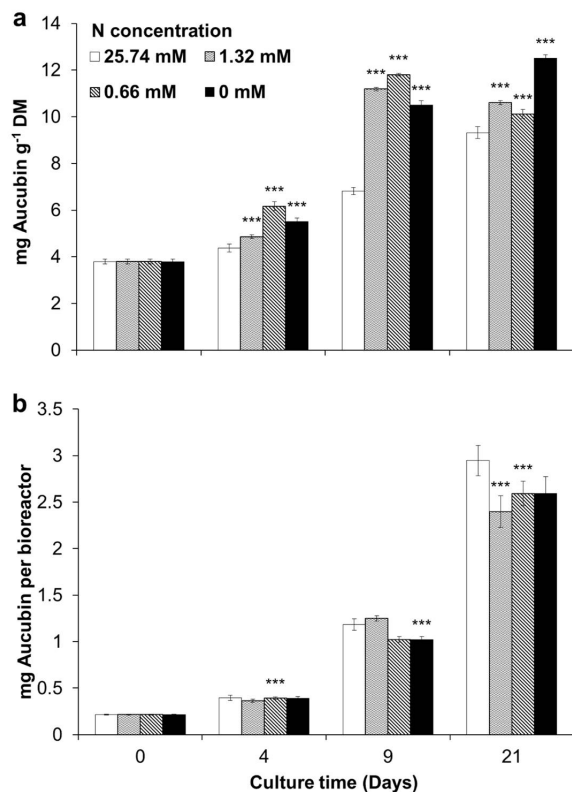
Fig 4 Specific (a) and total (b) concentration of aucubin in *C. tenuiflora* plants grown in temporary immersion system for 21 days under control (25.74 mM N) and nitrogen stress conditions. Data are means ± SD (*n* = 3). (***) Indicate significant difference between the control and each treatment in each of the assessment times, according to the comparison of Dunnett´s test (*P* < 0.05)

control (9.3 ± 0.3 mg g^{-1} DM). On the contrary, N-stress treatment did not enhance total concentration of aucubin per bioreactor compared with control.

4 Discussion

According to growth differentiation balance hypothesis plants have the ability to allocate resources from growth to defense according to the environmental conditions they face (Massad *et al*. 2012). In that context, the stress by N deficiency or starvation promotes significant reprogramming in plants from primary to secondary metabolism to ensure their survival and yield (Kumagai *et al*. 2009; Caretto *et al*. 2015). In the present study, we evaluated the effect

of nitrogen stress in *C. tenuiflora* grown in a temporary immersion system. Nitrogen stress negatively affected the growth and development of *C. tenuiflora* plants. All plants exposed to a prolonged N-deficiency or starvation resulted in a loss of 40% of matter (both fresh and dry) and the shoots length was reduced considerably compared with control. While the control plants generated on average 7 new shoots per explant (SMR), plants under N stress conditions just generated 2 (70% less) (Table 1). When the aerial parts of plants are reduced against low availability of N, C-based compounds are target to root construction to ensure efficient N uptake (Royer *et al*. 2013; Ncube *et al*. 2014). Here we observe that generated roots showed changes in their architecture; they were more numerous and 30% longer than those of control plants (Fig 1h-k). A similar behaviour was observed in plants of *Matricaria chamomilla*, *Arabidopsis thaliana* and *Zea mays* that were grown under N starvation, where plant matter was strongly affected and root length was greater than shoots, as compared with control (Kovacik and Backor 2007; Krapp *et al*. 2011; Gao *et al*. 2015). In contrast, *Castilleja tenuiflora* shoots cultured in bioreactor under 1.32 mM N deficiency showed strongly growth and fresh matter reduction but, there was no root generation (Medina-Perez *et al*. 2015).

Castilleja tenuiflora plants exposed to nitrogen deficiency stress developed strong chlorosis compared with control plants (Fig 1a-d). This appeared earlier when N stress was more severe, but chlorophyll content decrease was not associated with N stress level (Table 1). Furthermore, 15 days after N stress treatment, a purple pigmentation in stems could be observed; this pigmentation increased towards the end of culture time (day 21) and was even stronger in plants grown under N-starvation (Fig 1e-g). The same pigmentation was observed previously in *C. tenuiflora* under N deficiency (1.32 mM) and was identified as anthocyanin biosynthesis (Medina-Perez *et al*. 2015). When the chlorophylls are degraded, the cells become susceptible to oxidative damage by highlight and other photosynthetic pigments are exposed or *the novo* synthetized to dissipate excess energy, as anthocyanins (Carpenter *et al*. 2014; Kovinich *et al*. 2014). A decrease in proteins, chlorophylls, appearance of chlorosis and anthocyanin production has been also observed in *Arabidopsis thaliana* and *Solanum lycopersicum* under N stress (Larbat *et al*. 2012; De Gernier *et al*. 2016).

We observed a dramatic decline in the total N content of *C. tenuiflora* plants exposed to N stress,

and this decrease was N stress level dependent. In response to the lack of N, a progressive degradation of chloroplast proteins is needed for N remobilization. Thus, liberated amino acids can be translocated via the phloem from senescing leaves to the growing parts of plant (Hörtensteiner and Feller 2002; Guiboileau *et al*. 2013). We also note that,soluble protein content in plants treated with 1.32 mM N remained similar to that of control. However, it began to decline in plants treated with 0.66 mM N and declined even further in those treated with N starvation (Table 1).

Castilleja tenuiflora plants cultivated *in vitro* produce PhGs and iridoid glycosides as major compounds (Gómez-Aguirre *et al*. 2012; Sánchez *et al*. 2013 Cardenas-Sandoval *et al*. 2015). These secondary metabolites are known to be excellent radical scavengers (Hong-Yu *et al*. 2012; Alipieva *et al*. 2014). Against an abiotic stress as such as N deficiency, reactive oxygen species production is one of the first defense responses of plants (Rubio-Wilhelmi *et al*. 2011). In this study, secondary metabolism of *C. tenuiflora* plants was differentially affected in response to different N stress levels. PAL, the key enzyme that regulates the biosynthesis of phenolics as phenylpropanoids, flavonoids and anthocyanins (Dixon and Paiva 1995; Kováčik and Bačkor 2007; Lovdal *et al*. 2010) was enhanced by N deficiency and starvation (Fig 2a), consequently total phenolic compounds (Fig 2b) and PhGs concentration (Fig 3) was increased. Control plants showed a slight increase of PAL activity towards day 9 of culture, remaining similar until day 21. By the other side, from day 9 to the end of culture, PAL activity showed a differential increase in all treatments under N stress compared to control. The maximum PAL activity was observed at day 9 in the 0.66 mM N treatment, while at day 21 the PAL activity decreased in all N deficiency and starvation treatments (Fig 2a). This behavior coincides with anthocyanin accentuation in the stems of plants cultured under the same N stress treatment (Fig 1g), thus the accumulation of anthocyanins seems to be influenced by the availability of N indicating a regulation mechanism.

Similar to PAL activity, the content of total phenolic compounds showed an increase associated with culture time in all treatments assessed (also control). Of course, under N deficiency this increase was greater compared to control from day 4 to the end of the experiment. However, the phenolic content in the treatment under N starvation was lower than control on all evaluated days. Being free radical scavengers, phenolic compounds also serve

as precursors for lignin polymerization, which is necessary for root formation and for which reactive oxygen species are generated when synthesized (Cheniany *et al*. 2010). We observe an increase in the length of *C. tenuiflora* roots of plants exposed to stress by N deficiency and starvation (Table 1; Fig 1 i-k). It is possible that phenolic compounds were directed towards root construction to reduce the effect of oxidative stress. We also observed marked depletion of phenolic compounds in plants exposed to N starvation (Fig 2b). It is known that plants under N deficiency release phenolic compounds, which act as signal molecules for nitrogen-fixing microbes in the soil (mycorrhization), which indirectly allows plants to obtain N and other necessary elements (Badri and Vivanco 2009; Yoneyama *et al*. 2012). Juszczuk *et al*. (2004) reported higher concentrations of total phenolic compounds released to culture medium from bean plant roots exposed to prolonged N deficiency compared with control. Other experiments are needed to determine whether the decrease of total phenolic compounds that we observed in *C. tenuiflora* plants under N starvation was because they were released into culture medium as an adaptive response to nutritional deficiency.

It is well known that *in vitro* culture in temporary immersion bioreactors per se with the optimum time and frequency of immersion is a good method to increase the production of matter and secondary metabolites of plants with high commercial value (Ivanov *et al*. 2012; Georgiev *et al*. 2014). In this sense, it was logical that we observed an increase of secondary metabolites (PhGs and Aucubin) associated to the increase of matter and culture time in plants grown in the control medium (complete N supply). Nevertheless, the specific concentration of both PhGs and aucubin also showed an increase due to N-stress treatment (deficiency and starvation) which was associated with the increase of culture time (Fig 3, 4), total phenolic compounds and PAL activity (Fig 2).

The content of PhGs was greatest in all N stress treatments at day 21 of the experiment (Fig 3). In a similar study carried out by Medina-Perez *et al*. (2015), the verbascoside and isoverbascoside concentrations (113.88 ± 8.6 and 36.41 ± 2.3 mg g^{-1} BS respectively) were reported in 1.32 mM N condition. In this work, the concentrations of verbascoside and isoverbascoside at the same condition were 1.5 times higher. On the other hand, Jeong-Ae *et al*. (2015) performed a study in Vitis flexuosa plantlets grown in different culture systems at different NH_4^+: NO_3^- ratios, and reported

an optimal production of total phenolic compounds, total flavonoids and antioxidant capacity in plantlets cultured in temporary immersion bioreactors with a 10:20 (NH_4^+: NO_3^-) ratio.

We observed a decline of PhGs specific concentration in plants under N starvation (Fig 3a, c); similar to what occurred with total phenolic compounds content. We have previously mentioned that plants without N recorded the maximum PAL activity at day 21 (Fig 2a). It is possible that against prolonged stress from N starvation, PAL enzyme activity began to regulate the pathway toward anthocyanins biosynthesis to relieve oxidative damage. Although, the total concentration of aucubin per bioreactor in *C. tenuiflora* plants exposed to N stress shows values lower than control; the specific concentration of this compound was higher in all treatments under N stress; which indicates that the metabolic pathway of this type of iridoids was also stimulated because stress by N deficiency and starvation. These results are agree with those reported for *Deschampsia antarctica*, *Digitalis lanata* and *Leucojum aestivum*, where an *in vitro* culture in a temporary immersion bioreactors combined with the application of some biotic or abiotic factor to elicit and increase the content of secondary metabolites was used (Sequeida *et al*. 2012; Pérez-Alonso *et al*. 2012; Saliba *et al*. 2016).

PhGs are water-soluble compounds derived from phenylpropanoids. Verbascoside has been the most studied and has been shown to be a powerful antioxidant and photo-protector (Alipieva *et al*. 2014). There are no reports about iridoid glycosydes accumulation in response to stress from N deficiency. However, antioxidative and photo-protector effect of aucubin has been demonstrated previously (Ho *et al*. 2005; Hong-Yu *et al*. 2012). Because of the effectiveness of PhGs and IGs as free radicals scavengers, we deduce that verbascoside and isoverbascoside together with aucubin form part of the *C. tenuiflora* plants defense response to counteract the oxidative effect caused by N stress.

Conclusion

The stress by N deficiency and starvation promoted significant changes in growth, development, and phenylpropanoid metabolism in *C. tenuiflora* plants. PAL activity contributes to increase the accumulation of total phenolic compounds, phenylethanoid

glycosides and *de novo* synthesis of anthocyanins. Despite the fact that, the matter of *C. tenuiflora* plants exposed to N stress was decreased considerably and the total concentration per bioreactor of aucubin was lower than the control; the concentration of the phenylethanoids verbascoside and isverbascoside was greatly increased. Therefore, we can conclude that the use of a biotechnological tool such as temporary immersion system bioreactors and suitable manipulating of nutrients in the culture medium such as N deficiency and starvation is a good strategy to stimulate secondary metabolism of *Castilleja tenuiflora* plants and to increase PhGs with valuable pharmacological interest.

Nomenclature

CA	Cinnamic Acid
DM	Dry Matter
FM	Fresh Matter
GAE	Gallic Acid Equivalents
N	nitrogen
PAL	Phenylalanine Ammonia-Lyase
PhGs	Phenylethanoid Glycosides
SMR	Shoot Multiplication Rate
WC	Water Content

Acknowledgements

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