Received: 1 March 2019 | Revised: 6 September 2019 | Accepted: 9 September 2019

DOI: 10.1002/elsc.201900040

Engineering in Life Sciences

RESEARCH ARTICLE

Influence of spermine and nitrogen deficiency on growth and secondary metabolites accumulation in *Castilleja tenuiflora* Benth. cultured in a RITA® temporary immersion system

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The effect of exogenous spermine (SPM) on *Castilleja tenuiflora* shoots developing under nitrogen deficiency (ND) stress was evaluated. Shoots cultivated in a temporary immersion system were subjected to four experimental treatments: (1) control; (2) exogenous SPM; (3) ND; and (4) ND+SPM. Shoots were longer in the ND+SPM treatment (6.3 ± 0.5 cm) than in the ND treatment (4.2 ± 0.5 cm). The total chlorophyll content was similar in the control and SPM treatments (0.41 μg mg^{-1} FM) and the highest values of total phenolic content were detected at 21 days in the ND+SPM treatment (84.1 ± 0.05 GAE g^{-1} DM). In the ND+SPM treatment, the phenylalanine ammonia lyase activity increased earlier than in ND treatment, and reached its maximum at day 21 (3.9 ± 0.2 μmol E-CIN h^{-1} mg^{-1} protein). Compared with the control, the ND and ND+SPM treatments resulted in increased secondary metabolites contents in both root and aerial parts. The strongest effect was in the roots, where the SPM and ND+SPM treatments both resulted in increased quercetin content (4.3-fold that in the control). Our results showed that SPM partially counteract the damage caused by ND and results in increased contents of valuable bioactive compounds.

KEYWORDS
nitrogen deficiency, polyamine, secondary metabolism, spermine, temporary immersion system

1 | INTRODUCTION

Castilleja tenuiflora Benth. (Orobanchaceae) is a wild plant used in Mexican traditional medicine to relieve gastrointestinal diseases, and to "treat" nerves and tumors [1,2]. This species synthesizes secondary metabolites such as iridoid glycosides [3,4], phenylethanoid glycosides (PhGs) [5], flavonoids [6], and lignans [7]. These compounds have been shown to have valuable pharmacological activities such as cytotoxic [8], antioxidant [6], anti-inflammatory [4,9], antiulcer and anti-depressant effects [7,10].

Nitrogen (N) is essential for the growth and development of plants, as it is a major component of amino acids, proteins, nucleic acids, and chlorophyll. Thus, it is a major limiting macronutrient for plants. Nitrogen deficiency (ND) results in a common abiotic stress for plants and leads to

Abbreviations: ND, nitrogen deficiency; PAL, phenylalanine ammonia lyase; PhGs, phenylethanoid glycosides; SMR, shoot multiplication rate; SPM, Spermine; TPC, total phenolic content.

changes both in primary and secondary metabolism [11]. The main changes are the accumulation of reactive oxygen species [12] and an increase in the biosynthesis of secondary metabolites based on carbon compounds, mainly phenolic compounds such as hydroxycinnamic acids, flavonoids, and anthocyanins [13–15]. In a study with *Oryza sativa* L. it was shown that the levels of some phenolic acids were increased in plants with ND, in particular, the levels of *p*-coumaric acid and ferulic acid [16]. It has also been reported that in shoots and roots of cabbage (*Brassica rapa* L.) under ND, alternative biochemical pathways are activated that lead to the production of secondary metabolites such as quinate, which is a precursor of the shikimato pathway [17]. Some of these events are correlated with an increase of phenylalanine ammonia lyase (PAL) activity [18], a key enzyme in the biosynthesis of phenolic compounds. Other important consequences of ND in plants are the low biomass production and negative developmental effects, such as chlorosis, decreased stem and root elongation, and changes in leaf structure and root architecture [19].

Polyamines (putrescine, spermidine, and spermine (SPM)) are organic polycationic molecules that promote the growth and development of plants. They also participate in adaptation functions when transient changes in plant metabolism occur, thus avoiding disturbances in cellular homeostasis. Therefore, they have adaptation/defense roles against various kinds of abiotic stresses [20,21]. In particular, SPM (*N,N′*-bis(3-amino propyl)-1,4-diaminobutane) has been shown to promote multiple sprouting, division, and cell differentiation [22]. In previous studies, the addition of SPM positively affected shoot generation, root and shoot length, and rooting capacity in in vitro cultures of *Glycine max* L. [23] and *Cucumis anguria* L. [24]. Foliar application of SPM to *Echinaceae purpurea* L. increased the contents of caffeic acid derivatives [25] and the SPM addition in *Ruta graveolens* shoot cultures resulted in an increase of furanocoumarin content [26]. Exogenous polyamines have been shown to alleviate the damaging effects of various environmental stresses including drought [27], salinity [28], light [29], and heavy metals [30]. Exogenous SPM was shown to increase the expression of genes involved in biosynthesis and transport of their endogenous counterparts [31]. Polyamines have also been proposed as signaling molecules [32,33]. It has been reported that the maintenance of polyamines in the tobacco wild type plants would be correlated with the higher stress response to ND [14]. However, it is unknown whether polyamines can protect against low-N stress, and there is still much to learn about their roles as signaling molecules in plant secondary metabolism.

Our group has explored the application of different types of stresses as strategies to increase the production of valuable pharmacological compounds in *in vitro* cultures of *C. tenuiflora* [34,35]. Here, we investigated the effect of exogenous SPM on *C. tenuiflora* cultured under N-deficient conditions. The growth, developmental parameters, and secondary metabolite contents of *C. tenuiflora* treated with polyamines and/or ND were monitored in a temporary immersion bioreactor system.

PRACTICAL APPLICATION

The micropropagation of *Castilleja tenuiflora* plants in a temporary immersion system represents an appropriate alternative for the production of secondary metabolites of pharmacological interest. The addition of exogenous spermine (SPM) to a shoot culture allowed for normal shoot development under nitrogen deficiency (ND) conditions. Therefore, exogenous SPM could be used to counteract the damage to plants caused by an abiotic stress, in this case, the deficiency of an essential nutrient (nitrogen). Exogenous SPM also resulted in increased accumulation of important bioactive compounds (secondary metabolites), such as iridoid glycosides and phenolic compounds (phenylethanoid glycosides (PhGs), lignans, and flavonoids). The addition of exogenous SPM has the potential to increase the growth of *C. tenuiflora* under conditions that enhance the yields of valuable products.

2 | MATERIALS AND METHODS

2.1 | Plant material and experimental treatments

Shoot cultures of *C. tenuiflora* were initiated and propagated in vitro as described previously [36]. For experimenting 40 explants, three-week-old in vitro shoots of 2–2.5 cm size, were cultured in a Temporary Immersion System (RITA®; Sigma-Aldrich, St Louis, MO, USA) with 200 mL of B5 culture medium [37] and 3% of sucrose w/v, without plant growth regulators. The shoots were immersed for 5 min (immersion time) every 24 h (immersion frequency) and the airflow was controlled at 1 L min^{-1} in each bioreactor. All cultures used in this study were maintained in a growth chamber under the following controlled conditions: 25 ± 2°C with a 16-h light/8-h dark photoperiod and an irradiance of 77 μmol m^2 s^{-1} provided by 10 W LED lamps.

The effect of exogenous SPM on *C. tenuiflora* shoots under ND (The ND condition was induced by modifying the KNO_3 and $(NH_4)_2SO_4$ basal concentration in B5 medium without altering the nitrate:ammonium ratio 24:1) was evaluated in a completely randomized experimental design in four treatments as follows: (1) control: *C. tenuiflora* shoots

grown under non-stressed conditions with 25.5 mM total N (24.48 μM KNO_3 plus 1.02 μM $(NH_4)_2SO_4$); (2) SPM: *C. tenuiflora* shoots grown under non-stressed conditions with 25.5 mM total N plus 5 μM SPM; (3) ND: *C. tenuiflora* shoots grown under ND with 0.63 mM total N (0.605 μM KNO_3 plus 0.025 μM $(NH_4)_2SO_4$); and (4) ND+SPM: *C. tenuiflora* shoots grown under ND with 0.63 mM total N plus 5 μM SPM.

2.2 | Growth and shoot development

At the start (day 0) and at the end (day 21) of experiments, the plants were counted (number of shoots generated per reactor) and the shoot multiplication rate (SMR) was calculated as follows: number of shoots and buds at the end of culture period/number of shoots inoculated. The shoot height, length of the longest root (mm), and root formation efficiency were determined, and biomass (in fresh and dry basis) was measured gravimetrically.

The total chlorophyll content was determined as the sum of chlorophyll a and b concentrations, as described by Lichtenthaler [38], with minor modifications. Fresh tissue (15 mg) was frozen in liquid N, and ground to a fine powder, 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, using a spectrophotometer (UV-A 160, Shimadzu, Kyoto, Japan).

2.3 | Hydrogen peroxide content

The quantification of hydrogen peroxide (H_2O_2) was made accordingly [39]. Briefly, tissue (0.5 g FM) was grounded to a powder in liquid nitrogen, then homogenized in 1 mL of TCA (0.1% v/v) and centrifuged at 1400 *g* for 15 min at 4°C. The reaction mixture consisted of 0.5 mL supernatant, 1 mL 1 M potassium iodide, and 0.5 mL 10 mM phosphate buffer (pH 7). Then, it was incubated in the dark for 10 min before reading absorbance at 390 nm. The H_2O_2 content was calculated from a standard curve, and is expressed as μmol g^{-1} DM.

2.4 | PAL assay

The activity of PAL was determined by measuring cinnamic acid production from phenylalanine [40]. Fresh tissue (200 mg) was frozen in liquid nitrogen, pulverized in a cold mortar with 40 mg polyvinylpolypyrrolidone, 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. The mixture was centrifuged at 16,060 × *g* for 15 min at 4°C (Biofuge fresco, Heraeus®, Hanau, Germany), and the extract was used for the enzymatic reaction. The reaction mixture consisted of 550 μL buffer (50 μL mM Tris-HCl, pH 8.8), 250 μL L-phenylalanine 20 mM (pH 8.8), and 200 μL of enzyme extract (2–16 μg protein). After incubation for 60 min at 40°C with shaking at 600 rpm, the reaction was stopped by adding 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. The activity of PAL is expressed as nmol cinnamic acid h^{-1} mg^{-1} protein. The soluble protein content was determined by the Bradford assay [41].

2.5 | Extract preparation and determination of total phenolic content

The plant material from each treatment was collected and separated into aerial part and roots, and dried at 60°C for 48 h. Subsequently, a microextraction (0.2 g of dry matter per 1 mL of methanol) was performed by sonication for 30 min and the resulting mixture was vacuum-filtered through Whatman # 1 filter paper. The filtrate was concentrated under reduced pressure at 40°C, and then lyophilized.

Total phenolic content (TPC) were measured by a colorimetric method as follows: 40 μL methanol extract (1 mg mL^{-1}) was mixed with 100 μL Folin-Ciocalteu reagent (1:10). The solution was allowed to stand for 6 min, and then 100 μL sodium carbonate (3% w/v) was added and mixed vigorously. After 25 min, absorbance at $\lambda = 750$ nm was measured and the concentration of phenolic compounds was calculated by comparison with a calibration curve prepared with gallic acid (0–25 μg mL^{-1}; $R^2 = 0.997$). All samples were analyzed in quadruplicate. Results are reported as mg gallic acid equivalents per g dry matter (mg GAE g^{-1} DM).

2.6 | Identification of secondary metabolites by HPLC–photodiode array detector–MS

The chromatographic separation and mass spectrometric analysis of iridoids and phenolic compounds (PhGs, flavonoids, and lignans) was carried out using a Shimadzu LC-MS system (Shimadzu) consisting of a CBM-20A system controller, two LC-20AD pumps, a DGU-20 5R degasser, a SIL-20AC auto sampler, a CTO 20A column oven, a SPD-M20A UV–vis photodiode array detector, and a LC-MS2020 interfaced with an ESI source. Data were acquired and processed using LSMS solutions software v 5.0. The samples were eluted, and analyses were performed at 40°C using a reverse phase Chromolith® High Resolution RP-18 column (100 mm × 4 mm, 5 μm) (Merck, Darmstadt, Germany). The mobile phase consisted of a water (solvent A) and acetonitrile (solvent B). The gradient system was as follows: 0–3 min, 100%–0% A–B; 3–5 min, 90%–10% A–B; 5–11 min, 85%–15% A–B; 11–15 min, 80%–20% A–B; 15–19 min, 75%–25% A–B; 19–25 min, 70%–30% A–B; 25–28 min, 0%–100% A–B; 28–30 min, 100%–0% A–B. The sample injection volume was 20 μL and the flow rate was 1 mL min^{-1}. Fingerprints were analyzed as follows: the aucubine-type

iridoids at $\lambda = 205$ nm; geniposidic-type iridoid and lignans at $\lambda = 240$ nm; phenylethanoids at $\lambda = 330$ nm, and flavonoids at $\lambda = 360$ nm. The phenylethanoids content was estimated by interpolation of the peak areas and comparison with a calibration curve; absorbance was measured at 330 nm. The calibration curve was linear in the range of 7.8125–1000 μg mL^{-1} in methanol (y = 22659x − 64546, $R^2 = 0.9999$). Results are expressed as the mean from three determinations in mg g^{-1} of DM.

Anthocyanins were analyzed using the LC-MS systems described above. The samples were eluted, and analysis was performed at 40°C using a reverse phase Lichrospher RP-18 column (250 mm × 4 mm, 5 μm) (Merck, Darmstadt, Germany) connected to a guard column. The mobile phase consisted of water acidified with formic acid in a 95:5 ratio (solvent A) and methanol (solvent B). The gradient system was as follows: 0–10 min, 86%–14% A–B; 10–21 min, 83%–17% A–B; 21–38 min, 73%–27% A–B; 38–40 min, 61.2%–38.8% A–B; 40–44 min, 58%–42% A–B; 44–46 min, 55%–45% A–B; 46–60 min, 0%–100% A–B. The sample injection volume was 20 μL and the flow rate was maintained at 1 mL min^{-1}. Fingerprints were analyzed at $\lambda = 510$ nm.

The MS analyses were performed using the Shimadzu LC 2020 system comprising of UV-vis diode detector (SPD-M20A) coupled to a simple quadrupole MS (LCMS-2020) with an ESI. Chromatographic conditions were as described in the LC-PDA analysis. The MS conditions for these analyses were as follows: negative ionization mode, scanner between 0 and 900 mz^{-1}, N2 as drying gas (10 L min^{-1}), nebulizer gas flow of 1.5 L min^{-1}, 4.5 kV interface and 1.2 kV detection voltage, and 5 μL injection volume.

2.7 | Statistical analysis

Data were analyzed using one-way ANOVA. Tukey´s test with a significance level of 5% was performed to determine whether ND and/or SPM significantly affected each variable assessed with respect to the control. All tests were performed using Sigma Plot 12.0 (Systat Software, San Jose, CA, USA).

3 | RESULTS

3.1 | Growth and development of *C. tenuiflora*

The addition of SPM significantly promoted ($p < 0.05$) *C. tenuiflora* growth (Figure 1) and alleviated the negative impact of ND on its development (Figure 2). Shoot height (Figure 1B) and root length (Figure 1C) were greater in the SPM treatment than in the control. Plant height was 52% greater in the SPM treatment than in the control (2.1 ± 0.1 cm). Plant height was also greater in the ND+SPM treatment (6.3 ± 0.5 cm) than in the ND treatment

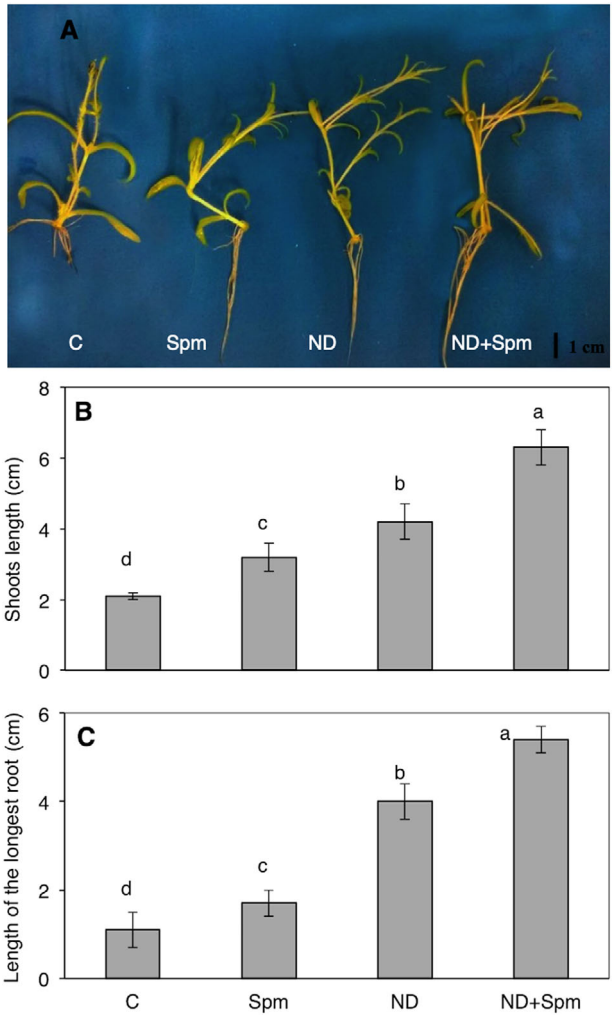

FIGURE 1 Growth and development of *C. tenuiflora* in a temporary immersion system. (A) Shoots grown under different treatments. (B) Shoot length. (C) Length of longest root. Treatments: C, control; ND, nitrogen deficiency; SPM, exogenous spermine; ND+SPM nitrogen deficiency plus exogenous spermine. Data are mean ± standard error of three replicates (10 plants each). Different letters above bars indicate significant difference ($p < 0.05$, Tukey's multiple range test)

(4.2 ± 0.5 cm). Plants in the SPM and ND+SPM treatments developed longer roots than those grown without exogenous SPM. The roots were longest in the ND+SPM treatment (5.4 ± 0.3 cm) and shortest in the control (1.1 ± 0.4 cm).

SPM reduced the negative impact of ND on SMR, biomass production, and chlorophyll content. The SMR was significantly affected by SPM addition: plants in the SPM treatment developed nine shoots per explant (360 plants per reactor) while those in the control developed eight shoots per explant (320 plants per reactor) (Figure 2B). Under N-stress treatments, SPM addition also increased the SMR to five to six shoots per explant (200–240 plants per reactor) compared with three shoots per explant in the ND treatment (120 plants per bioreactor). Neither ND nor SPM affected the relative

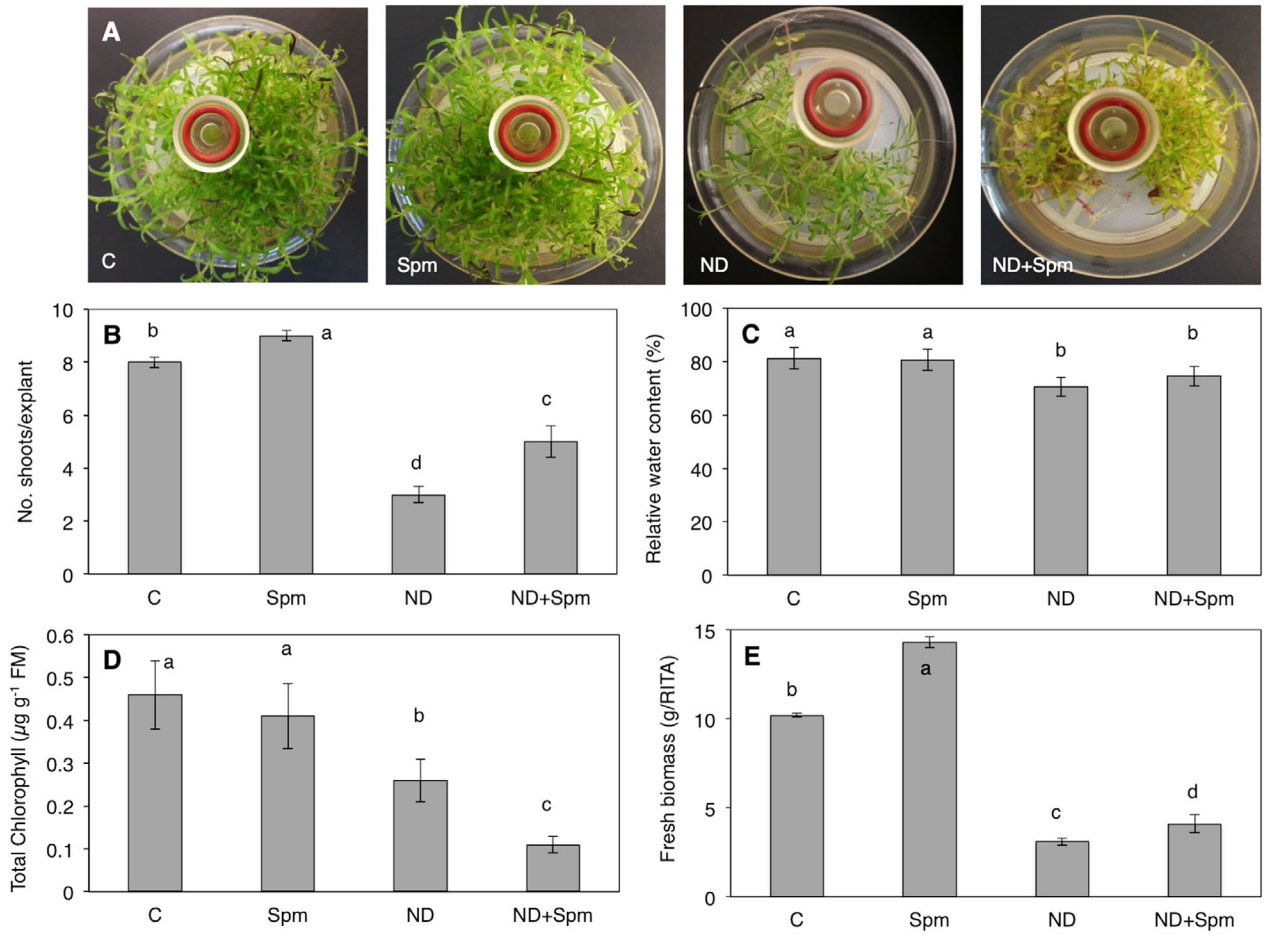

FIGURE 2 Growth and development of *C. tenuiflora.* (A) Shoots grown in a temporary immersion system under different treatments. (B) Number of shoots/explant. (C) Relative water content. (D) Total chlorophyll. (E) Fresh biomass. Treatments: C, control; ND, nitrogen deficiency; SPM, exogenous spermine; ND+SPM nitrogen deficiency plus exogenous spermine. Data are mean ± standard error of three replicates (10 plants each). Different letters above bars indicate significant difference ($p < 0.05$, Tukey's multiple range test)

water content of *C. tenuiflora* shoots (Figure 2C). The total chlorophyll content was similar in the control and SPM treatments (0.41 μg mg^{-1} FM). However, exogenous SPM did not prevent chlorosis (0.11 ± 0.1 μg mg^{-1} FM) induced by the ND treatment (0.26 ± 0.1 μg mg^{-1} FM) (Figure 2D). Biomass production reached 14.3 ± 0.3 g FM per reactor in the SPM treatment, which was 40% higher than that in the control (Figure 2E). Biomass was 32% higher in the ND+SPM treatment than in the ND treatment (3.1 ± 0.2 g per reactor) (Figure 2E).

3.2 | Hydrogen peroxide content

N deficiency and exogenous SPM induces oxidative stress (measured as H_2O_2) in *C. tenuiflora* culture, the results of all treatments were statistically different from the control and from each other (Table 1). At 72 h of culture time, *C. tenuiflora* shoot cultures showed increased H_2O_2 production, the highest content (31.41 ± 2.1 μmol g^{-1} DM) was obtained with the ND+SPM treatment, followed by the SPM (18.11 ± 1.75 μmol g^{-1} DM) and ND (10.08 ± 0.8 μmol g^{-1} DM) treatments.

3.3 | TPC and PAL activity

The TPC and PAL activity were measured in leaves at early (two and four days), mid- (nine days) and late stages (21 days) of culture (Figure 3). At two days, TPC was similar in the control, SPM, and ND treatments (4.50 ± 0.8 GAE g^{-1} DM), but much higher in the ND+SPM (8.84 ± 0.7 GAE g^{-1} DM). Differences among treatments were more evident at the mid-stage and greatest at the late stage. The highest TPC values were at 21 days in the ND (66.2 ± 0.02 GAE g^{-1} DM) and ND+SPM treatments (84.1 ± 0.05 GAE g^{-1} DM) (Figure 3A).

The activity of PAL at two days was 0.05) between them (1.1 ± 0.9 μmol E-CIN h^{-1} mg^{-1} protein). In the ND treatment, PAL activity increased from day nine and peaked at day 21 (2.4 ± 0.2 μmol E-CIN h^{-1} mg^{-1} protein). In the ND+SPM tratment, PAL activity increased from day four

TABLE 1 Hydrogen peroxide, total phenolics, phenylethanoids and anthocyanins contents in *C. tenuiflora* grown in a temporary immersion system with spermine and under nitrogen deficiency stress

Treatment*	Hydrogen peroxide** (μmol g^{-1} DM) Whole plant	Total phenolic content (mg GAE g^{-1} DM) Aerial part	Root	PhGs (mg g^{-1} DM) Aerial part	Root	Anthocyanins (mg mg^{-1} FM) Leaves
C	7.74 ± 0.9[a]	14.75 ± 0.6[d]	49.6 ± 3.5[d]	42.7 ± 0.03[c]	<10[c]	0.039 ± 0.02[c]
SPM	18.11 ± 1.75[b]	74.4 ± 1.9[c]	86.5 ± 4.6[c]	47.11 ± 0.01[b]	22.75 ± 0.02[b]	0.035 ± 0.07[c]
ND	10.08 ± 0.8[c]	107.2 ± 3.6[b]	113.7 ± 1.7[b]	18.14 ± 0.03[d]	<10[c]	0.267 ± 0.01[b]
ND+SPM	31.41 ± 2.1[d]	159.25 ± 3.6[a]	125.01 ± 2.6[a]	65.10 ± 0.02[a]	58.8 ± 0.02[a]	0.657 ± 0.01[a]

*C, SPM, ND and ND+SPM are the experimental treatments (described in Section 2).
**Measured on third day of cultivation.
Data represent mean ± standard error of three replicates (10 plants each). Values in each column followed by different letter are significantly different at $p < 0.05$ (Tukey's multiple range test).

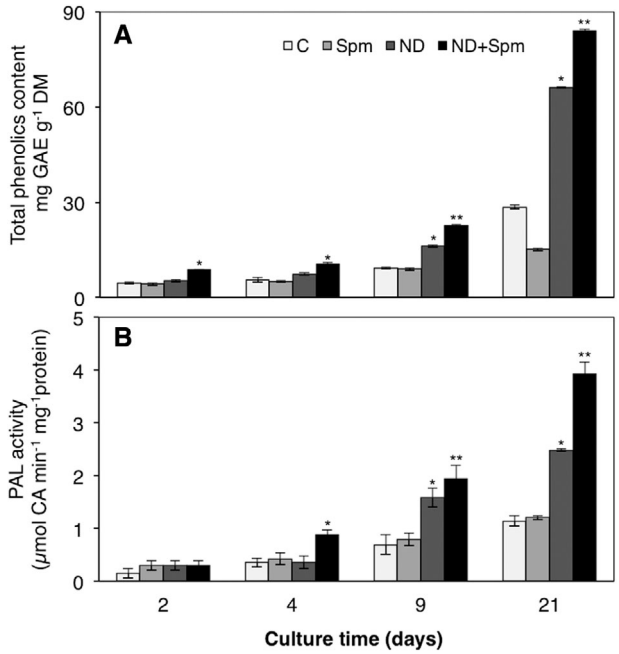

FIGURE 3 *C. tenuiflora* shoots cultured in a temporary immersion system for 21 days under different treatments. (A) Total phenolics content. (B) Phenylalanine ammonia lyase (PAL) activity. Treatments: C, control; ND, nitrogen deficiency; SPM, exogenous spermine; ND+SPM nitrogen deficiency plus exogenous spermine. Data are mean ± standard error of three replicates (10 plants each). Asterisk above bars indicate significant difference ($p < 0.05$, Tukey's multiple range test)

(0.8 ± 0.09 μmol E-CIN h^{-1} mg^{-1} protein) and peaked at day 21 (3.9 ± 0.2 μmol E-CIN h^{-1} mg^{-1} protein).

3.4 | Chemical analysis

At day 21, the TPC, PhGs, and anthocyanin contents were quantified in aerial parts and roots (Table 1) and LC-MS analyses were conducted (Table 2 and Figure 4). The TPC varied depending on the plant part and treatment. In the control, the TPC ranged between 14.75 and 49.6 mg GAE g^{-1} DM, and was higher in roots than in aerial parts. SPM addition resulted in a significant increase ($p \leq 0.05$) in TPC (74.4 to 86.5 mg GAE g^{-1} DM) compared with that in the control. Similarly, the TPC was significantly higher ($p \leq 0.05$) in the ND and ND+SPM treatments than in the control. The highest TPC was in aerial parts in the ND+SPM treatment (159.25 ± 3.6 mg GAE g^{-1} DM), and was 9-fold that in aerial parts of plants in the control (14.75 ± 0.6 mg GAE g^{-1} DM). The PhGs are a major class of phenolic compounds synthesized by *C. tenuiflora* [5]. As shown in Table 1, PhGs (sum of verbascoside and isoverbascoside) were more abundant in the aerial parts than in the roots and their concentration depended on the treatment. In the control, PhGs were only quantifiable in the aerial parts (42.7 ± 0.03 mg g^{-1} DM). The highest concentration of PhGs was in aerial parts in the ND+SPM treatment (65.10 ± 0.02 mg g^{-1} DM), which was 6-fold that in aerial parts in the ND treatment (18.14 ± 0.03 mg g^{-1} DM). Overall, isoverbascoside was more abundant than verbascoside.

C. tenuiflora develops purple colored stems under N stress because of anthocyanin accumulation [30]. The highest concentration of anthocyanins was in the ND+SPM treatment (0.657 ± 0.01 mg mg^{-1} FM), which was 2.5-fold that in the ND treatment (0.267 ± 0.01 mg mg^{-1} FM). The anthocyanin concentrations were not significantly different between the control and the SPM treatment (0.039 ± 0.02 and 0.035 ± 0.07 mg mg^{-1} FM, respectively) ($p > 0.05$). The LC-MS analysis of the anthocyanins extract had a major peak at Rt = 25 min (λ = 280, 370, and 522 nm), which was identified as cyanidin-3-*O*-α-arabinopyranoside (419 *m/z*).

The HPLC profile contained peaks representative of both iridoid glycosides and phenolic compounds (PhGs, lignans, and flavonoids) (Table 2). Most of the compounds were present in plants in all the experimental treatments, either in the aerial parts or roots or both. Overall, compared with the control, all the treatments resulted in an increase in the contents of secondary metabolites in both root and aerial parts

TABLE 2 Metabolic profile of *C. tenuiflora* grown under nitrogen deficiency stress and exogenous spermine in a temporary immersion system

Peak#	Rt (min)	Identification	[M-H]- (m/z)	Metabolite	Group	Occurrence
1	5.3	MS, spectrum, standard	345	Aucubin	Iridoids	All treatments
2	6.1	MS, spectrum	329	Bartsioside	Iridoids	Aerial part, all treatments
3	7.3	MS, spectrum	389	8-*epi*-loganin	Iridoids	All treatments
4	10.4	MS spectrum	463	Quercetin glucoside	Flavonoid	All treatments except SPM (R) and ND+SPM (R)
5	11.4	MS, spectrum, standard	623	Isoverbascoside	Phenylethanoid	All treatments
6	12.6	MS, spectrum, standard	623	Verbascoside	Phenylethanoid	All treatments
7	16.8	MS, spectrum	650	Tenuifloroside	Lignan	C (AP); SPM, ND (AP) and ND+SPM
8	20.4	MS, spectrum	299	Luteolin 5-methyl ether	Flavonoid	All treatments

Rt, retention time. C, SPM, ND and ND+SPM are the experimental treatments (described in Section 2). (AP) = aerial part; (R) = root.

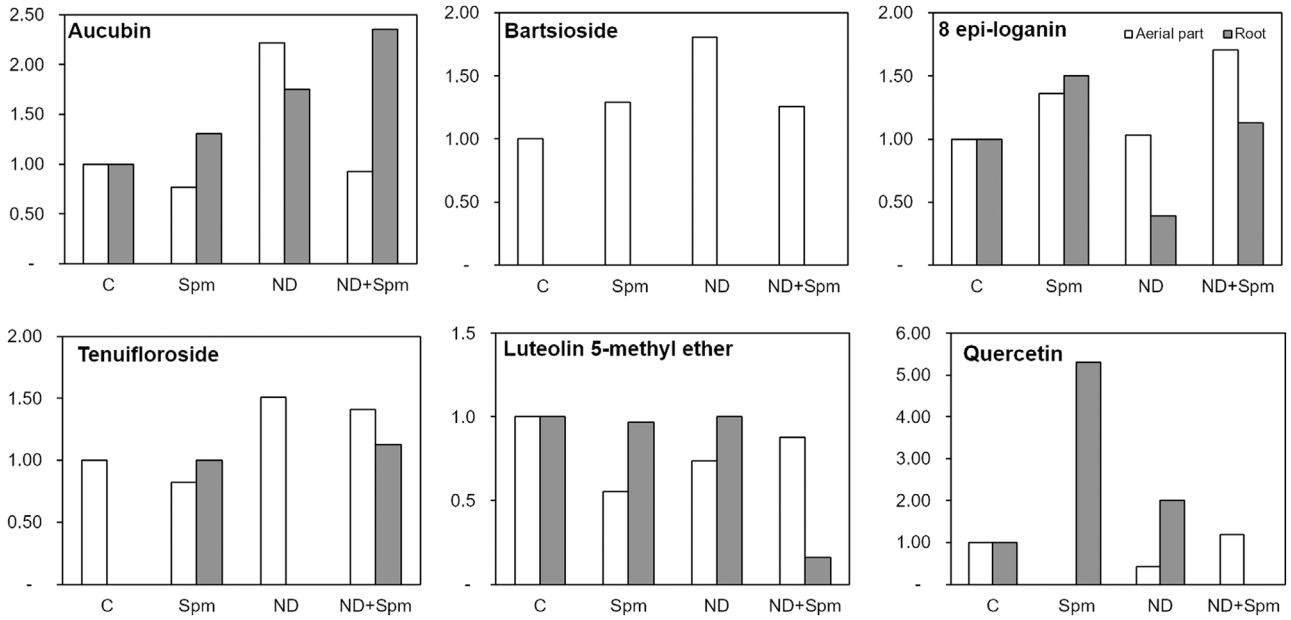

FIGURE 4 Relative contents of secondary metabolites produced by *C. tenuiflora* shoots cultured in a temporary immersion system for 21 days under different treatments: C, control; ND, nitrogen deficiency; SPM, exogenous spermine; ND+SPM nitrogen deficiency plus exogenous spermine

(Figure 4). Specific results by organ (root or aerial parts) and treatment are described below.

Compared with plants in the control, those in the ND treatment showed an increase in aucubin (0.75-fold) and quercetin (1.1-fold) contents and a decrease in 8-epi-loganin (0.6-fold) contents in the roots. The contents of luteolin 5-methyl ether; bartsioside and tenuifloroside were not affected. The aerial parts showed increased contents of aucubin (1.2-fold), bartsioside (0.8-fold), and tenuifloroside (0.5-fold), decreased contents of quercetin (0.6-fold) and luteolin 5-methyl ether (0.3-fold), and no change in iridoid 8-epi-loganin content.

In the SPM treatment, the roots showed increased contents of quercetin (4.3-fold), 8-epiloganin (0.5-fold), and aucubin (0.3-fold), no change in the contents of tenuifloroside and luteolin 5-methyl ether, and bartsioside was not detected. The aerial parts showed increased contents of 8-epi-loganin (0.36-fold) and bartsioside (0.21-fold), decreased contents of aucubin (0.23-fold), tenuifloroside (0.2-fold), and luteolin 5-methyl ether (0.4-fold), and quercetin was not detected.

In the ND+SPM treatment, the roots showed an increase in aucubin content (1.35-fold), a decrease in luteolin 5-methyl ether content (0.84-fold), no change in 8-epi-loganin and tenuifloroside contents, and bartsioside and quercetin were not detected. The aerial parts showed increased contents of 8 epi-loganin (0.71-fold), tenuifloroside (0.41-fold), and bartsioside (0.26-fold), and quercetin was not detected.

4 | DISCUSSION

ND is a stress that results in reduced growth and development and increased biosynthesis of phenolic compounds in *C. tenuiflora* [34]. Here, N stress was imposed by growing *C. tenuiflora* with only 0.63 mM total N in ND treatments (compared with 25.5 mM total N in the control), half the concentration used in a previous study (1.32 mM N).

In the present work, ND induced the elongation of *C. tenuiflora* shoots and roots but decreased its multiplication capacity, biomass production, and chlorophyll content. Secondary metabolism was stimulated at the level of PAL activity, and resulted in increased accumulation of TPC, PhGs, and anthocyanins. Iridoid glycosides production was affected by N stress, which led to decreased contents of 8-epiloganin in roots and increased contents of aucubin and bartsioside in both roots and aerial parts.

In plants, polyamines are involved in the regulation of developmental processes and in defense against environmental stresses [42]. Although their roles in defense responses are still not fully understood, the results of many studies point to a dual mode of action: as direct protective compounds and as signaling molecules that interact with multiple cellular pathways [20]. Previous studies suggest that polyamines play role as a source of organic N for growth and proteins synthesis in *Helianthus tuberosus* tissue [43]. There are also reports that putrescine has the ability to be used as sole nitrogen source for the growth of explants from dormant tubers of *Helianthus tuberosus* [44], however, when they using SPM as sole nitrogen source not growth occurred, despite used a similar concentration to that of nitrogen source in the cultured medium. Although in this work we used SPM, it cannot be considered as a supply of N for growth, basically because the concentration (5 μM) added does not significantly modify the total N content, however SPM allowed for normal shoot development under ND conditions.

Our results show that exogenous application of SPM affected the responses of *C. tenuiflora* to ND. The addition of SPM positively affected plant height, root formation, and multiplication capacity (Figures 1 and 2). This is consistent with the role of polyamines as positive regulators of cell growth [45]. Similarly, in previous studies, exogenous putrescine increased the contents of endogenous polyamines, promoted shoot elongation, and increased the number of shoots per explant in *Bixa Orellana* [22]. Exogenous spermidine (0.25 mM) enhanced the root biomass of tomato plants [46]. However, root growth was found to be inhibited by SPM in *Arabidopsis thaliana* [47] or soybean [48]. In the former, exogenous SPM (0.3 mM) inhibited root growth due to an increase in endogenous SPM content and H_2O_2 production. In the present study, the plant height and root length of *C. tenuiflora* increased under ND, consistent with increases in root length reported for *Brassica napus* and *B. carinata* under low-N conditions [49]. In response to ND, photo assimilates are directed preferentially to root growth to facilitate uptake from the deficient environment [50]. We expected that the addition of SPM would stimulate an increase in endogenous polyamines content, as has been observed in *Arabidopsis* [47] and *Cerasus humili* [27]. This may explain why *C. tenuiflora* plants grown with SPM under ND had the greatest plant height and root length.

The chlorophyll content in *C. tenuiflora* leaves was significantly lower in N-stressed plants than in control plants. Exogenous SPM not only did not alleviate chlorosis, in fact, promoted it (Figure 2D). Contrasting observations have been reported in soybean, where SPM (0.4 μM) enhanced chlorophyll a content in osmotically stressed leaves but slightly increased the content of chlorophyll b [51]. Our results show that ND and SPM treatments produce a significant increase in H_2O_2 content, which promotes oxidative stress in *C. tenuiflora* cultures, therefore the decrease in chlorophyll content under N deficiency might result from photooxidation or chlorophyll degradation. In another study, addition of exogenous polyamines increased H_2O_2 production leading to oxidative stress [47]. Oxidative stress resulting from ND stress and exogenous SPM may explain the strong chlorosis observed in *C. tenuiflora* in this study.

Our results show that ND and the SPM addition promote an oxidative stress generated by the increase in H_2O_2 content; this is significantly greater than the control when ND is combined with exogenous SPM (Table 1). It has been reported that ND in plants promotes an increase in reactive oxigen species, including H_2O_2, which triggers an oxidative stress [52]. It is also known that this increase in H_2O_2 concentration can be generated by the oxidation of polyamines [53]. There is evidence that under oxidative stress conditions there could be an increase in contents of both PA and H_2O_2, and that this process can occur in parallel or in succession with each other, causing these molecules to act synergistically or independently [21]. Therefore, we can hypothesize that the addition of SPM to the culture medium together with the ND promotes, in addition to the increase in the production of H_2O_2, a greater accumulation of PA in *C. tenuiflora,* and that both molecules participate in the activation of signaling cascades and transcription factors that promote the increase of bioactive compounds involved in the defense response of the plant [54].

As expected, N stress increased PAL activity in leaves, induced accumulation of anthocyanins, and increased the TP*C.* These observations were consistent with the results of our previous study on *C. tenuiflora* under a lower degree of stress [34] and with results from studies on *Matricaria chamomilla* [11] and *Achillea collina* [12]. We found that SPM alone did not stimulate PAL activity in *C. tenuiflora*, does not produce an increased in TPC and PhGs and neither does anthocyanin biosynthesis (Figures 3 and 4). Exoge-

nous SPM with N stress resulted in maximal PAL activity and the highest levels of total phenols, PhGs, and anthocyanins. In other studies, SPM treatments (0.1 and 1 mM) alone did not enhance PAL activity relative to the control in *Ocimum basilicum* L. [55]. Spermidine (10 μM) induced deposition of phenolics in roots of maize [46] and spermidine stress (0.05 mM) along with drought-stress increased the total phenols and flavonoids content in *Trifolium repens* [56].

Of the 11 secondary metabolites identified in *C. tenuiflora*, three had already been reported for both in vitro cultures and wild plants (aucubin, verbascoside and isoverbascoside) and the other eight have been detected only in wild plants. This is the first time that the accumulation of the lignan tenuifloroside, which has antidepressant activity [7], has been detected in in vitro cultures of *C. tenuiflora*.

In this work, SPM is not used as a source of inorganic N; however, complementing the N-deficient culture medium with exogenous SPM does not represent an increase in cost in relation to using the B5culture medium with 100% N.

5 | CONCLUDING REMARKS

In this work, a temporary immersion system was used to cultivate *C. tenuiflora* shoots under N-deficiency conditions. The shoots were subjected to this abiotic stress to enhance the production of bioactive compounds. Plant growth and development was affected by N stress, but exogenous SPM enhanced growth and resulted in increased accumulation of secondary metabolites with important pharmacological activities. Application of SPM partially counteracted the adverse effects of N-deficiency stress in *C. tenuiflora* by stimulating the synthesis of antioxidant compounds.

The increase in secondary metabolites in response to SPM an N-Deficiency offers a biotechnological system both for studies on the biosynthesis of these bioactive compounds and for its production.

ACKNOWLEDGMENTS

The authors received financial support from the Secretaría de Investigación y Posgrado of Instituto Politécnico Nacional (Grant no. 1776–20170461) and Consejo Nacional de Ciencia y Tecnología (CONACyT-México) (Grant no. CB-2013-01-220007). ERR is indebted to CONACyT for an awarded fellowship and to the Programa Institucional de Formación de Investigadores (BEIFIIPN). We thank Jennifer Smith, PhD, from Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

REFERENCES

1. Graham, J. G., Quinn, M. L., Fabricant, D. S. and Farnsworth, N. R., Plants used against cancer – an extension of the work of Jonathan Hartwell. *J. Ethnopharmacol.* 2000, *73*, 347–377.
2. Alonso-Castro, A. J., Villarreal, M. L., Salazar-Olivo, L. A., Gomez-Sanchez, M. et al, Mexican medicinal plants used for cancer treatment: pharmacological, phytochemical and ethnobotanical studies. *J. Ethnopharmacol.* 2011, *133*, 945–972.
3. Jiménez, M. E., Padilla, M., Reyes, C. R., Espinosa, L. M. et al., Iridoid glycoside constituents of *Castilleja tenuiflora*. *Biochem. Syst. Ecol.* 1995, *23*, 465-456.
4. Carrillo-Ocampo, D., Bazaldua-Gomez, S., Bonilla-Barbosa, J. R., Aburto-Amar, R. et al., AntiInflammatory activity of iridoids and verbascoside isolated from *Castilleja tenuiflora*. *Molecules* 2013, *18*, 12109–12118.
5. Gómez-Aguirre, Y. A., Zamilpa, A., González, M. and Trejo-Tapia, G., Adventitious root cultures of *Castilleja tenuiflora* Benth. as a source of phenylethanoid glycosides. *Ind. Crops Prod.* 2012, *36*, 188–195.
6. López-Laredo, A., Gómez-Aguirre, Y., Medina-Pérez, V., Salcedo-Morales, G. et al., Variation in antioxidant properties and phenolics concentration in different organs of wild growing and greenhouse cultivated *Castilleja tenuiflora* Benth. *Acta Physiol. Plant.* 2012, *34*, 2435–2442.
7. Herrera-Ruiz, M., López-Rodríguez, R., Trejo-Tapia, G., Domínguez-Mendoza, B. et al., A new furofuran lignan diglycoside and other secondary metabolites from the antidepressant extract of *Castilleja tenuiflora* Benth. *Molecules* 2015, *20*, 13127–13143.
8. Moreno-Escobar, J. A., Bazaldúa, S., Villarreal, M. L., Bonilla-Barbosa, J. R. et al., Cytotoxic and antioxidant activities of selected Lamiales species from Mexico. *Pharm. Biol.* 2011, *49*, 1243–1248.
9. Sanchez, P. M., Villarreal, M. L., Herrera-Ruiz, M., Zamilpa, A. et al., In vivo anti-inflammatory and anti-ulcerogenic activities of extracts from wild growing and in vitro plants of *Castilleja tenuiflora* Benth. (Orobanchaceae). *J. Ethnopharmacol.* 2013, *150*, 1032–1037.
10. López-Rodríguez, R., Herrera-Ruiz, M., Trejo-Tapia, G., Domínguez-Mendoza, B. E. et al., *In Vivo* Gastroprotective and Antidepressant Effects of Iridoids, Verbascoside and Tenuifloroside from *Castilleja tenuiflora* Benth. *Molecules* 2019, *24*, 1292–1298.
11. Kováčik, J. and Bačkor, M., Changes of phenolic metabolism and oxidative status in nitrogen deficient *Matricaria chamomilla* plants. *Plant and Soil* 2007, *297*, 255–265.
12. Giorgi, A., Mingozzi, M., Madeo, M., Speranza, G. et al., Effect of nitrogen starvation on the phenolic metabolism and antioxidant properties of yarrow (*Achillea collina* Becker ex Rchb.). *Food Chem.* 2009, *114*, 204–211.
13. Ncube, B., Finnie, J. F. and Van Staden, J., Quality from the field: the impact of environmental factors as quality determinants in medicinal plants. *South Afr. J. Bot.* 2012, *82*, 11–20.
14. Rubio-Wilhelmi, M., Sanchez-Rodriguez, E., Leyva, R., Blasco, B. et al., Response of carbon and nitrogen-rich metabolites to nitrogen deficiency in PSARK:IPT tobacco plants. *Plant Physiol. Biochem.* 2012, *57*, 231–237.
15. Guillén-Román, C. J., Guevara-González, R. G., Rocha-Guzmán, N. E., Mercado-Luna, A. et al., Effect of nitrogen privation on the phenolics contents, antioxidant and antibacterial activities in *Moringa oleifera* leaves. *Ind. Crops Prod.* 2018, *114*, 45–51.

16. Chishaki, N. and Horiguchi, T., Responses of secondary metabolism in plants to nutrient deficiency. *Plant Nutrition for Sustainable Food Production and Environment*. Springer, Dordrecht, 1997. 341–345.
17. Sung, J., Sung, J., Yun, H., Back, S. et al., Changes in mineral nutrient concentrations and C-N metabolism in cabbage shoots and roots following macronutrient deficiency. *J. Plant Nutr Soil SC.* 2018, *181*, 777–786.
18. Wang, G., Cao, F., Chang, L., Guo, X., et al., Temperature has more effects than soil moisture on biosynthesis of flavonoids in Ginkgo (*Ginkgo biloba* L.) leaves. *New For*. 2014, *45*, 797–812.
19. Kováčik, J. and Klejdus, B., Induction of phenolic metabolites and physiological changes in chamomile plants in relation to nitrogen nutrition. *Food Chem.* 2014, *142*, 334–341.
20. Pal, M., Szalai, G. and Janda, T., Speculation: Polyamines are important in abiotic stress signaling. *Plant Sci.* 2015, *237*, 16–23.
21. Alcazar, R., Altabella, T., Marco, F., Bortolotti, C. et al., Polyamines: molecules with regulatory functions in plant abiotic stress tolerance. *Planta* 2010, *231*, 1237–1249.
22. Parimalan, R., Giridhar, P., Ravishankar, G. A., Enhanced shoot organogenesis in *Bixa orellana* L. in the presence of putrescine and silver nitrate. *Plant Cell Tiss. Org. Cult.* 2010, *105*, 285–290.
23. Arun, M., Subramanyam, K., Theboral, J., Ganapathi, A. et al., Optimized shoot regeneration for Indian soybean: the influence of exogenous polyamines. *Plant Cell Tiss. Org. Cult.* 2014, *117*, 305–309.
24. Thiruvengadam, M., Chung, I.-M., Phenolic compound production and biological activities from in vitro regenerated plants of gherkin (*Cucumis anguria* L.). *EleC. J. Biotechnol.* 2015, *18*, 295–301.
25. Darvizheh, H., Zahedi, M., Abaszadeh, B., Razmjoo, J., Effects of irrigation regime and foliar application of salicylic acid and spermine on the contents of essential oil and caffeic acid derivatives in *Echinacea purpurea* L. *J. Plant Growth Regul.* 2018, *37*, 1267–1285.
26. Diwan, R. and Malpathak, N., Effect of polyamines on shoot multiplication and furanocoumarin production in *Ruta graveolens* cultures. *Nat Prod Commun.* 2012. *7*, 1934578X1200700723.
27. Yin, Z. P., Li, S., Ren, J., Song, X. S., Role of spermidine and spermine in alleviation of droughtinduced oxidative stress and photosynthetic inhibition in Chinese dwarf cherry (*Cerasus humilis*) seedlings. *Plant Growth Reg.* 2014, *74*, 209–218.
28. Shu, S., Yuan, L. Y., Guo, S. R., Sun, J. et al., Effects of exogenous spermine on chlorophyll fluorescence, antioxidant system and ultrastructure of chloroplasts in *Cucumis sativus* L. under salt stress. *Plant Physiol. Biochem.* 2013, *63*, 209–216.
29. Takacs, Z., Poor, P., Tari, I., Comparison of polyamine metabolism in tomato plants exposed to different concentrations of salicylic acid under light or dark conditions. *Plant Physiol. Biochem.* 2016, *108*, 266–278.
30. Zhao, H., Yang, H., Exogenous polyamines alleviate the lipid peroxidation induced by cadmium chloride stress in *Malus hupehensis* Rehd. *Sci. Hort.* 2008, *116*, 442–447.
31. Sagor, G. H., Berberich, T., Kojima, S., Niitsu, M. et al., Spermine modulates the expression of two probable polyamine transporter genes and determines growth responses to cadaverine in *Arabidopsis*. *Plant Cell Rep.* 2016, *35*, 1247–1257.
32. Mitsuya, Y., Takahashi, Y., Berberich, T., Miyazaki, A. et al., Spermine signaling plays a significant role in the defense response of *Arabidopsis thaliana* to cucumber mosaic virus. *J. Plant Physiol.* 2009, *166*, 626–643.
33. Paul, S., Roychoudhury, A., Effect of seed priming with spermine/spermidine on transcriptional regulation of stress-responsive genes in salt-stressed seedlings of an aromatic rice cultivar. *Plant Gene.* 2017, *11*, 133–142.
34. Medina-Pérez, V., López-Laredo, A., Sepúlveda-Jiménez, G., Zamilpa, A. et al., Nitrogen deficiency stimulates biosynthesis of bioactive phenylethanoid glycosides in the medicinal plant *Castilleja tenuiflora* Benth. *Acta Physiol. Plant.* 2015, *37*, 1–8.
35. Cardenas-Sandoval, B. A., Bravo-Luna, L., Bermúdez Torres, K., Trejo-Espino, J. L. et al., Enhancement of phenylethanoids glycosides in *Castilleja tenuiflora* Benth. shoot cultures whit cell wall oligosaccharides from *Fusarium oxysporum* f. sp. *lycopersici* race 3. *Rev. Mex. Ing. Quim.* 2015, 14.
36. Trejo-Tapia, G., Rosas-Romero, G., López-Laredo, A. R., Bermúdez-Torres, K., et al., *In vitro* organ cultures of the cancer herb *Castilleja tenuiflora* Benth. as potential sources of iridoids and antioxidant compounds, in: Orhan, I. (Ed.), *Biotechnological Production of Plant Secondary Metabolites*, Bentham Science Publishers 2012, pp. 87–106.
37. Gamborg, O. L., Miller, R. A., Ojima, K., Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res.* 1968, *50*, 150–158.
38. Lichtenthaler, H. K., Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes, in: Lester Packer, R. D. (Ed.), *Methods in Enzymology*, Academic Press 1987, Vol. 148, pp. 350–382.
39. Sergiev, I., Alexieva, V., and Karanov, E., Effect of spermine, atrazine and combination between them on some endogenous protective systems and stress markers in plants. *Compt Rend Acad Bulg Sci.* 1997, *51*, 121–124.
40. Yan, Q., Shi, M., Ng, J., Wu, J. Y., Elicitor-induced rosmarinic acid accumulation and secondary metabolism enzyme activities in *Salvia miltiorrhiza* hairy roots. *Plant Sci.* 2006, *170*, 853–858.
41. Bradford, M. M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 1976, *72*, 248–254.
42. Sequera-Mutiozabal, M., Antoniou, C., Tiburcio, A. F., Alcázar, R. et al., Polyamines: emerging hubs promoting drought and salt stress tolerance in plants. *Curr. Mol. Biol. Rep.* 2017, *3*, 28–36.
43. Fracassini, D. S., Bagni, N., Cionini, P. G. and Bennici, A. Polyamines and nucleic acids during the first cell cycle of *Helianthus tuberosus* tissue after the dormancy break. *Planta*, 1980, *148*, 332–337.
44. Bagni, N., Calzoni, G. L. and Speranza, A., Polyamines as sole nitrogen sources for Helianthus tuberosus explants in vitro. *New Phytol.* 1978, *80*, 317–323.
45. Minocha, R., Majumdar, R., Minocha, S. C., Polyamines and abiotic stress in plants: a complex relationship. *Front. Plant Sci.* 2014, *5*, 175.
46. Hu, X., Zhang, Y., Shi, Y., Zhang, Z. et al., Effect of exogenous spermidine on polyamine content and metabolism in tomato exposed to salinity-alkalinity mixed stress. *Plant Physiol. Biochem.* 2012, *57*, 200–209.
47. Liu, T., Dobashi, H., Kim, D. W., Sagor, G. H. et al., Arabidopsis mutant plants with diverse defects in polyamine metabolism show unequal sensitivity to exogenous cadaverine probably based on their spermine content. *Physiol. Mol. Biol. Plants.* 2014, *20*, 151–159.

48. Tisi, A., Federico, R., Moreno, S., Lucretti, S. et al., Perturbation of polyamine catabolism can strongly affect root development and xylem differentiation. *Plant Physiol.* 2011, *157*, 200–215.
49. Seepaul, R., George, S., Wright, D. L., Comparative response of Brassica carinata and B. napus vegetative growth, development and photosynthesis to nitrogen nutrition. *Ind. Crops Prod.* 2016, *94*, 872–883.
50. Nguyen, N. T., Nakabayashi, K., Mohapatra, P. K., Thompson, J. et al., Effect of nitrogen deficiency on biomass production, photosynthesis, carbon partitioning, and nitrogen nutrition status of Melaleuca and Eucalyptus species. *Soil Sci. Plant Nut.* 2003, *49*, 99–109.
51. Radhakrishnan, R., Lee, I.-J., Spermine promotes acclimation to osmotic stress by modifying antioxidant, abscisic acid, and jasmonic acid signals in soybean. *J. Plant Growth Regul.* 2012, *32*, 22–30.
52. Grossman, A. and Takahashi, H., Macronutrient utilization by photosynthetic eukaryotes and the fabric of interactions. *Annu. Rev. Plant Biol.* 2001, *52*, 163–210.
53. Groppa, M. D., Benavides, M. P. and Tomaro, M. L., Polyamine metabolism in sunflower and wheat leaf discs under cadmium or copper stress. *Plant Sci. J.* 2003, *164*, 293–299.
54. Gong, B., Wang, X., Wei, M., Yang, F. et al., Overexpression of S-adenosylmethionine synthetase 1 enhances tomato callus tolerance to alkali stress through polyamine and hydrogen peroxide cross-linked networks. *Plant Cell Tiss. Org.* 2016, *124*, 377–391.
55. Koca, N., Karaman, S., The effects of plant growth regulators and L-phenylalanine on phenolic compounds of sweet basil. *Food Chem.* 2015, *166*, 515–521.
56. Li, Z., Zhang, Y., Zhang, X., Peng, Y. et al., The alterations of endogenous polyamines and phytohormones induced by exogenous application of spermidine regulate antioxidant metabolism, metallothionein and relevant genes conferring drought tolerance in white clover. *Env. Exp. Bot.* 2016, *124*, 22–38.

How to cite this article: Rubio-Rodríguez E, López-Laredo AR, Medina-Pérez V, Trejo-Tapia G, Trejo-Espino JL. Influence of spermine and nitrogen deficiency on growth and secondary metabolites accumulation in *Castilleja tenuiflora* Benth. cultured in a RITA® temporary immersion system. *Eng Life Sci*. 2019;19:944–954. https://doi.org/10.1002/elsc.201900040