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Research Article

Comprehensive Characterization of Extractable Phenolic Compounds by UPLC-PDA-ESI-QqQ of *Buddleja scordioides* Plants Elicited with Salicylic Acid

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Buddleja scordioides has a rich phytochemical composition and reported medicinal properties. An increase in its secondary metabolites production could improve its functional properties. A strategy to enhance its biological potential is to subject the plant to elicitation with phytohormones such as salicylic acid under controlled environmental conditions. The present study explores the effect of exogenous application of three salicylic acid levels as elicitation treatment in *B. scordioides* plants. Phenolic profile, enzymatic activities, and antioxidant capacity were evaluated. Elicitation with 100 μM of salicylic acid resulted in the biosynthesis of phenolic compounds with recognized biological activity.

1. Introduction

A great diversity of common herbs has medicinal properties, being a natural source of bioactive compounds. It is important to indicate that approximately 80% of the world population depends on the use of these plants for treating illnesses and keeping up with their health. In developed countries, it is common to use pharmaceutical medicines; however, these practices are changing. In recent years, the use of treatments with natural supplements has increased, due mainly to the cost and side effects produced by conventional medication [1].

The flavonoids and other phytochemicals found in these sources have been considered in many studies by their biological activity, the role they play in the diet and to understand their mechanism of action. *Buddleja scordioides* (also known as salvilla) is a medicinal plant, with pharmacological properties associated with its composition of flavonoids, phenylpropanoids, and verbascosides, product of secondary metabolism [2].

In general, plants synthesize a variety of secondary metabolites that contain the phenolic group. The synthesis of these compounds is influenced by environmental factors. Considering a chemical approach, their structures vary from simple molecules such as phenolic acids, the so-called nonflavonoid phenols, to condensed polymers called tannins. The synthesis of these products is directly involved in plant-herbivorous interactions.

The phytochemicals present in these plants are associated with processes of germination and maturity of the plant. Several biochemical strategies are established to allow them to defend themselves from pathogens and predators, such as

phytoalexins. These strategies involve the synthesis of compounds capable of promoting and inhibiting environmental aggression through abiotic and biotic stresses. The production of bioactive compounds can be increased by the use of elicitors; the term "elicitor" is defined as a substance that, when introduced at very low concentrations into living systems, initiates or promotes the biosynthesis of specific compounds [3].

In this context, the activation of the plant defense mechanism is initiated by the recognition of the elicitor by specific receptors, followed by phosphorylation and dephosphorylation reactions of membrane and cytosolic proteins, ion flux, depolarization of the plasma membrane, extracellular alkalization, intracellular acidification, and genetic modification. These events occur within a period of time after administration of the elicitor by activating downstream responses. After these changes, NADPH oxidases and peroxidases that are activated lead to a massive accumulation of reactive oxygen species (ROS), in coordination with the production of specific signaling molecules such as salicylic acid, resulting in delayed defense responses associated with the synthesis of secondary metabolites.

The elements involved in the signal transduction pathways can be activated differentially in response to specific elicitors. There is also a cross-collaboration between the different signaling pathways that lead to specific responses to different stimuli, whether biotic or abiotic.

The type of defense reaction is different in both cases. In biotic stress, the concentration of ROS is increased to induce programmed cell death in infected cells. Under conditions of abiotic stress, it promotes the reduction of oxidative damage caused by ROS, producing a change in the redox balance of the plant. This induces a regulatory response with the participation of antioxidant enzymes such as superoxide dismutase, catalase, glutathione reductase and ascorbate peroxidase, as well as nonenzymatic antioxidants such as ascorbic acid and reduced glutathione [4, 5].

The intensity of the response varies depending on the strength of the stimulus to which the plant is subjected [6]. Salicylic acid (SA) is a chemical compound derived from the amino acid phenylalanine [7] that participates as a signal molecule in local defenses and regulation of the acquired systemic response [8]. This phytohormone is a powerful elicitor and signaling agent that plays a key role in the growth and development of plants. Metabolic alterations of *Buddleja scordioides* plants subjected to elicitation with salicylic acid and their impact on antioxidant activities have been explored in this study.

2. Materials and Methods

2.1. Chemicals. The salicylic acid, formic acid, ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid), fluorescein sodium, flavonoids (mangiferin, rutin, isoquercitrin, quercetin, kaempferol, naringin, naringenin, linarin, luteolin, acacetin, and apigenin), and phenolic acids (shikimic, protocatechuic, 4-hydroxybenzoic, vanillic, syringic, quinic, chlorogenic, caffeic, coumaric, synaptic, ferulic, and 4,5-dicaffeoylquinic acids) were all obtained from Sigma Aldrich (St. Louis, MO). All reagents used for the preparation of standards and samples were LC-MS grade.

2.2. Plant Culture. Salvilla plant (*B. scordioides*) was collected in Colonia Minerva, Durango, Dgo., Mexico, and fully identified by Socorro Gonzalez-Elizondo at CIIDIR-IPN Herbarium with voucher number 42018. The production of salvilla plants was made from seeds according to Mendoza-Hernandez [9]. Seeds collected from the plant were sown in containers with a mixture of carbon source (peat moss), minerals (perlite), organic fertilizer (vermicompost), and fermented manure (bokashi) at an appropriate ratio (2/1/0.5/0.5). A microenvironment (22–25°C, 50% humidity) was generated with photoperiod cycles with low light of 12 h. When reaching a height of 4-5 cm and the first shoots developed, the exposition time to light was increased, as well as its intensity. When the plants reached a height of 20 cm, they were transplanted individually where their development was allowed until obtaining mature plants in an atmosphere of 60 to 85% humidity and 25°C.

2.3. Elicitation. In order to characterize the effect of elicitation with salicylic acid at time zero, the wet weight and height of the plant were recorded, as well as the number of leaves. Subsequently, the plants were frozen with liquid nitrogen and subjected to lyophilization. To assess the influence of elicitation with salicylic acid, a single application by spraying to salvilla plants was done at several concentrations (0, 10, 100, and 1000 μM). The plants were kept in a humidified atmosphere of 60 to 85% and 25°C for 14 days. At the end of the experiment, the wet weight, the height, and the number of leaves produced were recorded. The plants were frozen with liquid nitrogen and lyophilized, determining moisture by weight difference.

2.4. Enzyme Activity Assays. A pulverized sample (50 mg) of frozen plant with liquid nitrogen was homogenized with acetone (20 : 1 w/v), the suspension was centrifuged at 5000 rpm for 10 min, and the supernatant was removed, repeating the operation a second time. For catalase activity, the remaining pellet was suspended in 1.5 mL of 0.05 M phosphate buffer at pH 7.0, mixed in vortex, and then centrifuged at 13000 rpm at 4°C for 15 min. For measuring the phenylalanine ammonia-lyase activity, the pellet was mixed in 0.1 M borate buffer (pH 8.8) through a vortex and centrifuged 16000 rpm for 15 min. The supernatants were recovered for the determination of enzymatic activities. Protein concentration was measured according to the method described by Bradford [10].

2.4.1. Catalase (CAT). The activity of catalase was determined spectrophotometrically, monitoring the oxidation of hydrogen peroxide (H_2O_2) at 240 nm, according to Chandlee et al. [11]. The reaction consists of 1 mL of plant extract and 1 mL of 0.022 M H_2O_2, using phosphate buffer

(pH 7.0) as a control. The enzymatic activity was evaluated calculating the area under the curve and expressing it as μmol (oxidized H_2O_2) mg protein^{-1}·min^{-1}.

2.4.2. Phenylalanine Ammonia-Lyase (PAL) Activity. The PAL activity was determined spectrophotometrically at 290 nm after the formation of *trans*-cinnamic acid. The supernatant was recuperated and mixed with 60 μM/mL of L-phenylalanine solution (1 : 1 v/v). In this case, borate buffer was used as control instead of extract sample. The mixture was incubated 1 h at 37°C, subsequently the reaction was stopped with 1 M of trichloroacetic acid. Enzyme activity was expressed by the amount of cinnamic acid produced in μmol/mg·protein/h.

2.5. Antioxidant Activity

2.5.1. Oxygen Radical Absorbance Capacity (ORAC) Assay. The ORAC assay was performed on salvilla leaves infusions as described by Ou et al. [12]. AAPH, as a peroxyl radical generator, was used as the free-radical initiator; trolox, as the baseline standard to measure the relative antioxidant activity; and fluorescein, as a fluorescent probe. A sample (20 μL), a blank, or trolox calibration solutions (5 to 50 μM) were mixed with 200 μL of fluorescein (0.108 μM). The plate was incubated at 37°C by at least 15 min in a Synergy HT Multi-Detection Microplate Reader (Bio-Tek, Winooski, Vt., U.S.A.). The reaction was started with the addition of 75 μL of AAPH radical (79.65 mM) and kept shaking for 10 s at maximum intensity. Filters were used to select an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The fluorescence was measured every minute for 25 h. All samples were analyzed in duplicate. The final ORAC values were calculated using the area under the decay curves and expressed as μM·TE/g.

2.5.2. Nitric Oxide Assay. Scavenging activity of nitric oxide (NO) was determined using sodium nitroprusside (SNP) as the NO donor. Briefly, 10 mM of SNP solution was incubated with 1.0 mL of several dilutions of salvilla infusion at 25°C. After 120 min, 0.5 mL of solution was mixed with 0.5 mL of Griess reagent [13]. The absorbance was measured at 546 nm, and results were reported as inhibition percentage.

2.5.3. DPPH (2,2-Diphenyl-1-picrylhydrazyl). Scavenging capacity of DPPH free radical was determined according to Brand-Williams et al. [14]. The absorbance was measured at 515 nm. The percentage of DPPH radical scavenging was calculated as follows:

$$\text{Inhibition}\,(\%) = \frac{A_{t=0} - A_t}{A_{t=0}} \times 100 \quad (1)$$

where $A_{t=0}$ and A_t are the initial (control) and 30 min absorbance readings, respectively.

2.6. Chemical Analysis. The lyophilized and milled samples (10 mg) were extracted with 1 mL of acetone/water (85 : 15 v/v). The mixture was subjected to homogenization by Ultra turrax® at 24000 rpm for one minute, then sonicated for 15 s with 45 s of delay, and finally repeated three times. The supernatants were mixed, dried at a Labconco CentriVap, suspended in 100 μL of methanol (HPLC grade), and filtered in nylon membrane (0.45 μm). The samples were then stored at −20°C until chemical analysis.

2.6.1. Phenolic and Phenylethanoids Analysis. Sample analysis was carried out by Ultra-High-Performance Liquid Chromatography (Waters Corp., Milford) coupled with a tandem Photodiode Array-Electrospray Ionization-Triple Quadrupole (Xevo TQS, Waters Corp., Wexford). The LC system consisted of a sample manager (6°C) and a quaternary solvent manager. The column used to separate the phenolic compounds was a C18, 150 mm × 2.1, 1.7 μm (Agilent), operated at 30°C. The elution profile included two solvents: acidified Milli Q water with 7.5 mM of formic acid (Solvent A) and acetonitrile LC-MS (Solvent B): initial-3% B, 1.88 min gradient to 9% B; 5.66 min gradient to 16% B; 16.90 min gradient to 50% B; 19.62 min gradient to 3% B; and 20.0 min 3% B for column stabilization at a flow rate of 210 μL/min. Ionization was carried out using as cosolvent methanol with 0.1% of formic acid (v/v) at a flow of 5 μL/min with the use of an isocratic solvent manager (Waters Corp., Wexford). Multiple reactions ionization mode was used for MS/MS assays. Electrospray ionization (ESI) in negative conditions were as follows: capillary voltage 2.5 kV, desolvation temperature 400°C, source temperature 150°C, desolvation gas flow 800 L/h, and cone gas flow 150 L/h; collision gas flow was 0.13 mL/min, MS mode collision energy 5.0, and MS/MS mode collision energy 20.0. For identification and quantification of phenolic profile, a mixture of standard (20 ng/μL) was used for monitoring retention time and m/z values and MS/MS transitions. Multiple reaction monitoring mode was recorded for samples and standards. For quantification of phenylethanoid derivatives, caffeic acid (20 ng/μL) was used to determine the relative concentration of this chemical group by LC-PDA (320 nm), and the identification of these compounds was confirmed by ESI-MS/MS. The UPLC and Tandem Xevo TQ-S triple quadrupole mass spectrometer control and data processing were performed using MassLinx v. 4.1 Software (Waters Corp.).

2.6.2. Amino Acid Analysis. Ultra-High-Performance Liquid Chromatography (Waters Corp., Milford) coupled with a tandem-electrospray ionization-triple quadruple (Xevo TQS, Waters Corp., Wexford) was used. Chromatographic separation was performed with an Acquity BEH Phenyl 2.1 mm × 100 mm × 1.7 μM operated at 40°C and autosampler at 6°C, an injection volume was 5 μL. The elution profile included two solvents: acetonitrile/5 mM $HCOONH_4$, pH = 3.0, 95/5 (v/v) as solvent A and acetonitrile/5 mM $HCOONH_4$, pH = 3.0, 40/60 (v/v) as solvent B. Elution was isocratic −50% B with a flow rate of 500 μL/min.

Detection was performed by multiple reaction monitoring with electrospray-positive ionization mode. The identification of amino acids was monitored with *m/z* values and MS/MS transitions as documented by Tsochatzis et al. [15]. Parameters in the source were set as follows: capillary voltage 3.0 kV, desolvation temperature 400°C, source temperature 150°C, desolvation gas flow 800 L/h, and cone gas flow 150 L/h; collision gas flow 0.13 mL/min, MS mode collision energy 5.0, and MS/MS mode collision energy 20.0.

2.7. Statistical Analysis. All results were expressed as the mean ± standard deviation. The data were subjected to one-way ANOVA, and differences among treatments were determined by comparison of mean values using Tukey and Dunnett tests ($p < 0.05$). Statistical analysis was made using the SigmaPlot software version 13.0 (Systat Software, Inc., San Jose, CA, USA). Twelve replicates for each experimental condition were used.

3. Results and Discussion

Salicylic acid has been documented to generate metabolic and physiological responses in plants, affecting their growth and development, allowing their acclimatization to different environmental conditions. Salvilla growth parameters at different levels of salicylic acid administration show that relative growth rate in height per day had statistically significant differences between concentrations of elicitor applied (Table 1). The most contrasting response was observed with 100 μM of salicylic acid (1.08 ± 0.07) compared against plants not elicited (0.22 ± 0.02). A similar trend was observed in the relative effect on foliar production per day; it resulted in an important influence on the increase of leaves in the plant, obtaining the highest efficiency in the 100 μM of salicylic acid treatment (2.33 ± 0.08) with a production greater than 8-fold compared with the control without treatment (0.28 ± 0.04). The application of 10 μM of salicylic acid induces an increase of 1.50-fold, while 1000 μM salicylic acid increased 7.43-fold compared to the plants without eliciting in a period of 14 days. The use of salicylic acid as elicitor has been demonstrated by several researchers [16, 17]; however, it has been reported that the influence of salicylic acid is dependent on the dose [18].

Under conditions of biotic or abiotic stresses, the accumulation of reactive oxygen species is induced, which may cause damage to cellular molecules and can interfere with biological processes. For its protection, the plant has non-enzymatic and enzymatic antioxidant systems that can act against oxidative stress, including catalase. It has been shown that salicylic acid provides protection against cadmium toxicity in maize [19]. Meanwhile, other studies have suggested that exogenous application of salicylic acid may combat adverse effects of arsenic in *Artemisia annua* L [20] with a strong reduction in catalase activity. Our results indicate that CAT levels decline in plants not subjected to stress, and this response is contrary to the leaf production on salvilla plants (Table 1). It is well documented that the physiological response to the administration of salicylic acid alters the antioxidant system by inhibiting the enzymatic activity of catalase and stimulating peroxidase enzymes such as superoxide dismutase [21]. However, contradictory effects have been related to the concentrations of salicylic acid administered and the accumulation levels of catalase in wheat plants exposed to lead stress [22].

The activation of secondary metabolism in plants, produced by application of chemical elicitors, provokes a series of mechanisms responsible for the synthesis of metabolites of interest such as phenolic compounds. Several studies have revealed that PAL regulates the phenolic pathway catalyzed by the deamination of phenylalanine to form *trans*-cinnamic acid. Our results showed that all treated groups with salicylic acid as abiotic elicitor could enhance PAL activity in salvilla (Table 1). This response promotes phenolic synthesis associated with enhancement of the defense resistance of the plant (Figure 1). Two basic routes are widely recognized in the biosynthesis of phenolic compounds: first, the shikimic acid pathway and second, the malonic acid pathway. The latter is related to fungi and bacteria, while the shikimic acid pathway is responsible for the synthesis of phenolic compounds in plants. In this sense, the metabolic production of phenolic acids in salvilla (*Buddleja scordioides*) promotes the synthesis of 5 hydroxybenzoic acids and 7 hydroxycinnamic acids (Table 2). Phytochemical analysis of salvilla extracts by ultra-high-performance liquid chromatography coupled to a photodiode array-electrospray ionization-triple quadrupole showed three subclasses of phenolic acids identified at 280 nm (hydroxyphenolic acids) and 320 nm (hydroxycinnamic acids and phenylethanoids), four subclasses of flavonoids at 280 nm (flavanones), 320 nm (xanthones and flavones), and 360 nm (flavonols) (Figure 2). The identified compounds, their retention times, as well as their parent m/z^- ions and transitions are summarized in Table 2. Five hydroxybenzoic, seven hydroxycinnamic acids, five phenylethanoids, one xanthone, four flavonols, two flavanones, and four flavones were identified and quantified. It is relevant to indicate that mangiferin was contrasted against retention time, UV-spectra, and MS-fragmentation pattern of pure standard. It is important to highlight that, in a period of 14 days, the phenolic acid synthesis showed high statistical significance on shikimic acid, increasing the levels of accumulation in 2.17-fold ($p = 0.0124$) in *Buddleja scordioides* plants not exposed to stress. Several hydroxybenzoic acids are formed from this phenolic acid, such as protocatechuic acid, compound detected in salvilla. One of the documented pathways for its synthesis is associated with the combination of erythrose-4-P, derived from the pathway of pentose phosphate and phosphoenolpyruvic acid (PEP), derived from the glycolysis. Consequently, this turns out in the formation of the metabolite 3-deoxy-O-arabino-heptulosonate phosphate, precursor of 3-dehydroquinate, and therefore, the formation of hydroxybenzoic acids in the plant [23]. Simultaneously, a sequence of reactions for the synthesis of shikimic acid and related derivatives, as well as the biosynthesis of aromatic amino acids such as phenylalanine, tyrosine, and tryptophan is activated. The accumulation of phenylalanine detected in salvilla plants at 14 days of preconditioning at 25°C was 1.28-fold ($p = 0.0029$), while the accumulation observed for

Table 1: Effect of different salicylic acid concentrations on growth parameter, phenylpropanoids biosynthesis, enzymatic activities, and antioxidant capacity in a period of 14 days at 25°C.

Parameter	Initial time (day zero)	Salicylic acid (μM)				p value
		0	10	100	1000	
Height (cm)	20.87 ± 2.69^{a}	24.00 ± 5.65^{a}	22.33 ± 1.52^{a}	36.00 ± 1.73^{b}	33.00 ± 3.00^{b}	0.001
Relative growth rate in height (day^{-1})	—	0.223 ± 0.02^{a}	0.104 ± 0.02^{b}	1.080 ± 0.07^{d}	0.86 ± 0.04^{c}	0.001
Number of leaves per plant	21.00 ± 3.02^{a}	25.00 ± 1.41^{ab}	27.00 ± 1.00^{b}	53.66 ± 3.05^{c}	50.66 ± 1.52^{c}	0.001
Relative effect on foliar production (leaves day^{-1})	—	0.285 ± 0.04^{a}	0.428 ± 0.02^{b}	2.333 ± 0.08^{c}	2.119 ± 0.12^{c}	0.0013
*Relative concentration of phenylethanoids (μg equivalent to caffeic acid g^{-1})**						
Forsythoside A	6.34 ± 0.08^{a}	1.05 ± 0.03^{b}	1.39 ± 0.06^{c}	3.44 ± 0.83^{d}	1.77 ± 0.08^{e}	0.001
Verbascoside	6.90 ± 0.00^{a}	1.56 ± 0.69^{b}	1.45 ± 0.06^{b}	2.62 ± 0.21^{c}	3.91 ± 0.00^{d}	0.0132
Alyssonoside	1.05 ± 0.09^{a}	0.29 ± 0.04^{b}	0.18 ± 0.02^{c}	*Nd*	*nd*	0.001
Acteoside	0.54 ± 0.00^{a}	0.05 ± 0.02^{b}	0.20 ± 0.00^{c}	0.50 ± 0.06^{a}	0.30 ± 0.03^{d}	0.0728
Leucoseptoside	0.69 ± 0.01^{a}	0.02 ± 0.01^{b}	0.07 ± 0.00^{c}	0.19 ± 0.01^{d}	0.07 ± 0.00^{c}	0.001
Enzymatic activities						
PAL (cinnamic acid produced in μmol·mg protein^{-1}·h^{-1})	222.65 ± 2.95^{a}	227.37 ± 1.91^{a}	239.52 ± 0.25^{b}	251.06 ± 0.55^{c}	264.22 ± 0.48^{d}	0.001
CAT (μM·mg·protein^{-1}·min^{-1})	11.80 ± 0.82^{a}	11.95 ± 0.08^{a}	8.18 ± 0.10^{b}	7.38 ± 0.21^{c}	7.70 ± 0.41^{c}	0.001
Antioxidant capacity						
ORAC (μM·TE/g)	37.51 ± 2.80^{a}	42.15 ± 5.04^{ba}	38.11 ± 0.73^{a}	42.87 ± 0.98^{b}	44.86 ± 2.02^{b}	0.001
NO (inhibition %)	17.04 ± 2.92^{a}	13.27 ± 0.02^{b}	19.87 ± 1.44^{a}	30.00 ± 1.82^{c}	31.54 ± 0.19^{c}	0.001
DPPH (inhibition %)	8.92 ± 2.40^{a}	6.61 ± 0.67^{a}	16.71 ± 1.21^{b}	21.97 ± 0.57^{c}	15.95 ± 0.62^{b}	0.001

Different letters in same row indicate statistical differences by Tukey test ($p \leq 0.05$), ($n = 12$), *calculated against caffeic acid at 320 nm.

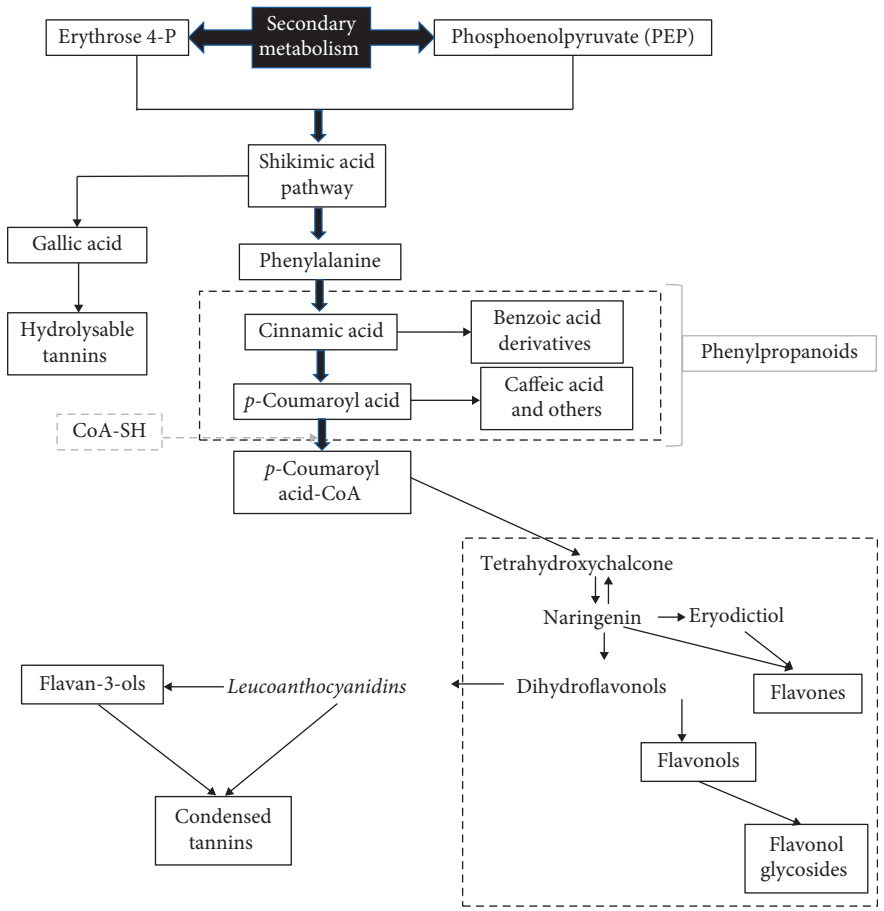

Figure 1: Biosynthesis of polyphenols in *Buddleja scordioides*.

tryptophan was 1.18-fold ($p = 0.0088$) and for tyrosine 1.26-fold ($p = 0.0049$) (Figure 3). Particularly, phenylalanine and tyrosine are important intermediaries in the synthesis of phenylpropanoids and flavonoids, so that the deamination of phenylalanine results in cinnamic acid and consequent conversion to coumaric acid, the latter also derived from

Table 2: Accumulation levels of phytochemical compounds in *Buddleja scordioides* plants preconditioned at 25°C.

No.	Compound	rt (min) PDA	rt (min) QqQ	λ_{max} (nm)	m/z^-	Transitions m/z^-	Precondition content (μg/g dry sample)		p value
							Initial	14 days	
Hydroxybenzoic acids									
(1)	Shikimic acid	1.50	2.35	297.53	173.18	111.07, 93.06	1.85	4.02 (↑2.17)*	0.0124
(2)	Protocatechuic acid	5.99	6.80	293.53	153.15	109.05, 91.04	0.02	0.29 (↑14.5)	0.2936
(3)	4-Hydroxybenzoic acid	8.05	8.82	254.53	137.04	93.05, 65.09	0.16	0.31 (↑1.93)	0.4037
(4)	Vanillic acid	9.04	9.82	294.53	167.18	152.18, 123.09	0.19	0.36 (↑1.89)	0.4701
(5)	Syringic acid	9.31	10.06	273.53	197.21	182.05, 153.11	0.07	0.08 (↑1.14)	0.1924
Hydroxycinnamic acids									
(6)	Quinic acid	1.51	2.28	--	191.20	93.06, 85.06	6.04	13.65 (↑2.25)*	0.0053
(7)	Chlorogenic acid	7.51	8.30	324.34	353.34	191.06, 85.05	0.00	0.02 (↑)	0.1403
(8)	Caffeic acid	9.09	9.85	322.53	179.19	135.08, 89.09	1.84	1.14 (↓1.61)*	0.0345
(9)	Coumaric acid	11.07	11.79	308.53	163.24	119.08, 98.07	0.16	0.24 (↑1.50)*	0.3354
(10)	Sinapic acid	11.65	12.36	323.53	223.24	164.06, 149.04	0.11	0.09 (↓1.22)	0.2171
(11)	Ferulic acid	11.73	12.43	322.53	193.24	178.07, 134.04	0.29	0.28 (↓1.03)	0.4865
(12)	4,5-Dicaffeoylquinic acid	11.75	12.45	324.53	515.43	353.20, 179.06, 173.01	0.00	0.00 (↓)	0.2723
Xanthones									
(13)	Mangiferin	8.83	9.62	317.53	420.89	332.02, 300.99	0.03	0.02 (↓1.5)*	0.0022
Flavonols									
(14)	Rutin	10.86	11.62	353.53	609.28	300.24, 271.13	0.02	0.00 (↓)*	0.0020
(15)	Isoquercitrin	11.34	12.10	353.53	463.36	300.42, 271.11	5.78	0.75 (↓7.70)	0.2829
(16)	Quercetin	15.25	16.01	370.53	301.20	179.04, 151.02	0.76	1.10 (↑1.44)	0.1062
(17)	Kaempferol	17.16	17.89	363.53	285.22	185.02, 151.02	0.02	0.01 (↓2.00)*	0.0021
Flavanones									
(18)	Naringin	12.07	12.82	282.53	579.32	271.12, 151.02	0.05	0.01 (↓5.00)*	0.0022
(19)	Naringenin	16.86	17.53	288.53	271.28	151.04, 119.06	0.06	0.09 (↑1.5)*	0.0026
Flavones									
(20)	Linarin	12.43	13.22	329.53	591.54	283.23, 268.10	11.31	3.33 (↓3.39)*	0.0498
(21)	Luteolin	15.08	15.85	347.53	285.21	151.05, 133.04	0.34	0.72 (↑2.11)*	0.0190
(22)	Acacetin	16.08	17.03	333.53	283.23	268.10, 211.10	0.00	3.69 (↑)	0.7988
(23)	Apigenin	16.68	17.38	335.53	269.27	148.27, 177.04	0.12	0.65 (↑5.41)*	0.0132

*Statistical differences vs initial precondition content of compounds by Dunnett test ($p \leq 0.05$); rt denotes retention time.

tyrosine. The coumaric acid precursor of the hydroxycinnamic acids was identified in *Buddleja scordioides* with an accumulation in two weeks at 25°C of 1.5-fold. From this phenolic acid, the synthesis of 6 additional hydroxycinnamic acids was modulated. In this group, the most abundant metabolite was quinic acid, which significantly increased its production (2.18-fold, $p = 0.0053$). Otherwise, accumulation levels occurred with caffeic, sinapic, ferulic, and 4,5-dicafeoylquinic acids, which were metabolites detected at concentrations lower than those evaluated in the controls at zero time. Of these metabolites, the most significant in the analysis was caffeic acid ($p = 0.0345$), whose levels of accumulation in the plant decreased by 1.65-fold. The above indicates that the acclimation of salvilla plants at 25°C induces synthesis pathways of flavonoid aglycones, detecting an increase in the accumulation levels of metabolites such as quercetin, naringin, luteolin, acacetin, and apigenin, accompanied by a decrease in glycosylated flavonoids such as rutin, isoquercitrin, naringin, linarin, and mangiferin, compounds that were not reported for *Buddleja* species in previous studies (Table 2). It is relevant to indicate that even when compounds derived from leucoanthocyanidins were explored, they were not detected in salvilla plants (Figure 1). Additionally, phenylethanoids that structurally are characterized by cinnamic acid (C_6-C_3), and hydroxyphenylethyl (C_6-C_2) entities linked to β-glucopyranose by glycosidic linkages were detected in salvilla. These compounds are considered as one of the major chemical groups present in *Buddleja scordioides*. It is also important to point out that the environmental conditions to which the plants were subjected are the main factors that induced a decrease in their biosynthesis. When comparing the experimental treatments against the control, it is observed a decrease in the accumulation levels for forsythoside A (↓6.03-fold), verbascoside (↓4.33-fold), alyssonoside (↓3.62-fold), acteoside (↓10.8-fold), and leucoseptoside (↓34.5-fold). Furthermore, the exogenous application of salicylic acid did not induce a significant biosynthesis of these chemical compounds.

One of the mechanisms involved in acclimation of plants is the accumulation of proline, which has been suggested as a general biomarker of stress. Its biosynthesis is controlled by the activity of two pyrroline-5-carboxylate synthetase genes in plants. Proline is activated and its catabolism repressed during dehydration, whereas rehydration triggers

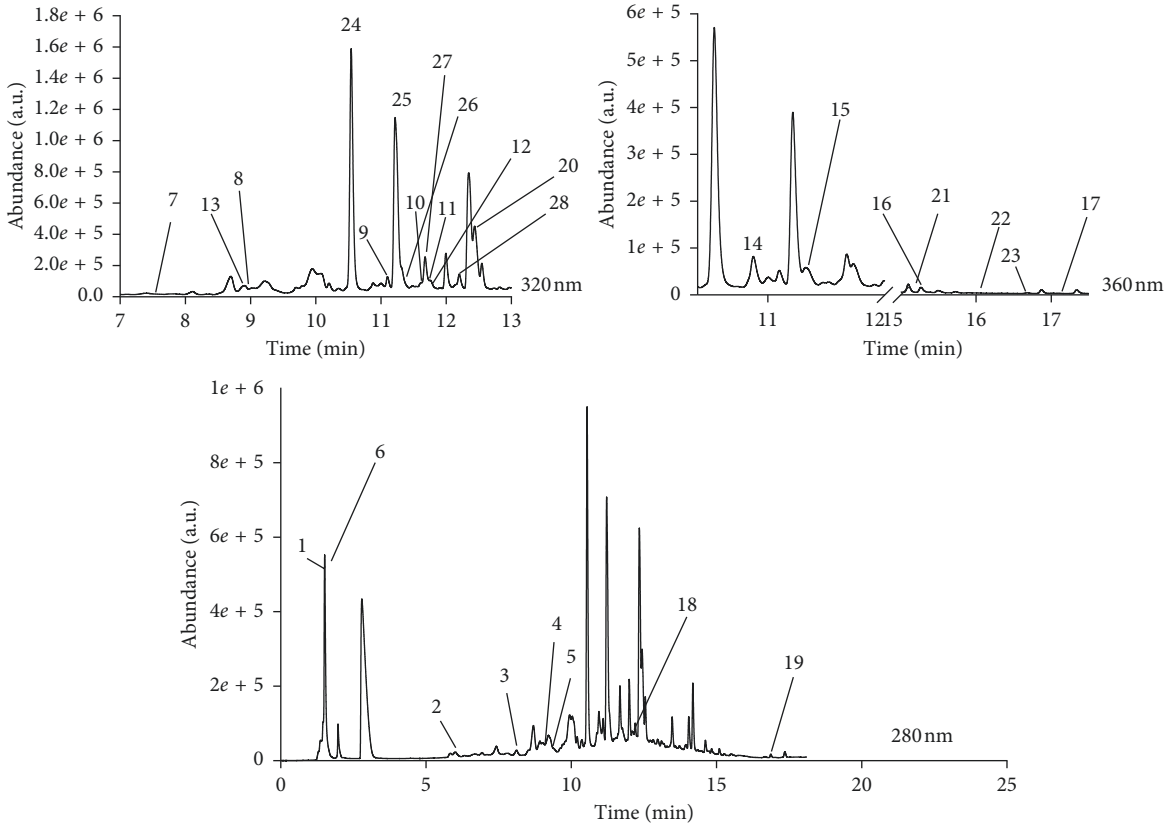

Figure 2: UPLC-UV chromatograms of *Buddleja scordioides* extract recorded at 280, 320, and 360 nm. Numbers correspond to the compounds listed in Table 2.

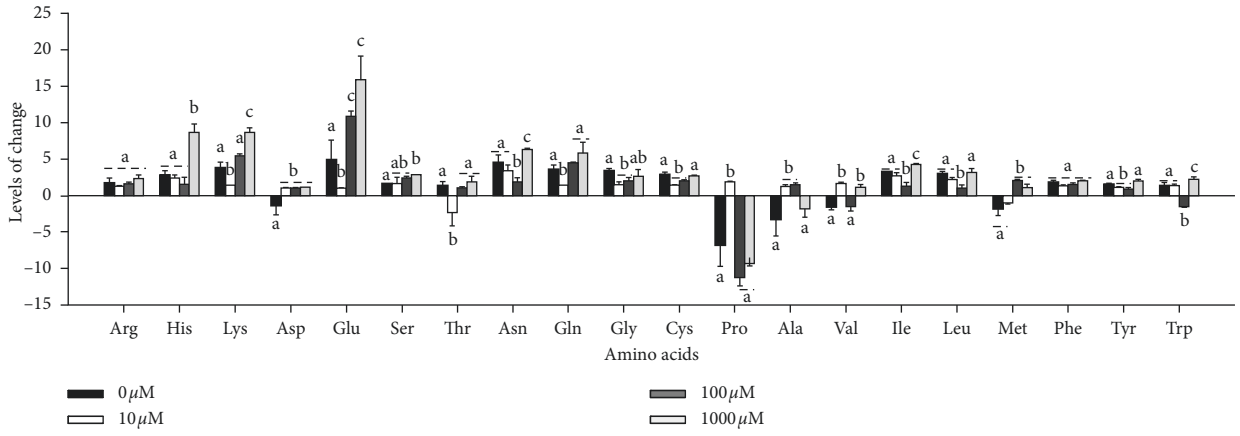

Figure 3: Exogenous application of salicylic acid on accumulation levels of amino acids in plants of *Buddleja scordioides* not subjected to stress. Precursor ion (m/z^+) and transitions registered for each amino acid were 175.12 > 70.31 (Arg), 156.10 > 110.23 (His), 147.14 > 84.29 (Lys), 134.10 > 88.27 (Asp), 147.97 > 84.02 (Glu), 106.10 > 60.4 (Ser), 120.10 > 74.3 (Thr), 133.07 > 87.21 (Asn), 147.09 > 130.18 (Gln), 76.12 > 30.56 (Gly), 122.06 > 59.33 (Cys), 116.12 > 70.33 (Pro), 90.11 > 44.48 (Ala), 118.12 > 72.32 (Val), 132.08 > 86.29 (Ile), 132.11 > 86.27 (Leu), 150.09 > 56.37 (Met), 166.09 > 120.19 (Phe), 182.09 > 136.18 (Tyr), and 205.09 > 188.13 (Trp) by LC-ESI-MS/MS. Different letters on each amino acid bar indicate statistical differences by Tukey test ($p \leq 0.05$).

the opposed regulation [24]. In tolerance to drought, proline plays a protective role in supporting osmotic adjustment. Proline content can be considered as a biochemical and physiological indicator in plants. In our study, the proline content decreased significantly, up to 6.21-fold change in plants not subjected to stress and with no exogenous application of salicylic acid in a period of 14 days. However, plants elicited with 10 μM of salicylic acid induced an increase of 1.86-fold, but not so the exogenous application of 100 μM and 1000 μM, whose doses caused a decrease in the levels of accumulation of this amino acid of 11.26-fold and 9.31-fold respectively, compared with the plant at time zero

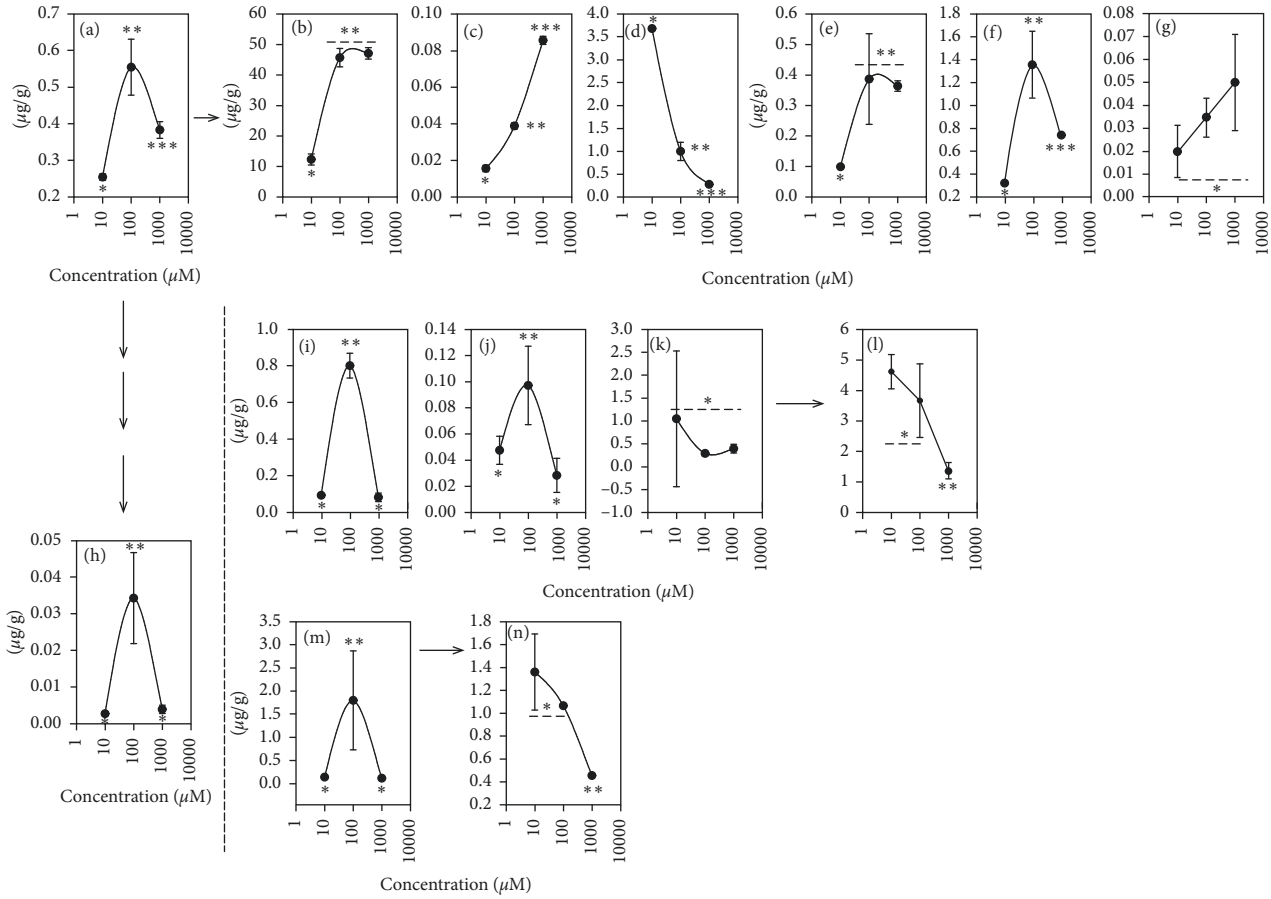

Figure 4: Effect of salicylic acid on accumulation levels of hydroxycinnamic acids and flavonoids in plants of *Buddleja scordioides*. (a) coumaric acid, (b) quinic acid, (c) chlorogenic acid, (d) caffeic acid, (e) sinapic acid, (f) ferulic acid, (g) 4,5-dicaffeoylquinic acid, (h) naringenin, (i) luteolin, (j) apigenin, (k) acacetin, (l) linarin, (m) quercetin, and (n) isoquercitrin. Different ∗ symbols in each graph indicate statistical differences by Tukey test ($p \leq 0.05$).

(Figure 3). In plants, the precursor for proline biosynthesis is glutamic acid whose level of accumulation was opposite to the levels detected of proline. Furthermore, glutamate levels were increased with the exogenous salicylic acid application at 100 μM and 1000 μM; this result allows us to postulate that concentrations greater than 10 μM of salicylic acid inhibit pyrroline-5-carboxylate synthetase genes [25].

The elicitation with phytohormones, considered an important tool to increase the production and accumulation of secondary metabolites under controlled environmental conditions, provokes physiological and morphological responses. In this research, the effect of different concentrations (10, 100, and 1000 μM) of salicylic acid in the synthesis of phenylpropanoids and flavonoids was explored. Salicylic acid is a metabolite derived from phenylalanine, modulator of kaempferol, quercetin, luteolin, caffeic acid, ferulic acid, chlorogenic acid, and coumaric acid synthesis [26]. The elicitation of salvilla plants at different concentrations significantly influenced the synthesis of the different chemical groups monitored in this investigation (Figure 4). Particularly, direct correlations were detected in the concentration of salicylic acid against the synthesis levels detected for quinic acid ($r = 0.88$, $p = 0.001$), chlorogenic acid ($r = 0.98$, $p \leq 0.001$), and 4,5-dicaffeoylquinic acid ($r = 0.76$, $p \leq 0.001$), while inverse correlations were observed in the levels of accumulation for caffeic acid ($r = -0.95$), linarin ($r = -0.90$), and isoquercitrin ($r = -0.92$). When analyzing the intermediaries of the shikimate pathway, specifically coumaric acid with the synthesis of phenylpropanoids, high level of correlation was detected with the accumulation of sinapic acid ($r = 0.85$) and ferulic acid ($r = 1.00$), following the pathway below, a high correlation with concentration of naringenin was detected ($r = 0.92$). In turn, this metabolite showed strong correlations with the synthesis of flavones such as luteolin ($r = 0.97$) and apigenin ($r = 0.93$), as well as flavonols such as quercetin. The relevance of the synthesis of these metabolites focuses on the bioactivity that is conferred on them in various studies, particularly luteolin, which is one of the main flavonoids present in vegetables sources and in medicinal plants such as *Buddleja scordioides*. This flavone can be extracted from leaves and stems of the plant. The synthesis of luteolin and its bioactive derivatives in plants is influenced by environmental and physiological factors. Luteolin exerts a variety of pharmacological and antioxidant activities associated with its ability to scavenging reactive oxygen and nitrogen species. Antioxidant capacity in general is associated to the presence of phenolic compounds and their ability to counteract the formation of free radicals.

Table 1 shows the antioxidant capacity of extracts from salvilla plants as determined by inhibition of NO and DPPH radicals expressed in percentage and oxygen radical absorbance capacity expressed in μM·TE/g. We found that exogenous application of salicylic acid induces the synthesis of chemical compounds capable of inducing greater inhibition of NO and DPPH radicals, but not so compounds able of scavenging reactive oxygen species, since no significant difference was found in the response determined by ORAC compared to the control without elicitation.

4. Conclusions

The phytochemical analysis of extracts from salvilla plants (*Buddleja scordioides*) subjected to elicitation with salicylic acid showed the presence of a wide variety of phenolic compounds, secondary metabolites that have diverse biological activities. The exogenous application of 100 μM of salicylic acid as elicitor induces the synthesis of luteolin, apigenin, and quercetin, with a strong decrease in flavonoid glycosides. Our study provides a complete description of phenolic content in salvilla, although some variability in phytochemical content was found in plants treated with salicylic acid in controlled environment conditions. Further studies would be needed to explore the effect of elicitation with this phytohormone in biotic or abiotic stress conditions to establish expected relationships of chemical contents and elicitation settings.

Data Availability

The experimental data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare no conflicts of interest.

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