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RESEARCH ARTICLE

In vivo elicitation is efficient in increasing essential oil yield with high anti-inflammatory sesquiterpene content in *Varronia curassavica* Jacq.

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ABSTRACT

Essential oils in plants are produced in very low concentrations and elicitation stands out among the techniques used to increase their productivity. This work evaluated the potential elicitor of salicylic acid (SA) or seaweed extract (SE) on the biomass yield, physiological parameters and essential oil production in *Varronia curassavica* Jacq. plants. The elicitors were applied through foliar spray in four serial applications (21, 34, 53 and 70 d after transplanting the seedlings to the pots - DAT), in concentrations of 1 mM (SA) and 5 mL L^{-1} (SE). The control plants were sprayed with water. Biometric measurements of plant height and number of branches were performed after 2, 3 and 4 elicitor applications. Plants were harvested at 91 DAT and biometric, biochemical and phytochemical parameters were evaluated. Application of SA resulted in increases in number of leaves (11.68%) and foliar concentrations of chlorophylls (57.67%), anthocyanins (73.80%), carotenoids (42.58%), total soluble sugars (19.48%) and essential oil (18%). The plants treated with SE had no changes in leaf biomass or essential oil production. The SA treatment increased by twice the amount of α-humulene and (*E*)-caryophyllene present in the essential oil while for SE treatment there was an average increase of 78.6%. It was concluded that the elicitation of *V. curassavica* plants by SA foliar pulverization is an efficient strategy for promoting the higher productivity of leaves and essential oil. Foliar pulverization of SA or SE modifies essential oil quality, inducing increases in the compounds of greatest interest for the pharmaceutical industry.

Key words: α-Humulene, (*E*)-caryophyllene, salicylic acid, seaweed extract, specific leaf area.

INTRODUCTION

Varronia curassavica Jacq. (Boraginaceae), popularly known as “erva-baleeira”, is a native Brazilian medicinal plant that produces an essential oil with great commercial potential. The essential oil (EO) is found in glandular trichomes in the leaf epidermis, and its yield varies between 0.85% and 3.2% among genotypes (Oliveira et al., 2020). The major components of its EO include α-humulene and *trans*-caryophyllene, which are responsible for their anti-inflammatory activity. These two sesquiterpenes are classified as the main chemical markers of the EO of *V. curassavica* (Marques et al., 2019).

Elicitation is a technique that has been widely studied and used in medicinal plants. It consists of the use of substances that promote an increase in the bioactive compound content in the plant, which can also be accompanied by an increase in the production of biomass depending on the type of plant elicitor used (Ramirez-Estrada et al., 2016). Elicitor molecules have long-lasting effects, are relatively low cost and

safe for the environment, are acceptable in organic production systems, and characterize themselves as a promising technology in terms of sustainable agricultural production (Ali et al., 2021). Elicitation alters the expression of genes encoding enzymes associated with the biosynthetic pathways of plant secondary metabolism. Responses to elicitation vary between plant species and types of elicitors, which can have different effects on the same species. Elicitors are initially tested under controlled conditions to select the most promising molecules, which are later tested under more realistic field conditions. Among the various elicitors, it is important to consider the concentration, number and interval of applications and the phenological phase of the culture at the time of stimulation (Gorni et al., 2020; Kandoudi and Németh-Zámboriné, 2022).

Salicylic acid (SA) is a phenolic plant hormone that acts in different physiological processes related to plant growth and development. It is considered an efficient elicitor capable of increasing the synthesis of secondary compounds of commercial interest (Abbaszadeh et al., 2020; Gorni et al., 2021). Seaweed extracts (SE) have been exploited in several products marketed as biostimulants in agriculture (Yakhin et al., 2017; de Saeger et al., 2019). The combined action of different organic molecules and nutrients present in SE produces the effects of stimulation of plant physiological processes (Ali et al., 2021) and elicitation for the synthesis of bioactive compounds (Mukherjee and Patel, 2020; Gorni et al., 2021).

Studies on *in vitro* and *in vivo* elicitation in *V. curassavica* are still scarce in the literature, in part because the commercial cultivation of this species is relatively recent in Brazil, and research efforts have thus far focused on studying the conditions of agricultural management and the ecophysiology of the species. Tonial et al. (2020) studied the effect of the application of the elicitor acibenzolar-*S*-methyl (an analogue of SA) and 1,6 β-D-glucan (a compound derived from the fungus *Lasidioplodia theobromae*) in *V. curassavica* plants produced in the field. Although elicitation modified biochemical aspects of the plant related to defense metabolism, it did not result in significant increases in EO production and did not change its composition, especially in relation to the percentages of α-humulene and β-caryophyllene.

In this context, it was evaluated the elicitation of *V. curassavica* plants by foliar application of SA and SE. The results of this study will provide subsidies for adopting the elicitation technique by producers and companies that cultivate these medicinal species, providing greater profitability.

MATERIALS AND METHODS

Place and conditions of experiment implementation

The experiment was carried out under greenhouse conditions. *Varronia curassavica* Jacq. plant material was obtained in the selection and breeding program from the Agronomic Institute of Campinas (IAC), Campinas, São Paulo, Brazil. In order to homogenize the material, the seedlings were selected in terms of height (around 15 cm) and minimum number of leaves (approximately 10 fully expanded leaves). The seedlings were planted in pots with a capacity of 3 L, filled with a mixture of white sand and commercial vegetable soil (6:4), which had the following chemical characteristics: Organic matter 68.5 g dm^{-3}; pH (1:2.5 soil/suspension $CaCl_2$ 0.01 mol L^{-1}) 6.2; P (resin) 305 mg dm^{-3}; K, Ca and Mg exchangeable from 10.6, 193 and 45.8 $mmol_c$ dm^{-3}, respectively, total acidity in pH 7.0 (H+Al) 17.2 $mmol_c$ dm^{-3}, total cation exchange capacity (CEC) 267.5 $mmol_c$ dm^{-3} and base saturation 93%. The pots were irrigated frequently, trying to keep the soil moist at field capacity.

The elicitors were applied to the plants by foliar spraying in four serial applications at 15 d intervals, at 21, 34, 53 and 70 d after transplanting (DAT) to pots. Salicylic acid (SA; Sigma Aldrich, St. Louis, Missouri, USA) was tested at 1 mM (Gorni et al., 2020; 2021) and the seaweed extract (SE; commercial product, Acadian Seaplants, Darmouth, Canada) was tested at 5 mL L^{-1} (Ali et al., 2021). Control plants were sprayed with water. The volume of solution applied per plant was estimated by previous spraying tests, based on the drip point, with the application of increasing volumes (mL) according to the increase in height and ramifications of the plants. A manual spray was used, adding Agral ([nonylphenoxy]poly[ethyleneoxy]) ethanol; Syngenta, Paulínia, São Paulo, Brazil) as an adhesive spreading agent in the proportion of 50 µL L^{-1} solution, in the case of treatment with SA and SE. The plants were irrigated with nutrient solution three times a week during the last month of the experiment.

Growth and biochemistry analyses

Biometric evaluations of plant height (cm) and number of branches per plant were performed after the application of elicitors (plants that received 2, 3 and 4 sprays, respectively). For the biochemical analysis, leaves were collected at 84 DAT (at the end of four sprays of the elicitors), with immediate freezing in an ultra-freezer for later determination of the concentration of chlorophylls *a* and *b*, carotenoids and anthocyanin (mg g^{-1}) in fresh leaf tissue added in acetone 80% (Sims and Gamon, 2002); and the content of total soluble carbohydrates (mg g^{-1}) by extraction with phenol and sulfuric acid and glucose standard curve (Dubois et al., 1956).

The harvest was performed at 91 DAT, collecting all the aerial parts of the plants (leaves + branches). Leaf area (cm^2) was evaluated using a portable meter (LI-3000A, LI-COR, Lincoln, Nebraska, USA) and the number of leaves per plant was also counted. The materials were placed to dry in an oven with air circulation at a temperature of 40 °C until constant weight was obtained to determine the dry mass (DM) of leaves and stems (g $plant^{-1}$). The specific leaf area (cm^2 g^{-1}) was determined by the relationship between leaf area and dry mass of leaves.

Essential oil analysis

The essential oil (EO) content of the leaves was determined in the dry leaves, through hydrodistillation in a Clevenger-type apparatus. Samples of 60 g dried leaves per replicate were ground together with 1000 mL distilled water in a blender. The mixture was transferred to a 1000 mL volumetric flask and the extraction period of 2.5 h (Marques et al., 2019). At the end of the extraction period, the EO was collected through a phase separation and weighed on an analytical balance to determine the mass (g) of oil (Marques et al., 2019). Subsequently, the EO were stored in amber glass flasks and kept in a freezer at 4 °C for characterization of the chemical composition.

The analysis of the chemical composition of the EO was carried out in a GC-MS system composed of a gas chromatograph (GC, TRACE 1310, Thermo Scientific, Waltham, Massachusetts, USA), a mass spectrometer (MS, ISQ 7000, Thermo Scientific) and a flame ionization detector (FIC) with 3:7 flow split, for MS and FIC, respectively. The injector was maintained at 220 °C with carrier gas (helium) flow division at a 1:20 ratio (split), interface at 240 °C and detector at 250 °C. The mass spectrometer (MS) operated in full scan mode, with electron impact (70 eV) and acquisition range from 40 to 450 m/z.

The substances were separated in an Rtx-5 MS capillary column (20 m × 0.18 mm × 0.2 μm; Restek Co., Bellefonte, Pennsylvania, USA), with a carrier gas flow of 1 mL min^{-1} at the following temperature program: 60-240 °C, 3 °C min^{-1}. The quantification of substances was performed using the area normalization method.

The identification of substances was carried out through a comparative analysis of the mass spectra with the National Institute of Standards and Technology (NIST 14) libraries; Flavor & Fragrance Natural & Synthetic Compounds (FFNSC3) and the linear retention rates of substances in the literature (Adams, 2007). Linear retention indices were obtained from the injection of a series of *n*-alkanes (C_9-C_{24}, Sigma-Aldrich) under the same chromatographic conditions as the samples, applying the equation of van Den Dool and Kratz (1963).

The experimental design adopted was completely randomized, with three treatments and eight replicates of three plants, with the mean of the three plants being determined. The data obtained were tested for normality and homogeneity of variance and submitted to ANOVA ($p \leq 0.05$) using Assistat Software. Subsequently, the means were compared using the LSD test ($p \leq 0.01$). Data on the number of leaves per plant were transformed into log x. The EO content and the α-humulene and (*E*)-caryophyllene contents present in the EO were compared by the Tukey's test ($p \leq 0.05$). The data on the chemical composition of the EO and the chemical groups formed were analyzed using principal component analysis (PCA). Both analyses were performed using the StatSoft program (TIBCO Software, Palo Alto, California, USA).

RESULTS

Biometric determination

The plants that received only two applications of the elicitors showed nonsignificant difference in height or number of branches. After the third application of the elicitors, the plants treated with SE were smaller in relation to SA but showed an increase in branches compared to the control. After four elicitor applications, the plants treated with SE showed greater height compared to the control, and plants treated with SA and SE had a greater number of branches (Table 1).

Table 1. Height and number of branches in *Varronia curassavica* plants treated with salicylic acid and seaweed extract elicitors after 2, 3 and 4 sprays. Means followed by the same letters express significantly equal values by the LSD test (n = 8). *Significant at 5% probability level **Significant at 1% probability level.

Treatments	Plants with 2 sprays	
	Height (cm)	Number of branches
Control	43.08 ± 1.04[a]	4.80 ± 0.19[a]
Salicylic acid	41.16 ± 1.24[a]	4.80 ± 0.27[a]
Seaweed extract	39.45 ± 1.62[a]	4.88 ± 0.36[a]
	Plants with 3 sprays	
	Height (cm)	Number of branches
Control	45.91 ± 1.71[ab*]	5.54 ± 0.14[b*]
Salicylic acid	46.38 ± 1.15[a]	5.37 ± 0.36[b]
Seaweed extract	43.66 ± 1.30[b]	6.00 ± 0.14[a]
	Plants with 4 sprays	
	Height (cm)	Number of branches
Control	47.00 ± 0.62[b*]	6.08 ± 0.28[c**]
Salicylic acid	48.20 ± 0.95[ab]	7.16 ± 0.30[b]
Seaweed extract	49.38 ± 0.70[a]	8.30 ± 0.20[a]

Biometric determinations at the time of harvest

The plants treated with SA showed an increase of 11.68% in the number of leaves and a greater specific leaf area when compared to the control plants (Table 2). However, the other variables analyzed such as leaf area, fresh leaf mass, leaf dry mass and stem dry mass did not show significant variation (Tables 2 and 3).

Table 2. Number of leaves per plant (NL), leaf area (LA) and specific leaf area (SLA) in *Varronia curassavica* plants treated with salicylic acid and seaweed extract elicitors. Means followed by the same letters express significantly equal values by the LSD test (n = 8). *Significant at 5% probability level **Significant at 1% probability level.

Treatments	NL	LA	SLA
		cm^2	$cm^2\ g^{-1}$
Control	118.33 ± 2.88[b*]	1.203.04 ± 26.96[a]	88.20 ± 2.12[b**]
Salicylic acid	132.16 ± 5.60[a]	1.237.14 ± 44.16[a]	98.51 ± 2.30[a]
Seaweed extract	118.08 ± 4.50[b]	1.125.07 ± 51.09[a]	83.52 ± 4.65[b]

Table 3. Fresh mass of leaves (FML), dry mass of leaves (DML) and dry mass of stem (DMS) in *Varronia curassavica* plants treated with salicylic acid and seaweed extract elicitors. Means followed by the same letters express significantly equal values by the LSD test at the 5% probability level (n = 8).

Treatments	FML	DML	DMS
		$g\ plant^{-1}$	
Control	36.31 ± 0.75[a]	13.68 ± 0.44[a]	10.43 ± 0.83[a]
Salicylic acid	37.08 ± 1.07[a]	12.61 ± 0.53[a]	9.54 ± 0.20[a]
Seaweed extract	36.98 ± 1.62[a]	13.54 ± 0.57[a]	9.57 ± 0.57[a]

Biochemical analyses

The SA-treated plants showed the highest increases in carotenoids, anthocyanin, chlorophyll *a* and chlorophyll *b*, with increments of 42.58%, 73.80%, 45.53% and 69.81%, respectively, in relation to the control. The plants treated with SE did not show variations in carotenoid concentrations compared to the control; however, there were significant increases in the concentrations of anthocyanin, chlorophyll *a* and chlorophyll *b* (52.96%, 30.66% and 36.14%, respectively) in relation to the control (Table 4). Both plants treated with SA and SE showed higher concentrations of total soluble sugars in the leaves, with increases of 19.48% and 23.11%, respectively, in relation to the control, with no difference between the types of elicitors (Table 4).

Table 4. Foliar concentration of carotenoids, anthocyanin, chlorophyll *a*, chlorophyll *b* and total soluble sugars (TSS) in *Varronia curassavica* plants treated with salicylic acid and seaweed extract elicitors. Means followed by the same letters express significantly equal values by the LSD test (n = 5). **Significant at the 1% probability level.

Treatments	Carotenoids	Anthocyanin	Chlorophyll *a*	Chlorophyll *b*	TSS
	μg g^{-1}			mg g FW^{-1}	
Control	$231.29 \pm 12.55^{b**}$	$352.06 \pm 24.52^{c**}$	$424.67 \pm 22.33^{c**}$	$174.02 \pm 3.75^{c**}$	$7.55 \pm 0.27^{b**}$
Salicylic acid	402.83 ± 11.86^{a}	1343.77 ± 60.80^{a}	779.73 ± 35.55^{a}	576.60 ± 19.75^{a}	9.25 ± 0.37^{a}
Seaweed extract	222.74 ± 6.95^{b}	748.508 ± 38.62^{b}	554.89 ± 29.61^{b}	272.51 ± 21.54^{b}	9.71 ± 0.49^{a}

Concentration and chemical composition of essential oil in response to elicitors

Regarding the concentration of EO (%) present in leaves, there was a significant increase of 18% in the plants treated with SA compared to the control (Table 5). Plants treated with SA had twice the α-humulene content compared to control plants and both elicitors induced increases of 103% (SA) and 78% (SE) in (*E*)-caryophyllene content.

Table 5. Essential oil content and mean relative proportion of α-humulene and (*E*)-caryophyllene in *Varronia curassavica* plants treated with salicylic acid and seaweed extract elicitors. Means followed by the same letters express significantly equal values by the Tukey test (n = 5). **Significant at the 1% probability level by the Tukey test. *Significant at the 5% probability level by the Tukey Test.

Treatments	Essential oil	α-Humulene	(*E*)-Caryophyllene
		%	
Control	$0.63 \pm 0.032^{b*}$	$1.91 \pm 0.19^{b*}$	$10.10 \pm 1.62^{b**}$
Salicylic acid	0.77 ± 0.034^{a}	4.02 ± 0.64^{a}	20.55 ± 2.50^{a}
Seaweed extract	0.62 ± 0.020^{b}	3.41 ± 0.67^{ab}	18.06 ± 1.74^{a}

The compounds identified in the EO and their average relative percentages in the samples are presented in Table 6. A mean of 91.14% of the compound composition was identified, with 27 substances identified. The substances with the highest relative proportion were α-pinene, (*E*)-caryophyllene, β-elemene and β-guaiene with means of 24.50%, 12.64%, 6.54% and 5.93%, respectively, among the treatments. These substances have been reported as majority in specimens of *V. curassavica* (Marques et al., 2019). Variations were observed in the relative proportions (%) of some compounds due to the elicitation treatments.

Table 6. Average chemical composition (%) of the essential oil of *Varronia curassavica* in response to elicitors treatments (salicylic acid and seaweed extract). RT: Retention time (min); SA: salicylic acid, SE: seaweed extract; RIc: linear retention index, experimentally determined using homologous series of C_9-C_{25} alkanes; RIl: linear retention index taken from Adams (2007); -: below detection level.

Compounds	Control	RT	SA	RT	SE	RT	RIc	RIl
α-Pinene	35.07	5.523	11.65	5.508	26.80	5.524	933	939
β-Pinene	0.76	6.663	1.38	6.663	2.64	6.668	977	979
Mircene	0.84	7.019	-	-	0.87	7.022	991	990
α-Terpinene	1.06	7.840	-	-	-	-	1017	1017
Limonene	1.51	8.236	-	-	1.24	8.239	1029	1029
1,8-Cineol	0.70	8.367	1.10	8.368	1.49	8.368	1032	1031
γ-Terpinene	0.87	9.271	-	-	-	-	1059	1059
Terpinolene	-	10.344	-	-	-	-	1090	1088
Terpinen-4-ol	0.57	14.363	-	-	0.50	14.363	1188	1177
Bornyl acetate	9.57	18.414	1.19	18.416	0.86	18.410	1290	1285
δ-Elemene	1.30	20.251	15.32	20.294	11.67	20.265	1337	1338
β-Elemene	12.62	22.555	3.88	22.560	3.14	22.556	1392	1390
(*E*)-Caryophyllene	0.62	23.648	19.66	23.667	17.66	23.683	1420	1419
6,9-Guaiadiene	2.15	24.579	0.93	24.574	0.78	24.580	1443	1444
α-Humulene	0.60	25.044	4.99	25.046	3.03	25.051	1454	1454
Allo-aromadendrene	1.39	25.306	1.58	25.306	0.77	25.306	1461	1460
cis-Cadin-1(6)-diene	2.33	25.496	0.94	25.500	0.79	25.499	1466	1463
trans-Cadin-1(6),4-diene	2.89	25.811	1.92	25.813	1.32	25.817	1474	1476
γ-Muurolene	2.07	25.913	4.41	25.917	1.88	25.918	1476	1479
β-Selinene	4.23	26.372	5.16	26.375	2.63	26.378	1487	1490
β-Guaiene	14.26	26.442	1.68	26.416	1.86	26.435	1490	1490
δ-Selinene	1.84	26.574	2.98	26.533	5.05	26.554	1493	1492
α-Selinene	0.85	26.734	4.12	26.714	3.31	26.729	1493	1492
trans-β-Guaiene	1.74	26.918	-	-	-	-	1502	1502
δ-Cadinene	0.92	27.814	4.38	27.812	2.45		1525	1523
Isospatulenol	0.57	30.797	1.86	30.796	1.03	30.797	1600	1628
Carophyllene acetate	-	34.500	-	-	-	-	1704	1701
Total identified (%)		Control		SA		SE		
		101.33		89.13		91.77		
Hydrocarbon monoterpenes		40.11		13.3		31.55		
Oxygenated monoterpenes		1.27		1.1		1.99		
Hydrocarbon sesquiterpenes		49.81		71.95		56.34		
Oxygenated sesquiterpenes		0.57		1.86		1.03		
Others		9.57		1.19		0.86		

Principal component analysis explained 99.38% of the variation in compounds, with most of this variation present on the horizontal axis (Figure 1). Three distinct groups were formed, where the control favored the formation of α-pinene, β-guaiene, δ-selinene and β-elemene. The treatment with SA favored the formation of δ-elemene and (*E*)-caryophyllene. And the treatment with SE favored the formation of α-humulene, β-selinene and γ-muurolene (Figure 1A). For the chemical groups, PCA explained 99.74% of the total variation, most of which corresponded to the horizontal axis (Figure 1B). Most of the control treatment was composed of the group of monoterpenes (40.11%) and sesquiterpenes (49.81%) hydrocarbons (Figure 1C). Whereas SA treatment favored the hydrocarbon sesquiterpenes (Figures 1B-2C). Oxygenated monoterpenes showed similar distribution among the three treatments (Figures 1B-2C).

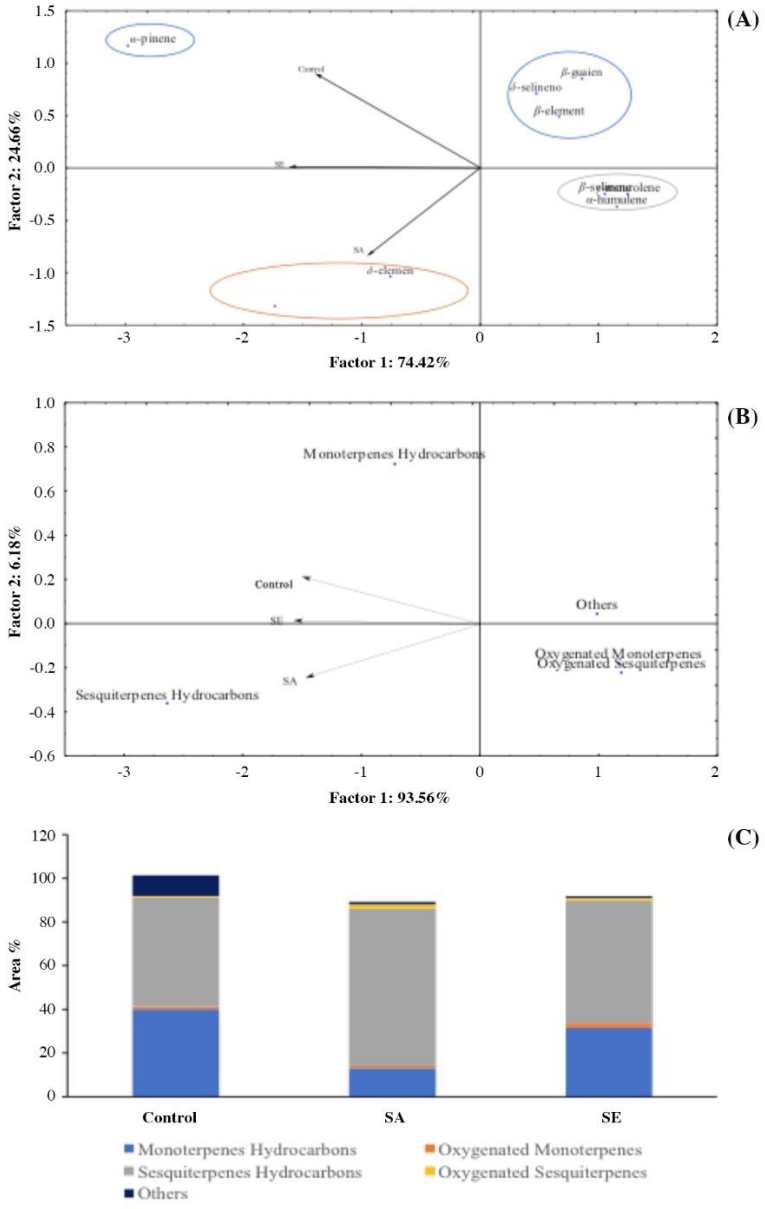

Figure 1. Analysis of principal components of chemical composition (A) and chemical groups (B) and percentage of chemical groups identified in *Varronia curassavica* leaves as a function of salicylic acid (SA) and seaweed extract (SE) elicitation treatments.

DISCUSSION

Varronia curassavica Jacq. is a plant originally from Brazil that has been gaining ground in the pharmaceutical industry due to the anti-inflammatory activity of its EO (Marques et al., 2019). In view of the scarcity of research results on elicitation in this species, the hypothesis that SA and SE could stimulate growth and essential oil production in *V. curassavica* plants was tested.

To obtain the desired responses in plant elicitation, several key factors must be observed, including the number and frequency of applications carried out (Kandoudi and Németh-Zámboriné, 2022). It is often observed that there is a need for various applications of elicitors to induce increases in biomass production (Dianat et al., 2016; Gorni et al., 2020). This can be confirmed in this study, where plants that received only two elicitor applications did not show significant variations in height or number of branches (Table 1), requiring four applications to promote an increase in these parameters. Seaweed extracts and their components can modulate the expression of genes responsible for the endogenous biosynthesis of growth hormones, including auxins, cytokinin and gibberellins, consequently increasing the height and number of branches of the plant (Ali et al., 2021). Salicylic acid is a phenolic compound of a hormonal nature that controls metabolic and physiological responses, regulating plant growth and tolerance to abiotic and biotic stress (Hayat et al., 2010; Gorni et al., 2021).

Salicylic acid application modifies the nutritional status of plants and increases the leaf area, fresh and dry mass, and number of leaves. This phytohormone also promotes an increase in the concentrations of carbohydrates and photosynthetic pigments (Saharkhiz and Goudarzi, 2014; Klessig et al., 2018). In this study, the plants that were treated with SA had more leaves but without concomitant increases in leaf area and fresh and dry mass of leaves (Table 2). These results differed from those obtained in peppermint (Saharkhiz and Goudarzi, 2014) and thyme (Mohammadi et al., 2019), explaining that the responses of different genotypes to SA foliar applications are interspecific. However, treatment with SA increased the specific leaf area (SLA) in *V. curassavica* plants (Table 3). The SLA is an indicator of the availability of leaf area in each gram of leaf; that is, it represents leaf thickness (Gorni et al., 2021). This response can be explained by the fact that SA takes part in chloroplast biogenesis and photosynthetic signaling, as well as in the stimulation of N metabolism (Hayat et al., 2010). In the case of erva-baleeira, a greater leaf thickness becomes interesting in terms of EO production since its leaves have glandular trichomes in the epidermis, but the mesophyll also contains essential oil (Zotti-Sperotto et al., 2020).

An increased photosynthetic pigment content in plants sprayed with SA, such as chlorophylls (*a* and *b*) and carotenoids, has been reported for different species (Chakraborty et al., 2016; Gorni et al., 2020). In this study, SA application to *V. curassavica* plants also resulted in increases in chlorophyll and carotenoid concentrations (Table 4), reflecting the positive effect of this hormonal elicitor on photosynthesis (Askari and Ehsanzadeh, 2015). Salicylic acid also exerts direct effects on photosystem II reaction centers and on the activity of the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Hayat et al., 2010).

The positive effects of SA application on photosynthetic activity in this study were also reflected in the higher carbohydrate content in the leaves (Table 4). Changes in total carbohydrate levels have been observed in both the shoot and underground organs in response to exogenous SA, especially in relation to soluble sugars, namely sucrose, glucose and fructose (Ghasemzadeh and Jaafar, 2012; Krasavina and Burmistrova, 2013). Sugar content regulation by SA occurs via changes in phloem translocation patterns (Amin et al., 2007) and enzymatic activation, such as sucrose phosphate synthase and sucrose synthase (Dong et al., 2011; Gadi and Vidhya, 2012).

Varronia curassavica presented an EO content ranging from 0.63% to 0.77% (Table 5). Based on a study by Marques et al. (2019), these values are within the limits established for accessions of *V. curassavica* collected in the state of São Paulo, which present contents of 0.56% to 0.77% EO in the leaves. The plants sprayed with SA had a higher EO content in relation to the control plants (Table 4), which was not observed in the SE treatment. This can be explained by the nature of the elicitor compound since the formation of further amounts of SA in treated plants may be stimulated, causing positive feedback in terms of terpenoid biosynthesis (Ramirez-Estrada et al., 2016). The SA-mediated increment in plant EOs might be due to SA-stimulated vegetative growth, the population of leaf oil glands, and the beneficial effect of SA on metabolism and enzyme activities responsible for mono- and sesquiterpene biosynthesis (Pirbalouti et al., 2019; Gorni et al., 2020).

Similar results of an increase in the EO concentration in response to SA elicitation were obtained in studies conducted with thyme (*Thymus kotschyanus* Boiss. & Hohen.) and basil (*Ocimum basilicum* L.) (Mirzajani et al., 2015; Mohammadi et al., 2019). In *Melissa officinalis* L., exogenous SA application did not change the amount of EO in the leaves, but it did change its chemical composition, particularly regarding the percentages of oxygenated monoterpenes and sesquiterpenes (Pirbalouti et al., 2019).

As SA influences different physiological and biochemical processes of the plant, this compound can increase the density of leaf essential oil glands, metabolism and enzymatic activity, and ultimately the biosynthesis of monoterpenes and sesquiterpenes (Nasiri et al., 2018).

The SE present varied organic compounds, such as polysaccharides (alginates, fucoidans and laminarins, among others), plant hormones, betaines, sterols, carotenoids, phenolic compounds, minerals, lipids, proteins and amino acids, which stimulate the defense response and the growth of plants. The positive effects of seaweed extracts on plant growth are credited to this mixture of components that act in synergism, promoting the expression of genes that regulate C and N metabolism, cellular metabolism, ion transport across membranes and various metabolic pathways involved in the synthesis of phytohormones (Ali et al., 2021). In this study, although SE stimulated a greater branching of the plants after four applications, there was no increase in the number of leaves per plant and in the production of EO in the whaling herb. However, plants treated with SE had higher concentrations of chlorophylls and carbohydrates (Table 4). The increase in soluble sugars is linked to the upregulation of genes related to polysaccharide degradation (9SEX1 and SEX4) and carbohydrate biosynthesis (GOLS2 and GOLS3), as well as to the downregulation of sucrose-degrading genes (Nair et al., 2012). Thus, the increase in the number of applications or the use of a higher concentration of the product could exert positive effects on leaf biomass and EO production in *V. curassavica* since these factors, among others, influence the response of plants to elicitation with SE (Ghatas et al., 2021; Ali et al., 2021).

The reaction of EO volatile terpenoid compounds to elicitation treatments is variable. In this study, alterations in *V. curassavica* oil components were observed in response to both elicitor treatments (Table 6). There were no relevant chemical connections among changed components since mono- and sesquiterpenes of different skeleton types were targets of either reduction or elevation. This is a normal response demonstrated for in vivo trials in various species (Kandoudi and Németh-Zámboriné, 2022). As chemical markers in the pharmaceutical industry for quality control of the EO of *V. curassavica*, the levels of α-humulene and (*E*)-caryophyllene (sesquiterpenes with anti-inflammatory activity) were used to analyze the variance to identify differences induced by SA and SE. Interestingly, there were marked increases in the concentrations of these target compounds in plants treated with both SA and SE (Table 5). Changes in the physiological and metabolic processes of plants caused by hormonal elicitors (such as SA) or natural ones (such as organic compounds present in seaweed extracts) can exert an indirect influence on the accumulation of volatiles in plants. Simultaneously, elicitor compounds can exert a direct influence on the biosynthetic pathway of terpene production due to the upregulation of genes, resulting in quantitative and qualitative variations in EOs. Enzymatic activities of the first and later steps of the terpenoid pathway may be induced by elicitor treatments (Deschamps and Simon, 2006).

CONCLUSIONS

The in vivo elicitation of *Varronia curassavica* using salicylic acid and seaweed extract is a promising technique for improving the yield and quality of essential oils. Further studies on field conditions should enable the use of both elicitors on the industrial-scale production of this medicinal plant.

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References
Abbaszadeh, B., Layeghhaghighi, M., Azimi, R., Hadi, N. 2020. Improving water use efficiency through drought stress and using salicylic acid for proper production of *Rosmarinus officinalis* L. Industrial Crops and Product 144:111893. doi:10.1016/j.indcrop.2019.111893.

Adams, R.P. 2007. Identification of essential oil components by gas chromatography/mass spectrometry (ed. 4.1). Allured Publishing Corporation, Carol Stream, Illinois, USA.

Ali, O., Ramsubhag, A., Jayaraman, J. 2021. Biostimulant properties of seaweed extracts in plants: Implications towards sustainable crop production. Plants 10(3):531. doi:10.3390/plants10030531.

Amin, A.A., Rashad, M., El-Abagy, H.M.H. 2007. Physiological effect of indole-3-butyric acid and salicylic acid on growth, yield and chemical constituents of onion plants. Journal of Applied Sciences Research 3:1554-1563.

Askari, E., Ehsanzadeh, P. 2015. Drought stress mitigation by foliar application of salicylic acid and their interactive effects on physiological characteristics of fennel (*Foeniculum vulgare* Mill.) genotypes. Acta Physiologiae Plantarum 37:4. doi:10. 1007/s11738-014-1762-y.

Chakraborty, N., Ghosh, S., Chandra, S., Sengupta, S., Acharya, K. 2016. Abiotic elicitors mediated elicitation of innate immunity in tomato: an ex vivo comparison. Physiology and Molecular Biology of Plants 22:307-320. doi:10.1007/s12298-016-0373-z.

de Saeger, J., Van Praet, S., Vereecke, D., Park, J., Jacques, S., Han, T., et al. 2019. Toward the molecular understanding of the action mechanism of *Ascophyllum nodosum* extracts on plants. Journal of Applied Phycology 32:573-597. doi:10.1007/s10811-019-01903-9.

Deschamps, C., Simon, J.E. 2006. Terpenoid essential oil metabolism in basil (*Ocimum basilicum* L.) following elicitation. Journal of Essential Oil Research 18:618-621. doi:10.1080/10412905.2006.9699183.

Dianat, M., Saharkhiz, M.J., Tavassolian, I. 2016. Salicylic acid mitigates drought stress in *Lippia citriodora* L.: Effects on biochemical traits and essential oil yield. Biocatalysis and Agricultural Biotechnology 8:286-293. doi:10.1016/j.bcab.2016.10.010.

Dong, C.J., Wang, X.L., Shang, Q.M. 2011. Salicylic acid regulates sugar metabolism that confers tolerance to salinity stress in cucumber seedlings. Scientia Horticulture 129:629-636. doi:10.1016/j.scienta.2011.05.005.

Dubois, M., Giles, K., Hamilton, J.K., Rebers, P.T., Smith, F. 1956. Colorimetric method for determination of sugars and relates substances. Analytical Chemistry 28:350-356. doi:10.1021/ac60111a017.

Gadi, B.R., Vidhya, L. 2012. Effect of salicylic acid and moisture stress on sugar content and sucrose synthase activity in *Ziziphus* seedlings. Biochemical and Cellular Archives 12:21-23.

Ghasemzadeh, A., Jaafar, H.Z. 2012. Effect of salicylic acid application on biochemical changes in ginger (*Zingiber officinale* Roscoe). Journal of Medicinal Plants Research 6:790-795. doi:10.5897/JMPR11.1459.

Ghatas, Y., Ali, M., Elsadek, M., Mohamed, Y. 2021. Enhancing growth, productivity and artemisinin content of *Artemisia annua* L. plant using seaweed extract and micronutrients. Industrial Crops and Product 161:113202. doi:10.1016/j.indcrop.2020.113202.

Gorni, P.H., Pacheco, A.C., Moro, A.L., Silva, J.F.A., Moreli, R.R., de Miranda, G.R., et al. 2020. Salicylic acid foliar application increases biomass, nutrient assimilation, primary metabolites and essential oil content in *Achillea millefolium* L. Scientia Horticulture 270:109436. doi:10.1016/j.scienta.2020.109436.

Gorni, P.H., Pacheco, A.C., Moro, A.L., Silva, J.F.A., Moreli, R.R., de Miranda, G.R., et al. 2021. Elicitation improves the leaf area, enzymatic activities, antioxidant activity and content of secondary metabolites in *Achillea millefolium* L. grown in the field. Journal of Plant Growth Regulation 40:1652-1666. doi:10.1007/s00344-020-10217-x.

Hayat, Q., Hayat, S., Irfan, M., Ahmad, A. 2010. Effect of exogenous salicylic acid under changing environment: A review. Environmental and Experimental Botany 68:14-25. doi:10.1016/j.envexpbot.2009.08.005.

Kandoudi, W., Németh-Zámboriné, É. 2022. Stimulating secondary compound accumulation by elicitation: Is it a realistic tool in medicinal plants in vivo? Phytochemistry Reviews 21:2007-2025. doi:10.1007/s11101-022-09822-3.

Klessig, D.F., Choi, H.W., Dempsey, D.A. 2018. Systemic acquired resistance and salicylic acid: Past, present, and future. Molecular Plant-Microbe Interactions 31:871-888. doi:10.1094/MPMI-03-18-0067-CR.

Krasavina, M.S., Burmistrova, N.A. 2013. Impact of salicylic acid on the transport and distribution of sugars in plants. p. 83-117. In Hayat, S., Ahmad, A., Alyemeni, M. (eds.) Salicylic acid. Springer, Dordrecht, The Netherlands.

Marques, A.P.S., Bonfim, F.P.G., Dantas, W.F.C., Puppi, R.J., Marques, M.O.M. 2019. Chemical composition of essential oil from *Varronia curassavica* Jacq. accessions in different seasons of the year. Industrial Crops and Product 140:111656. doi:10.1016/j.indcrop.2019.111656.

Mirzajani, Z., Hadavi, E., Kashi, A. 2015. Changes in the essential oil content and selected traits of sweet basil (*Ocimum basilicum* L.) as induced by foliar sprays of citric acid and salicylic acid. Industrial Crops and Product 76:269-274. doi:10.1016/j.indcrop.2015.06.052.

Mohammadi, H., Amirikia, F., Ghorbanpour, M. Fatehi, F., Hashempour, H. 2019. Salicylic acid induced changes in physiological traits and essential oil constituents in different ecotypes of *Thymus kotschyanus* and *Thymus vulgaris* under well-watered and water stress conditions. Industrial Crops and Product 129:561-574. doi:10.1016/j.indcrop.2018.12.046.

Mukherjee, A., Patel, J. S. 2020. Seaweed extract: biostimulator of plant defense and plant productivity. International Journal of Environmental Science and Technology 17: 553-558. doi:10.1007/s13762-019-02442-z.

Nair, P., Kandasamy, S., Zhang, J., Ji, X., Kirby, C., Benkel, B., et al. 2012. Transcriptional and metabolomic analysis of *Ascophyllum nodosum* mediated freezing tolerance in *Arabidopsis thaliana*. BMC Genomics 13(1):1-23. doi:10.1186/1471-2164-13-643.

Nasiri, Y., Zandi, H., Morshedloo, M.R. 2018. Effect of salicylic acid and ascorbic acid on essential oil content and composition of dragonhead (*Dracocephalum moldavica* L.) under organic farming. Journal of Essential Oil Bearing Plants 21:362-373. doi:10.1080/0972060X.2018.1453383.

Oliveira, B.M.S., Blank, A.F., Nizio, D.A.C., Arrigoni-Blank, M.D.F., Bacci, L., Nascimento, L.F.D.A., et al. 2020. Morpho-agronomic characterization of *Varronia curassavica* germplasm conservated "ex situ". Bioscience Journal 36(2):353-363. doi:10.14393/BJ-v36n2a2020-42275.

Pirbalouti, A.G., Nekoei, M., Rahimmalek, M., Malekpoor, F. 2019. Chemical composition and yield of essential oil from lemon balm (*Melissa officinalis* L.) under foliar applications of jasmonic and salicylic acids. Biocatalysis and Agricultural Biotechnology 19:101144. doi:10.1016/j.bcab.2019.101144.

Ramirez-Estrada, K., Vidal-Limon, H., Hidalgo, D., Moyano, E., Golenioswki, M., Cusidó, R.M., et al. 2016. Elicitation, an effective strategy for the biotechnological production of bioactive high-added value compounds in plant cell factories. Molecules 21(2):182. doi:10.3390/molecules21020182.

Saharkhiz, M.J., Goudarzi, T. 2014. Foliar application of salicylic acid changes essential oil content and chemical compositions of peppermint (*Mentha piperita* L.) Journal of Essential Oil Bearing Plants 17:435-440. doi:10.1080/0972060X.2014.892839.

Sims, D.A., Gamon, J.A. 2002. Relationships between leaf pigment content and spectral reflectance across a wide range of species, leaf structures and developmental stages. Remote Sensing of Environment 81:337-354. doi:10.1016/S0034-4257(02)00010-X.

Tonial, C.H., Rodrigues, M.F., Bosse, M.A., Sousa, I.M., de Lima, J.D., da Cunha, M.A.A., et al. 2020. Technical and economic evaluation of cultivation and obtaining of *Varronia curassavica* Jacq. essential oil. Industrial Crops and Product 154:112650. doi:10.1016/j.indcrop.2020.112650.

van Den Dool, H., Kratz, P.D. 1963. A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. Journal of Chromatography 11:463-471.

Yakhin, O.I., Lubyanov, A.A., Yakhin, I.A., Brown, P.H. 2017. Biostimulants in plant science: A global perspective. Frontiers in Plant Science 7:2049. doi:10.3389/fpls.2016.02049.

Zotti-Sperotto, N.C., Melo, E.D.C., de Souza, M.I., Fonseca, M.C., Gonzaga, D.A., de Ávila, M.B.R., et al. 2020. Effect of drying with ultrasonic pretreatment on the yield and quality of the essential oil of *Varronia curassavica* Jacq. and *Ocimum gratissimum* Linn. Industrial Crops and Products 147:112211. doi:10.1016/j.indcrop.2020.112211.