



Effect of methyl jasmonate and salicylic acid on the production of metabolites in cell suspensions cultures of *Piper cumanense* (Piperaceae)



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ABSTRACT

Elicitation of cell suspensions culture is a strategy that could increase the production of secondary metabolites under controlled conditions. This research evaluated the effect of methyl jasmonate-MeJA and salicylic acid-SA as elicitors on the production of metabolites in cell suspensions of *P. cumanense*. The type of elicitor (MeJA or SA), the concentration of elicitor (10 μ M and 100 μ M), and time of exposition (3, 12, 24 h) on cell suspension were evaluated. Metabolic profiles of intracellular and extracellular extracts were analyzed by UHPLC-DAD and GC-MS. Differential production of metabolites was dependent on the type of elicitor, its concentration, and the time of exposition. Treatments with 100 μ M SA were conducted to high production of 5-hydroxymethylfurfural (6.3%), phenol (6.5%), and (Z)-9-octadecenamide (8.8%). This is the first report of elicitation on cell suspensions in the *Piper* genus and contributes to understanding the effect of MeJA and SA on metabolite production in plant cell culture.

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1. Introduction

Piper is the richest genus of the Piperaceae family with around 2000 species, and one of the most diverse lineages of basal angiosperms [1,2]. *Piper* species are characterized by producing a great diversity of metabolites of agricultural, medicinal, and ecological importance, such as alkaloids and amides, propenyl-phenols, lignans, neolignans, and terpenes, several of them with anticancer, antiparasitic, and antibacterial activity [3,4].

Piper cumanense is a species of the genus. It is distributed in Colombia, Ecuador, Peru, and Venezuela, and produces metabolites of interest, such as, benzoic acid derivatives and chromones. The extracts or isolated compounds of this species have antifungal

activity, insecticidal activity, and activity against leishmaniasis amastigotes. However, the low yield in the obtention of its compounds has prevented further research at an experimental or commercial level, which has encouraged the evaluation of strategies to increasing the production of its metabolites in controlled, permanent, and sustainable conditions [5–8].

One of the most widely used biotechnological strategies to increase the production of secondary metabolites is the elicitation of cell suspensions [9]. Elicitation refers to the induction of metabolite biosynthesis using compounds or environmental conditions that trigger the expression of defense genes that promote the production of secondary metabolites [10,11]. The most commonly used elicitors in this type of strategy are methyl jasmonate (MeJA), which, for example, has increased the production of vindoline, cathartine and ajmalicine in suspensions of *Catharanthus roseus* (Apocynaceae), and salicylic acid (SA), which use has increased taxol production in *in vitro* cultures of *Taxus baccata* (Taxaceae) [12,13].

In the genus *Piper*, some biotechnological strategies focus on increasing the production of compounds of interest are in preliminary stages. There are important advances in the

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic; BAP, 6-benzyladenine; GC-MS, Gas chromatography-mass spectrometry; MeJA, Methyl jasmonate; MS, Murashige & Skoog medium; PCA, Principal Component Analysis; PDVF, Polyvinylidene fluoride; SA, Salicylic acid; SI, Similarity index; UHPLC-DAD, Ultra-high performance liquid chromatography coupled to a diode array detector.

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establishment of *in vitro* cultures (micropropagation, callogenesis, and cell suspensions) in some species of the genus. Furthermore, the elicitation is seen as an important strategy to increase the production of secondary metabolites of interest [14–17].

Due to the potential of the metabolites present in *P. cumanense* and the interest to increase the production of their compounds, the objective of this study was to determine the effect of methyl jasmonate and salicylic acid on the production of metabolites in cell suspensions of *P. cumanense* (Piperaceae).

2. Materials and methods

2.1. Chemicals

Plant growth regulators 6-benzyladenine and 2,4-dichlorophenoxyacetic and elicitors methyl jasmonate 95 % and salicylic acid were purchased from Sigma-Aldrich Chemicals. Ethyl acetate 96 % and methanol for HPLC ≥ 99.9 % were purchased from Merck.

2.2. Callus culture

Petioles of 1 cm length were excised from *in vitro* plants of 45 days after *in vitro* germination and were placed horizontally to MS medium supplemented with the following components at a final concentration of 1 mg/L of thiamine, 100 mg/L of inositol, 30 g/L of sucrose, 7.5 g/L of agar and a combination of 2,4-D (2,4-dichlorophenoxyacetic) and BAP (6-benzyladenine). The media were adjusted to pH 5.8 with 0.1 M HCl and 0.1 M NaOH and then autoclaved at 120 °C for 15 min. Every 15 days, callus white and friable obtained, were sub-cultured for its maintenance. Cultures were incubated at 25 \pm 2 °C in constant light under white-fluorescent light bulbs with an intensity of 4500 lx.

2.3. Plant cell suspensions establishment

Cell suspensions were initiated by transferring 2 g of white and friable callus to 100 mL Erlenmeyer flasks which were cultured for 30 days. These bottles contained 20 mL of MS medium supplemented with the following components at a final concentration of 1 mg/L of thiamine, 100 mg/L of inositol, 30 g/L of sucrose, 1 mg/L of 2,4-D, and 0.5 mg/L of BAP. The liquid medium was adjusted to pH 5.8 with 0.1 M HCl and 0.1 M NaOH and then autoclaved at 120 °C for 15 min. On the 15th day, the medium was changed by removing 10 mL of old medium and then adding 10 mL of fresh MS medium supplemented with the components described before. Cell suspensions were placed in continuous orbital agitation at 110 rpm, under constant white light at 25 \pm 2 °C [15,18].

2.4. Cell suspensions elicitation

Cell suspensions previously established were homogenized in a flask and aliquots of 10 mL were taken. Each aliquot was added to 100 mL Erlenmeyer flasks contained 10 mL of MS liquid medium, supplemented with the components described in sub section 2.3. Each suspension had an initial concentration of 90 g of fresh weight per liter (FW/L). On the 15th day, MeJA and SA were added, separately, to the cell suspensions at a final concentration of 10 μ M and 100 μ M. Elicitor solutions were prepared in a 1 mL of a 0.25 % ethanol solution and filtered on a 0.22 μ m membrane filter. The effect of elicitors was evaluated at 3 h, 12 h, and 24 h after its addition to cell suspensions. Controls were suspensions without elicitors added. At times of evaluation (6 h, 12 h, and 24 h) suspensions were harvested and cells were separated from the medium by vacuum filtration. Cells were stored at -80 °C and medium at -20 °C to further metabolites extraction. All experiments were carried out in triplicate.

2.5. Metabolites extraction

2.5.1. Intracellular metabolites

Cells were pulverized in presence of liquid nitrogen and lyophilized at Labconco FreeZone[®]-2.5 L. From the powder obtained, 150 mg was separated, extracted with 2 mL of ethyl acetate, and ultrasonicated for 10 min. This solution was placed in continuous orbital agitation at 110 rpm for 12 h at 25 \pm 2 °C. Then, it was centrifuged at 8500 rpm for 8 min, the supernatant was separated, and its solvent was removed by distillation under reduced pressure. This extraction process was repeated three times. The extract obtained was solubilized in 500 μ L methanol, for HPLC, using ultrasound. Finally, the resulting solution was filtered on 0.22 μ m polyvinylidene fluoride (PDVF) polymer membrane filters of 0.22 μ m and stored at -20 °C.

2.5.2. Extracellular metabolites

Medium (20 mL) was extracted with 20 mL of ethyl acetate and vortexed for 2 min. The mixture was put in a separatory funnel and the organic phase was separated, dried with anhydrous sodium sulfate, and filtered with a qualitative filter paper grade (11 μ m). Then, the solvent was removed by distillation under reduced pressure. This extraction process was repeated three times. The extract obtained was solubilized in 500 μ L methanol for HPLC using ultrasound, filtered, and stored as described in section 2.5.1.

2.6. Analysis of metabolic profiles

Intracellular and extracellular metabolites were tentatively identified and analyzed using UHPLC-DAD and GC-MS chromatographic techniques as described below.

2.6.1. UHPLC-DAD analysis

Metabolic profiles of intracellular and extracellular extracts were analyzed by ultra-high-performance liquid chromatography (UHPLC) using the Thermo Fisher Scientific UPLC Ultimate 3000 equipment coupled to a diode array detector (UHPLC-DAD) and a C18 Kinetex column (50 \times 2.1 mm d.i.; 2.6 μ m). Solvents used as mobile phase were methanol (MeOH): water (0.1 % formic acid) with a gradient elution of 60–100 % MeOH in 30 min and 100 % MeOH in 10 min and the temperature of the oven was 30 °C. The injection volume was 2 μ L with a constant flow of 0.2 mL/min. Chromatograms were recorded at 254 nm and UV spectra were obtained in a range between 200 and 380 nm. The number of peaks, area, and retention time from each sample were analyzed and compared with data (retention time and UV spectra) of peaks from compounds previously isolated and identified by the research

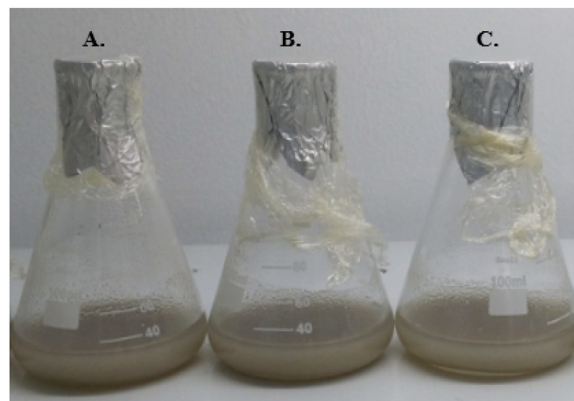


Fig. 1. Cell suspensions of *P. cumanense*: A. Control. Elicited with B. methyl jasmonate (MeJA) and C. Salicylic acid (SA).

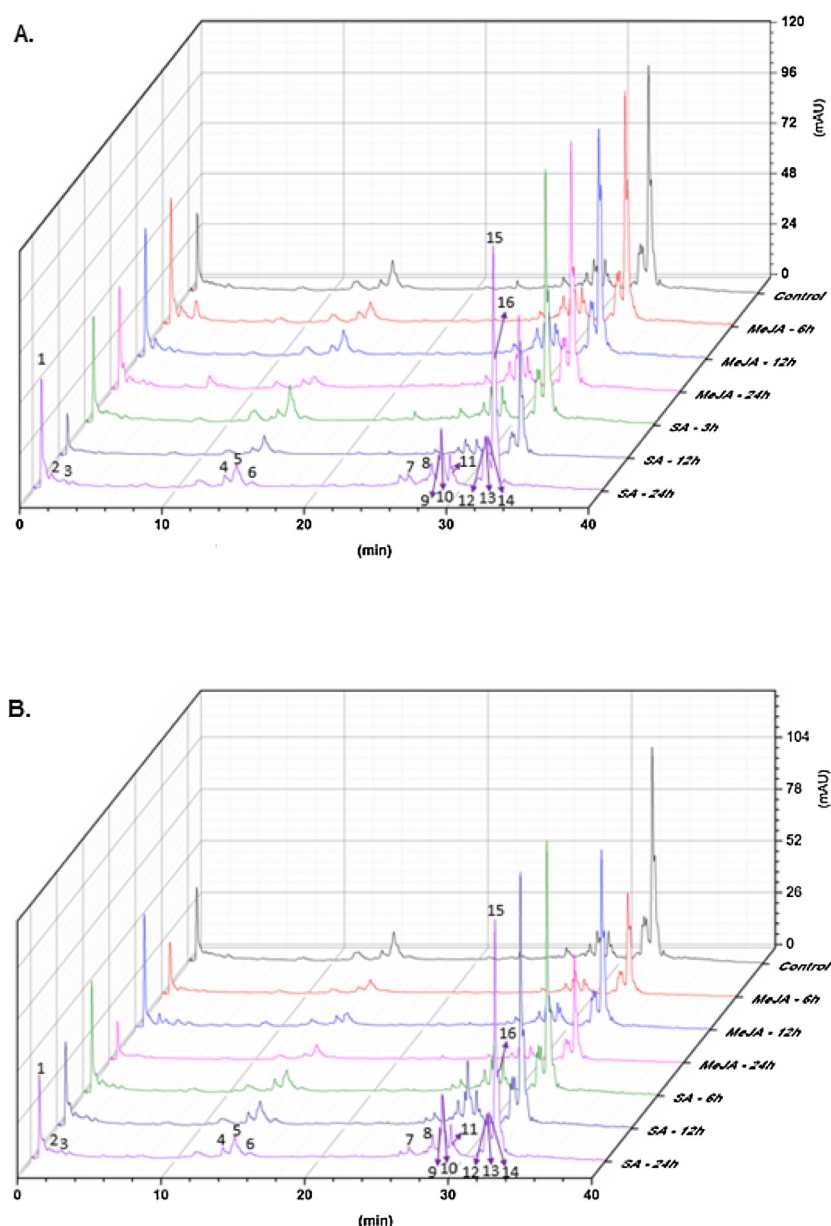


Fig. 2. UHPLC-DAD chromatographic profiles of intracellular extracts from elicited cell suspensions with SA and MeJA at an exposure time of 6 h, 12 h, and 24 h recorded at 254 nm. At the concentration of elicitor evaluated **A.** 10 μM and, **B.** 100 μM .

group QuiProNaB on the extract of inflorescences and infructescences from wild individuals of *P. cumanense*.

2.6.2. GC-MS analysis

Metabolic profiles of intracellular and extracellular extracts were obtained by direct injection without derivatization on a Shimadzu GC 2010 Plus chromatograph coupled to a Shimadzu GCMS-TQ 8040 selective mass detector, equipped with a split/splitless injection port (250 °C, 1:5 Split ratio), a Shimadzu AOC-20i autoinjector and Shimadzu AOC-20s autosampler. A HP-5MS capillary column with a stationary phase of 5% -phenyl-poly (methylsiloxane), 60 m \times 0.25 mm, ID \times 0.25 μm , df, was used. The carrier gas used was helium, with an inlet pressure at the top of the column of 117.6 kPa, a linear velocity of 25.7 cm^{-1} , and a constant flow of 1 mLmin^{-1} . The oven temperature was programmed from 50 °C (5 min) - 20 °C min up to 150 °C (3 min) and finally at 4 °C/min up to 310 °C (5 min). The mass spectra were obtained by

electron impact (EI) with an energy of 70 eV. The temperatures of the ionization chamber and transfer line were maintained at 290 °C and 310 °C, respectively. The mass spectra and total ion chromatogram (TIC) were obtained in a quadrupole, by automatic frequency scanning (full scan), in the mass range of m/z 45–600.

Peaks obtained were tentatively identified by comparing their mass spectrum with the library of the National Institute of Standards and Technology 14 (NIST, USA). Compounds with a similarity index (SI) \geq 90 % were selected and peaks such as silanes and siloxanes related to column bleeding were excluded.

2.7. Analysis of metabolite distribution (statistical analysis)

To analyze the effect of elicitors on cell suspensions Venn diagrams were performed with the InteractiVenn program [19], and multivariate statistical analysis like PCA biplot and heatmap were constructed in RStudio version 1.2.1335 using the pcomp,

Table 1

Retention times and absorption maximums of selected peaks of intracellular profiles, as well as patterns of compounds previously isolated of *P. cumanense* inflorescences and infructescences analyzed by UHPLC-DAD. **RT:** Retention time. **SEM:** Standard error of the mean. Peaks from intracellular profiles were not identified.

	Peak No. / Compound	RT (min) ± SEM	Absorption maximums (nm)
Peaks of intracellular profile	Peak 1	0.62 ± 0.00	200.0 ^a ; 258.8
	Peak 2	1.32 ± 0.02	200.9; 273.8
	Peak 3	2.39 ± 0.04	205.0; 239.7; 313.6
	Peak 4	13.55 ± 0.03	279.5
	Peak 5	14.38 ± 0.03	233.3 ^a ; 277.8
	Peak 6	25.82 ± 0.02	200.0 ^a ; 226.2; 276.6
	Peak 7	26.43 ± 0.02	216.0, 252.9 ^a
	Peak 8	28.04 ± 0.02	245.0
	Peak 9	28.56 ± 0.02	238.3
	Peak 10	28.75 ± 0.02	251.8
	Peak 11	29.35 ± 0.02	237.5
	Peak 12	31.69 ± 0.02	242.4
	Peak 13	31.82 ± 0.01	242.1 ^a ; 321.1
	Peak 14	31.97 ± 0.01	285.8
	Peak 15	32.39 ± 0.02	242.1
	Peak 16	32.58 ± 0.02	241.9
Patterns	Oxocumenic acid	15.85 ± 0.61	204.0; 236.0 ^a ; 327.0
	Gaudichaudianic acid	20.50 ± 0.60	247.0 ^a ; 284.0; 367.0
	Cumenic acid	23.11 ± 0.35	203.0; 242.0 ^a ; 270.0
	Caryophyllene oxide	24.86 ± 0.38	201.0 ^a ; 288.0

^a Corresponds to the maximum absorption in the spectrum.

factoextra, and pheatmap packages [20]. The heatmap was clustered by Euclidean distance.

3. Results and discussion

3.1. UHPLC-DAD analysis

Cell suspensions (control and treated) are shown in Fig. 1, there were no appreciable differences in color or viscosity. Intracellular and extracellular extracts from cell suspensions were analyzed by UHPLC-DAD. Chromatograms obtained were compared considering the presence/absence and relative area of selected peaks. Additionally, it was determined if previously isolated and identified compounds from wild *P. cumanense* plants were present in the extracts obtained in this study.

3.1.1. UHPLC-DAD: intracellular metabolites

Fig. 2 shows the chromatographic profiles of intracellular extracts obtained from suspensions elicited with MeJA and SA. The chromatographic profiles showed that in all the samples analyzed there was a production of metabolites of different polarities. However, most of the compounds eluted in the first 15 min and the largest number of metabolites eluted between minutes 25 and 33. The samples of both controls and treatments had similar chromatographic profiles. The most representative signals were selected and highlighted in Fig. 2 and their retention times and maximum absorbance were summarized in Table 1. Additionally, the table includes the retention times and maximum absorbance of the compounds previously isolated and identified from wild *P. cumanense* individuals. However, those compounds were not detected in the chromatographic profiles either of the control nor the treatments, indicating that the cells in suspensions did not produce this type of compounds under the evaluated *in vitro* culture conditions.

Elicited cells with SA at the two concentrations evaluated tended to increase the area of some peaks: Exposition to 10 μM SA for 24 h increased the area of peak 6 (2.6 times) concerning the control, while 6 h and 12 h exposition increased the intensities of the peaks 7 (7.6 and 11 times, respectively) and 9 (2.5 and 2.7, respectively). Treatments with 100 μM SA and 6 h exposition increased the area of peaks 6 (2.7 times) and 7 (8.7 times), while

24 h exposition only increased the intensity of peak 7 (8.6 times) regarding control.

On the other hand, MeJA treatments also increased the area of some peaks in the chromatographic profiles. Elicitation with 10 μM MeJA and an exposure time of 12 h increased the area of peak 2, 2.9 times compared to the control. Treatments with 100 μM MeJA and 6 h of exposition caused increases in peak 13, in this case, 3 times more than the control. A 24 h exposition of this elicitor (100 μM MeJA) caused an increase in peak 7 (13.7 times), which is higher compared with the observed in SA treatments.

3.1.2. UHPLC-DAD: extracellular metabolites

The chromatographic profiles from media of suspensions are shown in Fig. 3. A peak with a high polarity was detected in 0.62 min which represented around 85 % of the total relative area. However, the greatest number of peaks eluted between 24 and 34 min. As well as the observed in intracellular extracts, the samples of media of both controls and treatments had similar chromatographic profiles. The most representative peaks are highlighted in Fig. 3 and their retention times and maximum absorbance are listed in Table 2. Likewise, in this table, the data of the compounds previously isolated of *P. cumanense* are presented. However, those compounds were not detected in the profiles of media under the evaluated conditions which indicates cells neither produced those compounds nor expelled them to media after its contact with the elicitors.

Some peaks in the profiles from the media of elicited suspensions with SA decreased or increased its area. Treatment with 100 μM SA increased the area of peak 2, while in suspensions elicited with 10 μM SA peak 8 decreased (0.4 times) compared with control. It is highlighted that peak 2 was produced only by suspensions treated with 100 μM SA and exposed to it during 6 h, 12 h, and 24 h.

On the other hand, MeJA treatments reduced the relative areas of some peaks. Elicitation with 100 μM MeJA during 6 h caused a decrease in peak 8 (0.4 times) compared with control. Suspensions exposed to 10 μM MeJA caused a decrease in peaks 1 and 8, 0.9, and 0.5 times, respectively. Furthermore, the exposure of the suspensions to 10 μM MeJA during 12 h generated the greatest increases in peak areas of extracellular extracts. It was detected increases of 2.5, 3.1, and 2.0 times in peaks 5, 6, and 7, respectively.

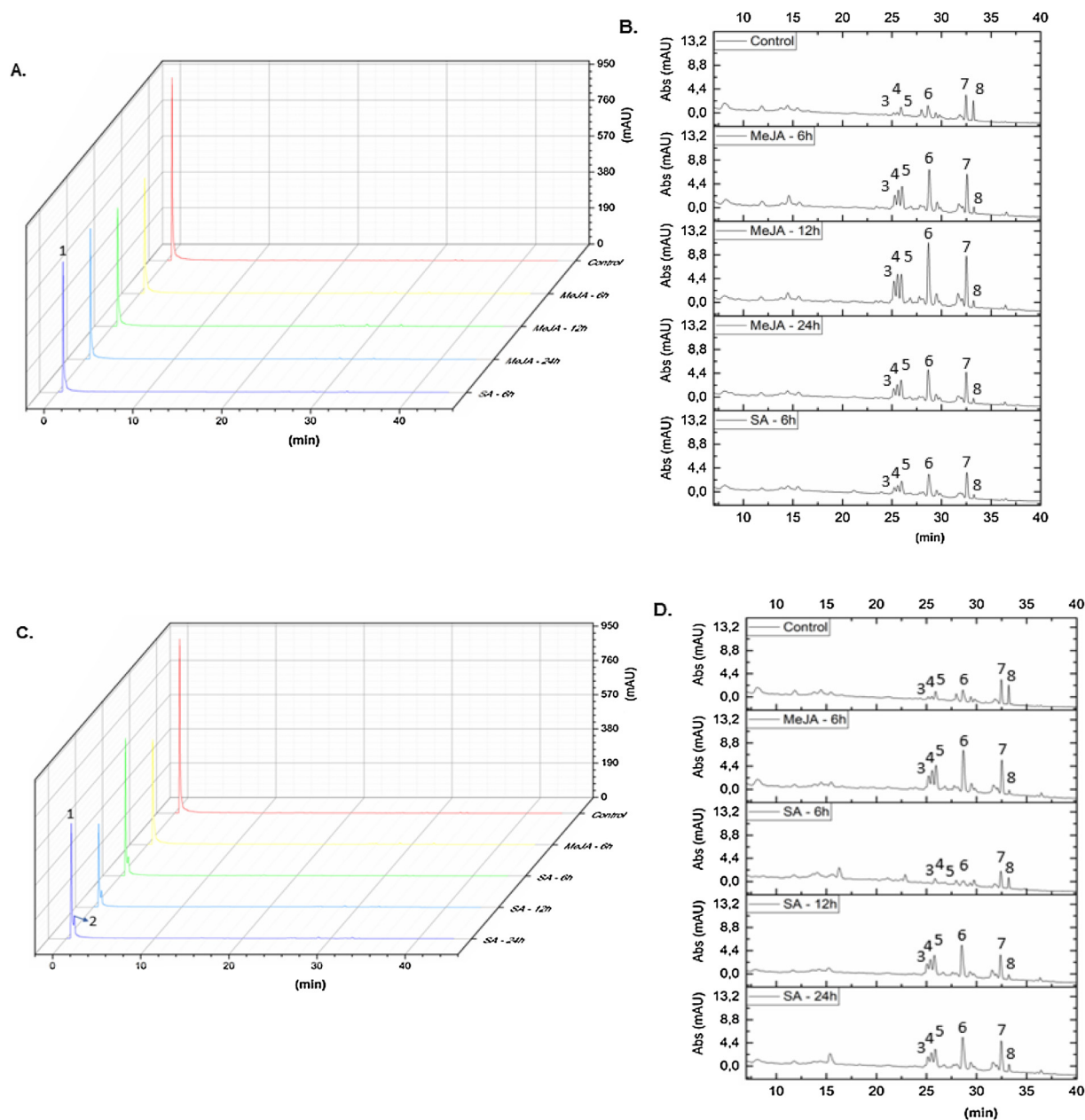


Fig. 3. UHPLC-DAD chromatographic profiles of extracellular extracts from cell suspensions elicited with SA and MeJA at an exposure time of 6 h, 12 h, and 24 h recorded at 254 nm. At the concentration of elicitor evaluated: **A-B**, 10 μM and, **C-D**, 100 μM . Chromatograms shown in **B**, and **D**, are presented from retention time: 4 min. In media, peak 2 is only observed in treatments with elicitors at a concentration of 100 μM .

3.1.3. UHPLC-DAD: Comparison between intracellular and extracellular metabolites

Compounds previously isolated and identified from *P. cumanaense* wild individuals were found neither in intracellular nor in extracellular extracts from suspensions elicited. This could be because the type of elicitor and times of exposure used in treatments did not induce the production of those compounds, at least not in amounts detectable by the UHPLC-DAD equipment. Furthermore, the production of these secondary metabolites may be associated with the state of differentiation of specific tissues, such as leaves or inflorescences, from which the compounds were isolated in wild plants.

It has been found that the state of cell differentiation is an important factor for the activation of biosynthetic pathways to produce specific secondary metabolites. For example, Langhansová

& Maršík (2005) found that root cultures of *Panax ginseng* led to the production of a greater variety of ginsenosides (saponins) compared to callus or cells *in vitro* cultures. This tissue-dependent production is related to the state of tissue differentiation that could be due to differential expression of genes involved in the biosynthesis of compounds [21,22].

On the other hand, the absence of compounds previously identified in *P. cumanaense* in the cell suspensions could also be due to the absence of endophytes, a term that refers to microorganisms such as bacteria or fungi that are closely associated with tissues of some plants in a symbiotic relationship. It has been found that endophytes produce intermediate molecules that use plants to synthesize some secondary metabolites or even produce them. For example, taxol, an important compound used for cancer treatment, was first isolated from the plant species *Taxus brevifolia*. However,

Table 2

Retention times and absorption maximums of selected peaks of extracellular profiles, as well as patterns of compounds previously isolated of *P. cumanense* inflorescences and infructescences analyzed by UHPLC-DAD. **RT:** Retention time. **SEM:** Standard error of the mean. Peaks from intracellular profiles were not identified.

	Peak No. / Compuesto	TR (min) \pm SEM	Absorption maximums (nm)
Peaks of extracellular profile	Peak 1	0.62 \pm 0.00	208.3 ^a ; 262.3
	Peak 2	0.94 \pm 0.00	202.4 ^a ; 205.5 ^a ; 235.4; 303.3
	Peak 3	25.62 \pm 0.01	200.0 ^a ; 250.0
	Peak 4	25.26 \pm 0.01	252.5
	Peak 5	25.92 \pm 0.01	200.0 ^a ; 224.5; 281.8
	Peak 6	28.66 \pm 0.01	240.8
	Peak 7	32.49 \pm 0.01	242.2
	Peak 8	33.23 \pm 0.00	200.0 ^a ; 212.5; 272
Patterns	Oxocumenic acid	15.85 \pm 0.61	204.0; 236.0 ^a ; 327.0
	Gaudichaudianic acid	20.50 \pm 0.60	247.0 ^a ; 284.0; 367.0
	Cumenic acid	23.11 \pm 0.35	203.0; 242.0 ^a ; 270.0
	Caryophyllene oxide	24.86 \pm 0.38	201.0 ^a ; 288.0

^a Corresponds to the maximum absorption in the spectrum.

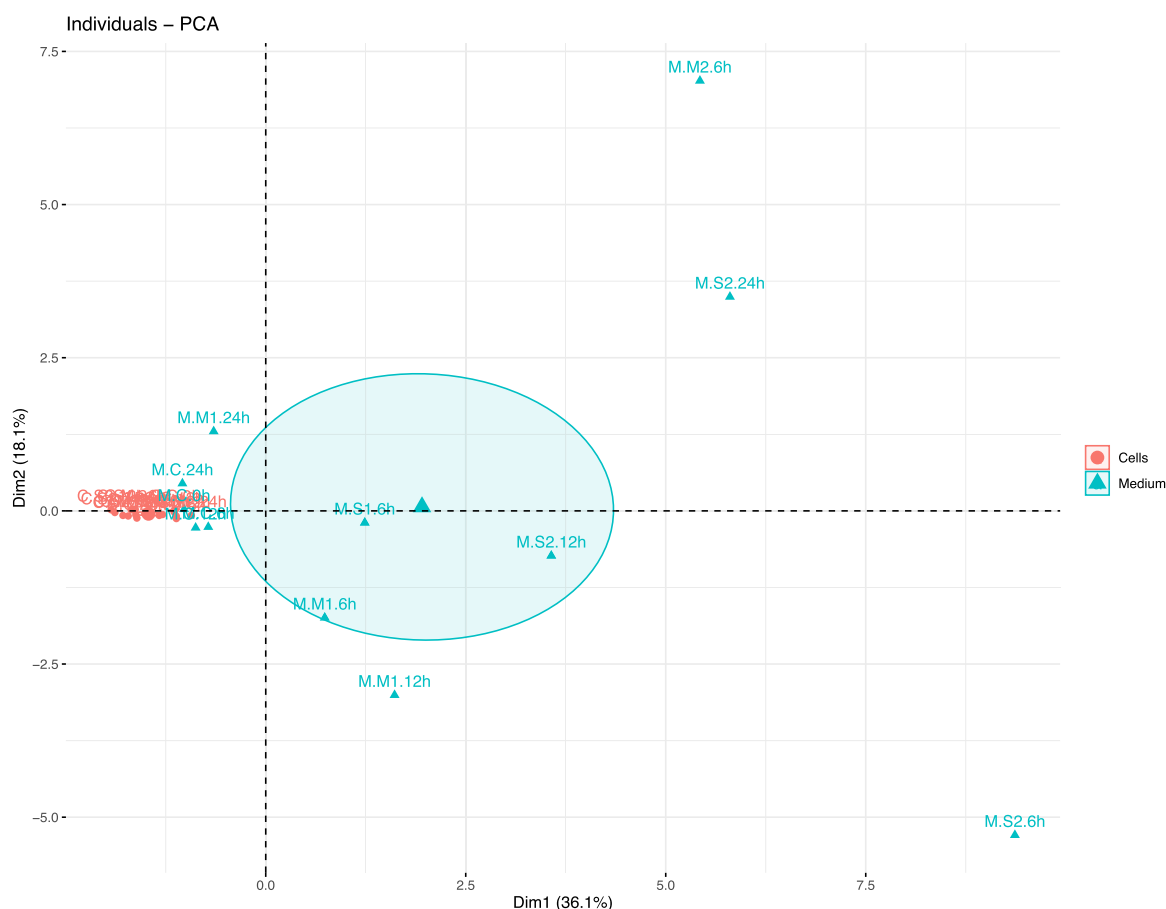


Fig. 4. PCA biplot of the relative areas of the metabolites tentatively identified in intracellular and extracellular extracts from cell suspensions elicited with MeJA and SA.

taxol production has been discovered also in a wide variety of fungal species like the endophytic fungi *Pestalotiopsis microspora* and *Stemphylium sedicola* which have been isolated from plant species of the genus *Taxus* and *Taxodium*. Likewise, the compounds azadirachtin A and B, which are widely used as natural insecticides and traditionally isolated from neem tree, *Azadirachta indica*, are also produced by the endophytic fungus *Eupenicillium parvum* isolated from neem tree [23–27].

Furthermore, the UHPLC-DAD profiles obtained (Figs. 2 and 3) show greater changes in peaks areas in extracts of media compared to

extracts of cells. This indicates that under the stress conditions evaluated cells tended to excrete metabolites to medium rather than keep them internally. The expulsion of the metabolites to the medium is desirable in this type of strategy because it favors the extraction procedure and, cells can continue growing in new media. In some species, it has been possible to induce cells to excrete secondary metabolites of interest to medium. For example, cell suspensions of *Vitis labrusca* elicited with methyl jasmonate tended to expulse predominantly trans-resveratrol, pallidol, δ -viniferin, and ϵ -viniferin to the medium [28,29].

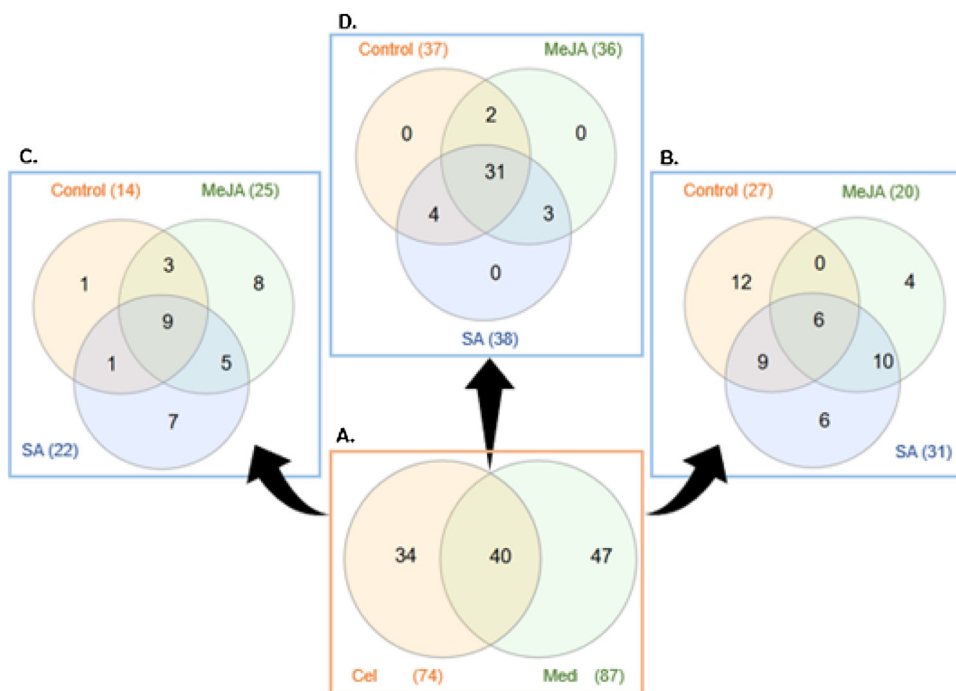


Fig. 5. Venn diagrams with the distribution of metabolites in intracellular and extracellular extracts from cell suspensions elicited with MeJA and SA. **A.** Total metabolites in cells and medium. **B.** Exclusive metabolites of the medium. **C.** Exclusive metabolites of the cells. **D.** Metabolites shared by cells and medium.

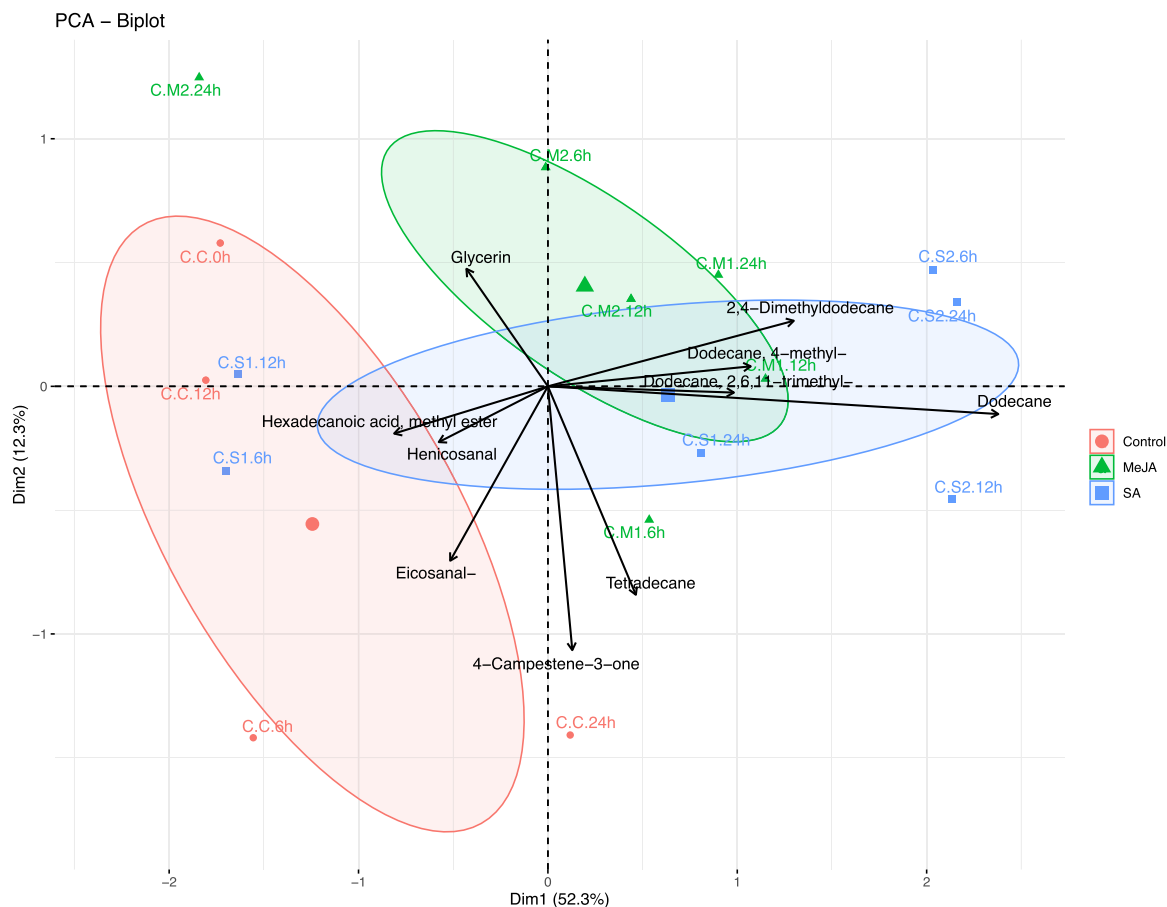


Fig. 6. PCA biplot of intracellular extracts. The letters of the sample labels refer to, in order of appearance: **C** to Cell extract. Next letter: **C, M, or S** to Control samples (**C**) or treated with methyl jasmonate (**M**) or salicylic acid (**S**). Finally, **1** or **2**: Concentration of 10 or 100 μ M, respectively, and **6 h, 12 h** or **24 h** to the exposure time.

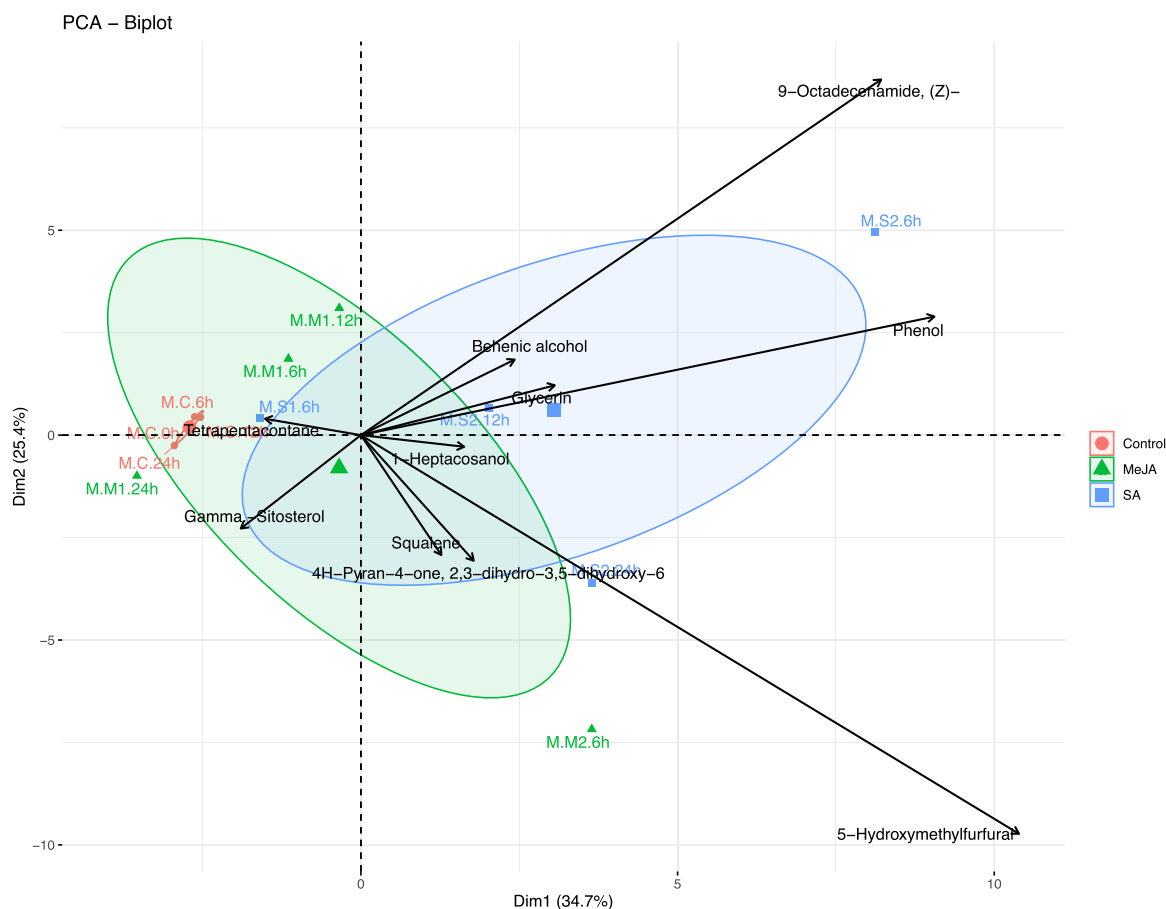


Fig. 7. PCA biplot of extracellular extracts. The letters of the sample labels refer to, in order of appearance: **M** to Media extract. Next letter, **C**, **M** or **S** to Control samples (C) or treated with methyl jasmonate (M) or salicylic acid (S). Finally, **1** or **2**: Concentration of 10 or 100 μ M, respectively, and **6 h**, **12 h** or **24 h** to the exposure time.

3.2. GC-MS analysis

GC-MS analysis of intracellular and extracellular extracts led to tentatively identify a total of 121 compounds. These metabolites correspond mostly to primary metabolites, mainly to hydrocarbons and esters. Also, some secondary metabolites such as terpenes, amides, and phenol were detected (see supplementary file).

3.2.1. Analysis of metabolites distribution: PCA of intracellular and extracellular metabolites

Fig. 4 shows the principal component analysis (PCA) of intracellular and extracellular extracts. PCA explains in the first two dimensions 54 % of the variance. Intracellular samples are grouped (orange color) and present a low variance. On the other hand, extracellular samples are grouped separately (aquamarine color) and have greater variance. This could be due to the number of metabolites present and their relative areas. PCA shows that intracellular and extracellular extracts have divergent characteristics.

However, some samples, particularly controls of both types of extracts, are overlapped. This suggests that compounds present in controls are mainly constitutive while compounds in samples, within the groupings, could be produced by the effect of treatments with elicitors.

3.2.2. Analysis of metabolites distribution: venn diagram

Venn diagrams were constructed to determine the distribution of the metabolites present in the intracellular and extracellular extracts (Fig. 5). A greater number of compounds were found in media (87) in comparison with cells (74), which indicates that cells tended to excrete the metabolites (Fig. 5.A). Some metabolites (40)

were present in both types of extracts, which represents about 50 % of the total metabolites present in each type of extract.

Fig. 5.B shows the distribution of the exclusive metabolites of extracellular extracts (media). Some metabolites were excreted to media when cell suspensions were exposed to the elicitors. Treatments with

MeJA induced the exclusive production of 4 compounds, with SA of 6 compounds, and 10 compounds were produced by both elicitors. Moreover, 12 metabolites were present only in controls and 15 in controls and media of elicited suspensions. This distribution suggests that the elicitors induced the synthesis of compounds that were excreted to the media. Additionally, the presence of some metabolites only in controls indicate that elicitors may be inhibiting the production of some compounds, causing their degradation or transformation into new metabolites [30].

Fig. 5.C shows the distribution of metabolites present exclusively in intracellular extracts (cells). In this case, MeJA induced the biosynthesis of 8 compounds, SA of 7 compounds, and the number of metabolites induced by both elicitors was lower compared with media extracts (5 versus 10 in the media). Furthermore, only 1 metabolite was exclusive of control, and 13 were produced in both, elicited cells, and controls.

Finally, Fig. 5.D shows the distribution of the 40 metabolites shared between intracellular and extracellular extracts. Most of the metabolites were present in both controls and samples of elicited suspensions. Furthermore, only 3 compounds were present in both MeJA and SA treatments, and no compound was exclusive to controls, MeJA, or SA. This indicates that metabolites shared by cells and media were mostly constitutive because they were present in control and treatments with at least one elicitor.

as found in this study in elicited suspensions of *P. cumanense* [15]. However, to our knowledge, this study is the first report of elicitation in *Piper* genus cell suspension which can serve as a basis to the understanding of the production of secondary metabolites in *Piper* species.

3.2.5. Analysis of metabolites distribution: Heatmap

A heatmap was constructed to analyze the production of the metabolites concerning the relative percentage area in the different treatments (Fig. 8). To construct this diagram, the 121 compounds tentatively identified were classified into 14 groups according to the main functional group. Two large groupings defined by the type of sample (cells or medium) were obtained in this diagram. The group of cells had a cluster established mainly by elicited cells, and another cluster composed mainly of controls of cells and media samples. Finally, there was a third, poorly defined grouping composed of media from suspensions exposed to elicitors and characterized by the presence of the highest relative areas of some compounds.

In this diagram, it was observed that the samples were composed mainly of primary metabolites such as hydrocarbons and esters, although secondary metabolites like terpene, phenol, and amide were also detected. Hydrocarbons were characteristic of all samples and formed blocks that defined the groupings in cells and media. For example, in the listing of compounds from dodecane to 2,6,10-trimethyl-pentadecane (on the right of Fig. 8), those metabolites defined the grouping of elicited cell samples. Moreover, dodecane was an important metabolite with a high weight in the biplot of cells (Fig. 6), the same as seen in this heatmap, in which this compound had a high relative area in elicited cells with SA.

On the other hand, media from elicited suspensions were characterized by having a higher intensity in some metabolites, mainly secondary metabolites. It indicates that elicitors induced the activation of secondary metabolic pathways in cell suspensions, and in this case, cells tended to preferentially excrete these metabolites to media. It is highlighted the compounds 5-hydroxymethylfurfural and phenol which were mainly detected in media from elicited suspension with SA, and (Z)-9-octadecenamamide which were present in elicited suspensions with both, MeJA and SA treatments. The production of this amide is interesting because the biosynthesis of this compound has been reported in *Piper* species like *P. boehmeriaefolium*, *P. maclueri*, and *P. guineense* [32,33]. However, the production of (Z)-9-octadecenamamide has not been reported yet in *P. cumanense* wild species, but interestingly in this study, the biosynthesis of this compound was induced by MeJA and SA elicitors under *in vitro* conditions. The study of the effect of these and other elicitors in various concentrations and exposure times could contribute to the understanding of the factors that cause the production of metabolites in *P. cumanense* and the implementation of strategies focused on obtaining compounds of interest in larger scales.

4. Conclusion

This research represents the first report on cell suspensions elicitation in *Piper* genus. Cell suspensions elicited with MeJA and SA had a differential production of metabolites dependent on the type and concentration of elicitor as well as the time of exposition to the cell suspension. Treatments with SA 10 μ M and 100 μ M induced the greatest metabolic changes highlighting the production of 5-hydroxymethylfurfural, (Z)-9-octadecenamamide, and phenol. However, under the elicitation conditions evaluated, the production of any of the secondary metabolites isolated from wild *P. cumanense* species was not achieved. This research can serve as a basis for understanding the effect of abiotic elicitors on *Piper* cell suspensions and on the application of strategies to improve metabolite production in cell suspensions culture.

Supplementary data

Supplementary material related to GC–MS data is provided.

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CRedit authorship contribution statement

Laura Katherine Rodríguez-Sánchez: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft. **Jorge Enrique Pérez-Bernal:** Methodology, Software, Validation, Investigation, Data curation. **Mary Andrea Santamaría-Torres:** Methodology, Formal analysis, Data curation. **Xavier Marquínez-Casas:** Validation, Formal analysis, Writing - review & editing. **Luis Enrique Cuca-Suárez:** Methodology, Formal analysis. **Juliet Angélica Prieto-Rodríguez:** Conceptualization, Formal analysis, Writing - review & editing, Supervision. **Oscar Javier Patiño-Ladino:** Conceptualization, Formal analysis, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.btre.2020.e00559>.

References

- [1] M.A. Jaramillo, R. Callejas, Current perspectives on the classification and phylogenetics of the genus *Piper* L., in: L. Dyer, A. Palmer (Eds.), *Piper A Model Genus Stud. Phytochem. Ecol. Evol.*, Kluwer Academic/Plenum Publishers, 2004, pp. 179–198.
- [2] G. Singh, *Plant Systematics. An Integrated Approach*, Science Publishers, 2010, doi:<http://dx.doi.org/10.1017/CBO9781107415324.004>.
- [3] L. Dyer, A. Palmer, *Piper: a Model Genus for Studies of Phytochemistry, Ecology, and Evolution*, Kluwer Academic/Plenum Publishers, 2004, doi:<http://dx.doi.org/10.1007/s13398-014-0173-7.2>.
- [4] E.E. Mgbeahuruike, T. Yrjönen, H. Vuorela, Y. Holm, Bioactive compounds from medicinal plants: focus on *Piper* species, *S. Afr. J. Bot.* 112 (2017) 54–69, doi:<http://dx.doi.org/10.1016/j.sajb.2017.05.007>.
- [5] J.E. Parra, O.J. Patiño, J.A. Prieto, W.A. Delgado, L.E. Cuca, A new benzoic acid derivative isolated from *Piper* cf. *Cumanense* Kunth (Piperaceae), *Phytochem. Lett.* 6 (2013) 590–592, doi:<http://dx.doi.org/10.1016/j.phytol.2013.07.014>.
- [6] J. Parra, W. Delgado, L. Cuca, Cumanensic acid, a new chromene isolated from *Piper* cf. *cumanense* Kunth, Piperaceae), *Phytochem. Lett.* 4 (2011) 280–282, doi:<http://dx.doi.org/10.1016/j.phytol.2011.04.015>.
- [7] J. Parra, L. Cuca, A. González-Coloma, Antifungal and phytotoxic activity of benzoic acid derivatives from inflorescences of *Piper cumanense*, *Nat. Prod. Res.* (2019) 1–9, doi:<http://dx.doi.org/10.1080/14786419.2019.1662010>.
- [8] G. Garavito, J. Rincón, L. Arteaga, Y. Hata, G. Bourdy, A. Gimenez, R. Pinzón, E. Deharo, Antimalarial activity of some Colombian medicinal plants, *J. Ethnopharmacol.* 107 (2006) 460–462, doi:<http://dx.doi.org/10.1016/j.jep.2006.03.033>.
- [9] H.N. Matsuura, S. Malik, F. de Costa, M. Yousefzadi, M.H. Mirjalili, R. Arroo, A.S. Bhambra, M. Strnad, M. Bonfill, A.G. Fett-Neto, Specialized plant metabolism

- characteristics and impact on target molecule biotechnological production, *Mol. Biotechnol.* 60 (2017) 169–183, doi:<http://dx.doi.org/10.1007/s12033-017-0056-1>.
- [10] M. Ochoa-Villareal, S. Howat, S. Hong, M. Jang, Y. Jin, E. Lee, G. Loake, Plant cell culture strategies for the production of natural products, *BMB Rep.* 49 (2016) 149–158, doi:<http://dx.doi.org/10.5483/BMBRep.2016.49.3.264>.
- [11] H. Murthy, E. Lee, K. Paek, Production of secondary metabolites from cell and organ cultures: strategies and approaches for biomass improvement and metabolite accumulation, *Plant Cell Tissue Organ Cult.* (2014), doi:<http://dx.doi.org/10.1007/s11240-014-0467-7>.
- [12] P. Zhou, J. Yang, J. Zhu, S. He, W. Zhang, R. Yu, J. Zi, L. Song, X. Huang, Effects of β -cyclodextrin and methyl jasmonate on the production of vindoline, catharanthine, and ajmalicine in *Catharanthus roseus* cambial meristematic cell cultures, *Appl. Microbiol. Biotechnol.* 99 (2015) 7035–7045, doi:<http://dx.doi.org/10.1007/s00253-015-6651-9>.
- [13] M. Sarmadi, N. Karimi, J. Palazón, A. Ghassempour, M. Mirjalili, The effects of salicylic acid and glucose on biochemical traits and taxane production in a *Taxus baccata* callus culture, *Plant Physiol. Biochem.* 132 (2018) 271–280, doi:<http://dx.doi.org/10.1016/j.plaphy.2018.09.013>.
- [14] G. Delgado-Paredes, K. Massou, C. Rojas-Idrogo, Cellular suspension and production of secondary metabolites in *in vitro* cultures of *Piper* sp, *Boletín Latinoam. y Del Caribe Plantas Med. y Aromáticas.* 12 (2013) 269–282.
- [15] T. Balbuena, C. Santa-Catarina, V. Silveira, M. Kato, E. Floh, *In vitro* morphogenesis and cell suspension culture establishment in *Piper solmsianum* C. DC, Piperaceae, *Acta Bot. Brasílica.* 23 (2009) 274–281, doi:<http://dx.doi.org/10.1590/S0102-33062009000100029>.
- [16] N. Ahmad, B. Abbasi, I. Rahman, H. Fazal, *Piper nigrum*: micropropagation, antioxidative enzyme activities, and chromatographic fingerprint analysis for quality Control, *Appl. Biochem. Biotechnol.* 169 (2013) 2004–2015, doi:<http://dx.doi.org/10.1007/s12010-013-0104-7>.
- [17] V. Prajapati, M. Patel, S. Jha, K. Makwana, *De novo* organogenesis from leaf explants in *Piper longum* L, *J. Pharmacogn. Phytochem.* 8 (2019) 483–485.
- [18] L. Szabados, L.A. Mroginski, W.M. Roca, Suspensiones celulares: descripción, manipulación y aplicaciones, in: *cultiv. Tejidos En La Agric. Fundam. y Apl.*, Centro Int (1991) 174–195.
- [19] H. Heberle, G.V. Meirelles, F.R. Silva, G.P. Telles, R. Minghim, InteractiVenn: A Web-based Tool for the Analysis of Sets Through Venn Diagrams, (2015), pp. 1–7, doi:<http://dx.doi.org/10.1186/s12859-015-0611-3>.
- [20] RStudioTeam, RStudio, Integrated Development for R. Studio, (2020) . <http://www.rstudio.com>.
- [21] C. Giri, M. Zaheer, Chemical elicitors versus secondary metabolite production *in vitro* using plant cell, tissue and organ cultures: recent trends and a sky eye view appraisal, *Plant Cell, Tissue Organ Cult.* 126 (2016), doi:<http://dx.doi.org/10.1007/s11240-016-0985-6>.
- [22] Y. Wei, M. Qian-liang, L. Bing, R. Khalid, Z. Cheng-Jian, H. Ting, Q. Lu-ping, Medicinal plant cell suspension cultures: pharmaceutical applications and high-yielding strategies for the desired secondary metabolites, *Crit. Rev. Biotechnol.* 36 (2016) 215–232.
- [23] S. Kusari, V. Verma, M. Lamshoef, M. Spittler, An endophytic fungus from *Azadirachta indica* A. Juss. That produces azadirachtin, *World J. Microbiol. Biotechnol.* 28 (2012) 1287–1294, doi:<http://dx.doi.org/10.1007/s11274-011-0876-2>.
- [24] J. Ludwig-Müller, Plants and endophytes: equal partners in secondary metabolite production, *Biotechnol. Lett.* 37 (2015), doi:<http://dx.doi.org/10.1007/s10529-015-1814-4>.
- [25] R. Nicoletti, A. Fiorentino, Plant bioactive metabolites and drugs produced by endophytic fungi of spermatophyta, *Agriculture* 5 (2015) 918–970, doi:<http://dx.doi.org/10.3390/agriculture5040918>.
- [26] K. Subban, R. Subramani, V. Priya, M. Srinivasan, M. Johnpaul, C. Jayabaskaran, Salicylic acid as an effective elicitor for improved taxol production in endophytic fungus *Pestalotiopsis microspora*, *PLoS One* (2019) 1–17, doi:<http://dx.doi.org/10.1371/journal.pone.0212736> February.
- [27] N. Rustamova, K. Bozorov, T. Efferth, A. Yili, Novel secondary metabolites from endophytic fungi: synthesis and biological properties, *Phytochem. Rev.* 19 (2020) 425–448, doi:<http://dx.doi.org/10.1007/s11101-020-09672-x>.
- [28] Z. Cai, A. Kastell, D. Knorr, I. Smetanska, Exudation: an expanding technique for continuous production and release of secondary metabolites from plant cell suspension and hairy root cultures, *Plant Cell Rep.* 31 (2012) 461–477, doi:<http://dx.doi.org/10.1007/s00299-011-1165-0>.
- [29] C. Lambert, J. Lemaire, H. Auger, A. Guilleret, R. Reynaud, C. Clément, E. Courot, B. Taidi, Optimize, modulate, and scale-up resveratrol and resveratrol dimers bioproduction in *Vitis labrusca* L. cell suspension from flasks to 20 L bioreactor, *Plants* 8 (2019), doi:<http://dx.doi.org/10.3390/plants8120567>.
- [30] M. Narayani, S. Srivastava, Elicitation: a stimulation of stress in *in vitro* plant cell/tissue cultures for enhancement of secondary metabolite production, *Phytochem. Rev.* 16 (2017) 1227–1252, doi:<http://dx.doi.org/10.1007/s11101-017-9534-0>.
- [31] A.P. Danelutte, M.B. Costantin, G.E. Delgado, R. Braz-Filho, M.J. Kato, Divergence of secondary metabolism in cell suspension cultures and differentiated plants of *Piper cernuum* and *P. Crassinervium*, *J. Braz. Chem. Soc.* 16 (2005) 1425–1430, doi:<http://dx.doi.org/10.1590/S0103-50532005000800022>.
- [32] L.D. Hieu, T.D. Thang, T.M. Hoi, I.A. Ogunwande, Chemical composition of essential oils from four vietnamese species of *Piper* (Piperaceae), *J. Oleo Sci.* 63 (2014) 211–217, doi:<http://dx.doi.org/10.5650/jos.ess13175>.
- [33] E. Kpomah, D. Monday, B. Kpomah, GCMS analysis of leaves and seeds of *Piper guineense* shumach & thoon, *African Sci.* 20 (2019) 127–138.