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The metabolomics reveals intraspecies variability of bioactive compounds in elicited suspension cell cultures of three *Bryophyllum* species

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ABSTRACT

In this work, the combination of elicited plant suspension cultured cells (PSCCs) with untargeted metabolomics establishes a powerful, cutting-edge strategy to unravel the effects of elicitors on the biosynthetic potential of medicinal plants, as *Bryophyllum* sp. The PSCC technology constitutes a successful biotechnological system for the study and production of bioactive compounds throughout the inclusion of elicitors with the ability to modulate secondary metabolism. The use of methyl jasmonate (MJ) and salicylic acid (SA) as abiotic elicitors on bryophyllum PSCCs, resulted in differential effects on cell growth and secondary metabolism, depending on the species, including synergistic and antagonistic effects. This fact suggests that both elicitors play a pleiotropic effect on plant secondary metabolism, showing complex interactions, according to the UHPLC-QTOF mass spectrometry profiling. Thus, the combination of both elicitors induced a strong synergistic on *B. daigremontianum* PSCCs, with 2272 putatively annotated compounds, whereas it caused a negative effect on the secondary metabolism of *B.* × *houghtonii* PSCCs, being MJ the only elicitor driving a positive effect, presenting 2972 annotated compounds. Meanwhile, *B. tubiflorum* PSCCs did not show a significant modulation of secondary metabolism, with 1521 annotated compounds. The metabolite annotation indicated that three families of secondary metabolites were mainly affected by elicitation: phenolic compounds constituted the most affected family by elicitation, mainly represented by flavonoids and lignans; N-containing compounds included glucosinolates, amines, and alkaloids, reported to *Bryophyllum* sp. for the first time; and terpenoids included mainly phytoalexins and saponins. The results depict a deep genotype-dependent metabolomic reprogramming of secondary metabolism in response to elicitors, thanks to the application of untargeted metabolomics. This knowledge will allow the consideration of *Bryophyllum* sp. as a valuable source of bioactive compounds, with the potential associated to PSCCs, for being included in food, cosmetic, and pharmaceutical applications.

1. Introduction

Plants constitute a reliable source of bioactive compounds which impact is reflected in the fact that a quarter part of drugs approved currently by US Food and Drug Administration (FDA) has a plant-based origin (Marchev et al., 2020). Thus, in the last decades, increasing interest regarding medicinal plants has focused on the design of biotechnology-based strategies to enhance the production of bioactive compounds with health-enhancing properties, acting as antimicrobial, antioxidant, and anticancer agents (Ahmadu and Ahmad, 2021; Kumar et al., 2020). Among the wide variety of medicinal plant species known nowadays, *Bryophyllum* constitutes a subgenus within the *Kalanchoe* genus (Crassulaceae family), well-known for its associated phytochemical properties, largely exploited in traditional medicine across Africa, Asia, and South America (García-Pérez et al., 2019a), and its constitutive species are commonly known as "bryophyllum" (García-Pérez et al., 2020a). As medicinal plants, different bryophyllum-derived formulations have been successfully used for the treatment of chronic ailments, such as diabetes, infections, and neurological, cardiovascular, and neoplastic diseases (García-Pérez et al., 2020a). Phytochemical and pharmacognostical analyses have highlighted that such bioactivities are mainly driven by two families of secondary metabolites: phenolic

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compounds and bufadienolides (Stefanowicz-Hajduk et al., 2020a). However, being secondary metabolites, they are found in very scarce amounts within plant tissues and their biosynthesis is highly influenced by pedoclimatic conditions (García-Pérez et al., 2020a), so new alternative strategies should be undertaken to overcome the limitations associated with the characterization of the phytochemical potential of *Bryophyllum* sp.

Although secondary metabolites do not participate directly in the maintenance physiology of plant organisms, they contribute to their adaptation to environmental conditions and protection against different threats (Tajner-Czopek et al., 2020). Secondary metabolism is tightly linked to plant stress, so novel approaches in plant bioactive compounds research should be addressed to the efficient establishment of plant stress under controlled conditions. To that aim, plant tissue culture stands out as a reliable methodology, additionally enabling the protection against pathogenic and climatic threads (García-Pérez et al., 2020a). More specifically, plant suspension cultured cells (PSCC) technology gained much attention in the last decades as the most convenient biotechnological tool for the production of secondary metabolites throughout the inclusion of elicitors (Moon et al., 2020). Elicitors are stress inductive agents, from both biotic and abiotic origins, with the ability of causing significant changes in the expression of plant defense-related genes, which lead to the accumulation of secondary metabolites (Salehi et al., 2019). This way, elicitation constitutes the most effective approach for secondary metabolite production to meet the current biotechnological requirements for the industrial exploitation of plant by-products with added-value (Eibl et al., 2018; Ramirez-Estrada et al., 2016). Among the great variety of elicitors applied to PSCCs, jasmonates and salicylates have been widely used for the production of bioactive compounds (Naik and Al-Khayri, 2016). These abiotic elicitors develop hormone signaling responses faced to the induction of several pathogenesis-related gene families, tightly associated to secondary metabolite biosynthesis throughout the induction of an integrative multicomponent network mediated by sequential signal transduction cascades (Irigoyen et al., 2020).

Methyl jasmonate (MJ) is a naturally-occurring phytohormone, which has been reported as the most elicitor used in PSCCs for the accumulation of secondary metabolites (Giri and Zaheer, 2016). Concerning its mechanism of action, MJ is a pleiotropic signal molecule considered as a vital cell regulator, due to its role on a plethora of both developmental events, such as germination, fertility, and senescence, and plant defense-associated processes, closely related to the modulation of plant responses against abiotic and biotic stresses (Yu et al., 2019). Another phytohormone, salicylic acid (SA), is also considered a key regulator on plant resistance against pathogens, as it is involved in the activation of systemic acquired resistance (SAR) system in plants (Tripathi et al., 2019). The interaction between both elicitors remains unclear, since differential results have been obtained on cell systems and other molecules, such as other phytohormones and chemical agents, may interfere with their mechanisms of action (Thakur et al., 2019). Nevertheless, the combination of elicitors is an outstanding approach that has been successfully applied to PSCCs because it promotes some advantages, as the occurrence of synergistic effects that enhance secondary metabolite production, and a more comparable system to real conditions, in which plants must face to several stresses at the same time (Li et al., 2019).

The accumulation of plant secondary metabolites by PSCCs is mainly driven by stress-induced stimuli, which lead to both quantitative and qualitative changes in the metabolite pool of these biological *in vitro* systems. Hence, the identification of the set of biosynthesized metabolites is critical to understand the biochemical status induced by stress. As a solution, metabolomics constitutes a powerful high throughput technology with the capability of analyzing the broad metabolic stage of biological samples under different conditions, throughout the identification and quantification of all their metabolites (Rocchetti et al., 2018; Zhang et al., 2020). More precisely, the application of untargeted metabolomics drives to the characterization of the metabolic fingerprinting associated to a given process (Ghatak et al., 2018), as it is the case of elicitation, in order to depict a deep and integrative approach of its effect on bryophyllum PSCCs. In addition, untargeted metabolomics constitutes a holistic, rational approach to be applied when dealing with poorly studied plant species (Hasanpour et al., 2020), such as those belonging to *Bryophyllum* subgenus., as it allows the annotation of compounds from different metabolite families at the same time, thus providing highly valuable information throughout the characterization of the metabolic profiling of many species (Marchev et al., 2020).

In this work, a combinatorial approach, including the elicitation of PSCCs with untargeted metabolomics will be applied to decipher the effect of two abiotic elicitors, MJ and SA, on the secondary metabolism of bryophyllum cell suspension cultures from three unexplored different species widely used in ethnomedicine: *Bryophyllum daigremontianum* Raym.-Hamet et Perr., *Bryophyllum* × *houghtonii* D.B. Ward, and *Bryophyllum tubiflorum* Harv. Overall, the metabolic fingerprinting obtained will shed light on the elicitation of the secondary metabolism of these unexploited medicinal plant species, providing insight about their biotechnological potential, facing their large-scale applications in different sectors, such as food, cosmetic and pharmaceutical industries.

2. Materials and methods

2.1. Plant material and in vitro culture establishment

Epiphyllous plantlets from three different species were used for the establishment of *in vitro* culture: *Bryophyllum daigremontianum* Raym.-Hamet et Perr. (BD), *Bryophyllum* × *houghtonii* D.B. Ward (*Bryophyllum daigremontianum* × *tubiflorum*, BH), and *Bryophyllum tubiflorum* Harv. (BT). Plantlets were harvested from a local greenhouse, disinfected, and micropropagated, as previously reported (García-Pérez et al., 2019b).

Leaves from 12-week-old bryophyllum plants cultured *in vitro* were subjected to callus induction using six different culture media formulations based on MS basal medium (Murashige and Skoog, 1962), as indicated elsewhere (García-Pérez et al., 2020b, 2020c; Pandey et al., 2019). Full strength MS medium and half-macronutrient MS (1/2MS) were combined with three different phytohormonal concentrations, including the auxin 2,4-dichlorophenoxyacetic acid (2,4-D) at the concentrations 0.5 and 1 mg L^{-1}; and the cytokinin 6-benzylaminopurine (BAP) at the concentrations 0.5, 1, and 2 mg L^{-1}. All media were supplemented with 3% (w/v) sucrose, solidified with 0.8% (w/v) agar at pH = 5.8 and autoclaved at 121 °C and 1.1 atm for 20 min. Finally, autoclaved media were plated by 20-mL aliquots into Petri dishes.

Foliar disks (~1 cm^2) were excised from *in vitro*-cultured plants and placed by groups of six explants onto Petri dishes. Five dishes were used for each species and medium and they were transferred to a growth chamber in the dark at 25 °C for 4 weeks. After this induction period, callus formation was determined by means of health and consistency parameters since healthy, friable calli are required for further experiments. Calli were subcultured every 21 days and after three subcultures, optimal media composition to get healthy and friable calli were selected: MS supplemented with 0.5 mg L^{-1} 2, 4-D and 1 mg L^{-1} BAP was selected for BD, 1/2MS supplemented with 0.5 mg L^{-1} 2, 4-D and 1 mg L^{-1} BAP was selected for BH and MS supplemented with 1 mg L^{-1} 2, 4-D and 0.5 mg L^{-1} BAP was selected for BT (García-Pérez et al., 2019c).

2.2. Establishment of plant cell suspension culture

Bryophyllum plant cell suspension cultures (PSCCs) were initiated as previously described (García-Pérez et al., 2019c) by transferring 3-week-old calli into 250-mL Erlenmeyer flasks with 50 mL of the same culture media used for callus induction without agar at an initial cellular density of 100 g of fresh cells per liter. Suspension cultures were transferred to an orbital shaker (Labwit ZWY-111C, Labwit® Australia)

at 25 °C and 120 rpm in the dark and were subcultured every 9 days by the half-dilution method, being used as batch cultures for the subsequent experiments.

2.3. Elicitation experiments

Two abiotic elicitors were used for elicitation experiments: methyl jasmonate (MJ) at the concentrations 0, 50, and 100; and salicylic acid (SA) at the concentrations 0, 100, 500, and 1000 μM, that made a total of 12 treatments. Both elicitors are considered the most largely applied to PSCCs for the production of secondary metabolites, either independently or in combination, at the concentrations tested in this study (Giri and Zaheer, 2016). Stock solutions of both elicitors were prepared in ethanol at the concentrations of 1 M MJ and 0.1 M SA. Both solutions were filter sterilized using 0.22 μm-pore size syringe filters and stored at 4 °C until use.

Eight-day-old Bryophyllum PSCCs (3th subculture) were subjected to elicitation experiments, as it represents the time required by suspension cultures to reach the stationary phase, as previously determined (García-Pérez et al., 2019c). Specifically, batch cultures were filtered in vacuum and the cellular fraction was transferred to 100-mL Erlenmeyer flasks containing 20 mL of fresh medium, maintaining the same cellular density of 100 g L^{-1}. Later, the elicitors were incorporated, and cultures were incubated under the same conditions described above. Three replicates were used for each treatment and species.

Afterwards, cell growth parameters were determined. For the purpose, cell fractions were filtered, separated from the culture medium, and immediately stored at −20 °C. Once frozen, cells were lyophilized, mechanically powdered, and weighted to determine their dry biomass, which was expressed as mg of dry weight. In parallel, the culture medium was collected and used for the determination of conductivity (using an electrical conductivity meter CM 35+, Crison®), expressed as mS cm^{-1}, in order to monitor nutrient consumption.

2.4. Sample extraction

Lyophilized cell samples were extracted with the solvent mixture MeOH/HCOOH/H_2O (80/0.1/19.9, v/v/v) at a concentration of 20 mg mL^{-1} and homogenized using a high-speed rotor (Polytron PT 1600-E) for two minutes. Samples were then centrifuged at 8000 × *g* for 10 min at 4 °C (Eppendorf 5810R, Hamburg, Germany). Finally, the supernatants were collected and filtered with 0.22 μm syringe filters, and extracts were transferred into vials for analysis or stored at −20 °C until use.

2.5. Metabolite screening via untargeted metabolomics

Plant metabolites were subjected to screening through an untargeted metabolomics approach using a hyphenated system based on a quadrupole-time-of-flight (QTOF) mass spectrometer coupled to an ultra-HPLC chromatographic system (UHPLC/Q-TOF), as indicated in previous works (Lucini et al., 2018). Briefly, a JetStream electrospray ionization source and a G6550 QTOF coupled to a 1290 ultra-high-performance liquid chromatograph (Agilent technologies, Santa Clara, CA, USA) were used. The chromatographic separation was achieved by a reverse phase Agilent PFP column (2.0 × 100 mm, 3 μm), and a binary gradient was applied combining water and acetonitrile (from 6% to 94% organic phase in 33 min, flow rate 200 μL min^{-1}). The mass spectrometer was set in positive polarity and SCAN mode, selecting the extended dynamic range mode in the range 100−1200 *m/z*. Injection volume was 6 μL. Quality control pooled samples (QCs) were also analyzed in MS/MS mode (data-dependent, 12 precursors per cycle at 1 Hz, 50–1000 *m/z*, positive polarity, active exclusion after 2 spectra), with collision energies of 10, 20, and 40 eV.

The annotation of compounds was achieved by their monoisotopic accurate mass and isotopes patterns (accurate spacing and isotopes ratio) and expressed by means of overall identification score. Metabolite annotation was performed by the Profinder B.07 software tool (Agilent Technologies, USA), using the database exported from PlantCyc 9.6 (Plant Metabolic Network, available in http://www.plantcyc.org). Identification was carried out in compliance with Level 2 (putatively annotated compounds) as set out by the COSMOS Metabolomics Standards Initiative (http://cosmos-fp7.eu/msi). MS-DIAL 3.98 software provided a higher degree of confidence in annotation using MS/MS spectra of QCs to confirm features obtained by Profinder B.07 (Tsugawa et al., 2015). For this purpose, publicly available MS/MS experimental spectra built in the software (e.g., MoNA) were used.

2.6. Statistical analysis

Metabolomics-derived data were statistically analyzed and interpreted by the Mass Profiler Professional v. 12.6 software tool (Agilent Technologies, USA). Compounds abundances were transformed into their log2 values and normalized at the 75th percentile before the establishment of a baseline against the median values obtained for each compound in all samples, as reported previously (Salehi et al., 2018). Next, multivariate unsupervised hierarchical cluster analysis (HCA) was performed (Euclidean distance, Ward's linkage rule) to describe similarities and dissimilarities among treatments by the construction of a fold change heat map from compounds abundance. In addition, supervised modeling by Orthogonal Projection to Latent Structure Discriminant Analysis (OPLS-DA) was performed by SIMCA 16 software tool (Umetrics, Sweden) in order to discriminate the effect of elicitation among treatments, according to their metabolic fingerprint, and to further identify markers responsible of the discrimination throughout the analysis of variable importance in projection (VIP). The obtained multivariate model was then cross-validated by Cross Validation-Analysis of Variance (CV-ANOVA, $\alpha = 0.05$) and its fitness and prediction were evaluated by R^2Y and Q^2Y parameters, respectively, assuming a threshold of >0.5 for Q^2Y. In order to highlight those compounds with the highest discriminant contribution, metabolites were selected according to their VIP score (>1.30). Finally, a Volcano analysis ($\alpha = 0.05$, Benjamini-Hochberg multiple testing correction; fold change, FC > 2) was carried out and the output results were imported to PlantCyc Pathway Tools software (Karp et al., 2009) to computationally detect the biosynthetic pathways involved in the metabolic response against elicitation treatments.

Cell growth and conductivity data from PSCCs were analyzed by one-way ANOVA followed by Duncan's post-hoc test ($\alpha = 0.05$). Pearson's correlation was performed to correlate the data obtained for dry biomass and conductivity ($\alpha = 0.001$). In both cases, the software SPSS 25 was used (IMB Corporation, 2017).

2.7. Chemicals and reagents

All chemicals for the development of plant tissue and cell cultures and elicitation experiments were of the highest purity available and supplied by Sigma Aldrich. All chemicals used for extraction and chromatographic separations were LC–MS grade and supplied by VWR (Milan, Italy).

3. Results

3.1. Effect of abiotic elicitors, MJ and SA, on Bryophyllum PSCCs growth

The results obtained for bryophyllum PSCCs growth are shown in Fig. 1. Results for dry biomass indicate that BH cell line presented a ~60% higher growth than BD and BT in all treatments (Fig. 1A). Concerning different treatments, the BH line did not show any alteration in growth under low concentration elicitor treatments, 100 μM SA and 50 μM MJ (Fig. 1A). In the case of BD, the lowest MJ treatment (50 μM) caused a significant increase in cell growth, whereas SA caused a

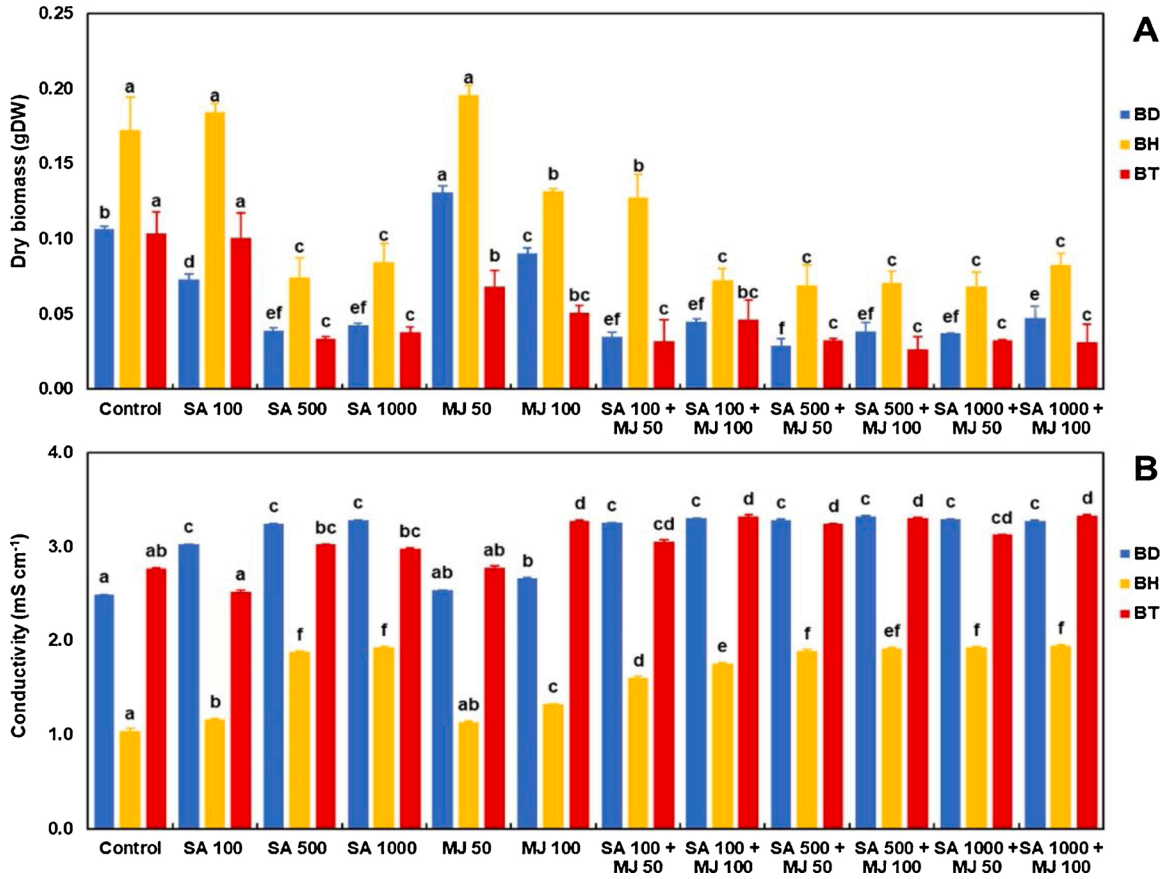

Fig. 1. A. Biomass accumulation of Bryophyllum cell suspension cultures (BH, BD and BT) elicited with salicylic acid (SA) and methyl jasmonate (MJ), expressed as g of dry weight, gDW. **B.** Conductivity of culture medium after elicitation, expressed as mS cm^{-1}. In both cases, elicitor concentrations are expressed in μM. Vertical bars indicate the standard deviation of three independent replicates. Different letters indicate significant differences between treatments within the same species ($\alpha = 0.05$).

decrease in all cases, proportional to SA concentration ($p < 0.05$). Furthermore, BD cell lines showed a similar growth independently of elicitor concentrations between all combined treatments (Fig. 1A). Finally, for BT cell line, the lowest SA treatment (100 μM) did not affect cell growth, as similar values were observed with respect to control. In contrast, the lowest MJ concentration caused a decrease in cell growth, which was emphasized by the combined MJSA treatments (Fig. 1A).

Overall, the results for conductivity reflected that the highest rates of cell growth were associated with the lowest conductivity values ($p < 0.05$; Fig. 1B). Both dry biomass and conductivity presented a highly negative correlation ($\alpha = 0.001$), with Pearson's coefficients of -0.94, -0.94, and -0.75 for BD, BH and BT, respectively. This indicates that plant cell growth was accompanied by the consumption of the mineral nutrients presented in the culture medium, thus suggesting that bryophyllum PSCCs constitute an effective biological system for biomass accumulation. It is noteworthy that BH cell lines had lower conductivity values because of the composition of culture medium employed, MS medium containing half-macronutrient concentrations, as stated earlier. Due to the differential effect found for biomass accumulation, the treatments containing 100 μM SA and 50 μM MJ and their combinations, were selected for the subsequent metabolomic analysis, together with the control.

3.2. Elicitation-mediated metabolic fingerprinting of bryophyllum PSCCs by UHPLC/Q-TOF-MS

The untargeted metabolomic analysis by UHPLC/Q-TOF-MS was performed, aiming at deciphering the effects of elicitation on bryophyllum PSCCs. The total compound annotation accounted for 2272 compounds associated to BD, 2972 for BH, and 1521 for BT cell lines, that were used for the subsequent modeling and statistical analysis. The whole compound list obtained after annotation was provided as supplementary material, also including the abundance and composite mass spectra of each metabolite and the list of metabolites identified by MS/MS (Table S1). The first approach applied was an unsupervised fold change-based hierarchical cluster analysis (HCA) in order to achieve a description of the relations between treatments, based on their metabolic profiles. As stated above, treatments were identified as control (no elicitors), SA (100 μM SA), MJ (50 μM MJ), and MJSA (combining 100 μM SA with 50 μM MJ). The results for the HCA were shown in Fig. 2.

In the case of BD, the combined MJSA treatment was grouped apart from the rest presenting a vast number of compounds up-accumulated, with respect to other treatments (red region, Fig. 2A). The results for BH (Fig. 2B) showed a different pattern for the distribution of treatments, grouping apart SA from the rest of them. Finally, concerning BT, the results showed that all treatments were separated from the control, which exhibited a strong down-regulation (Fig. 2C).

Afterwards, in order to determine the net differences between the control and treatments, the supervised Orthogonal Projection of Latent Structures Discriminant Analysis (OPLS-DA) was performed for each species. The results for the OPLS-DA for BD and BH are shown in Figs. 3A and 3B, respectively. In contrast, in the case of BT, the corresponding model showed low predictability, making impossible its generation.

The supervised modeling of results for BD (Fig. 3A) demonstrated that all treatments were statistically different between them and the control, showing high values for linearity between the experimental and predicted results ($R^2X = 0.807$), which allowed the construction of a highly predictive model ($Q^2Y = 0.751$). Similar results were obtained

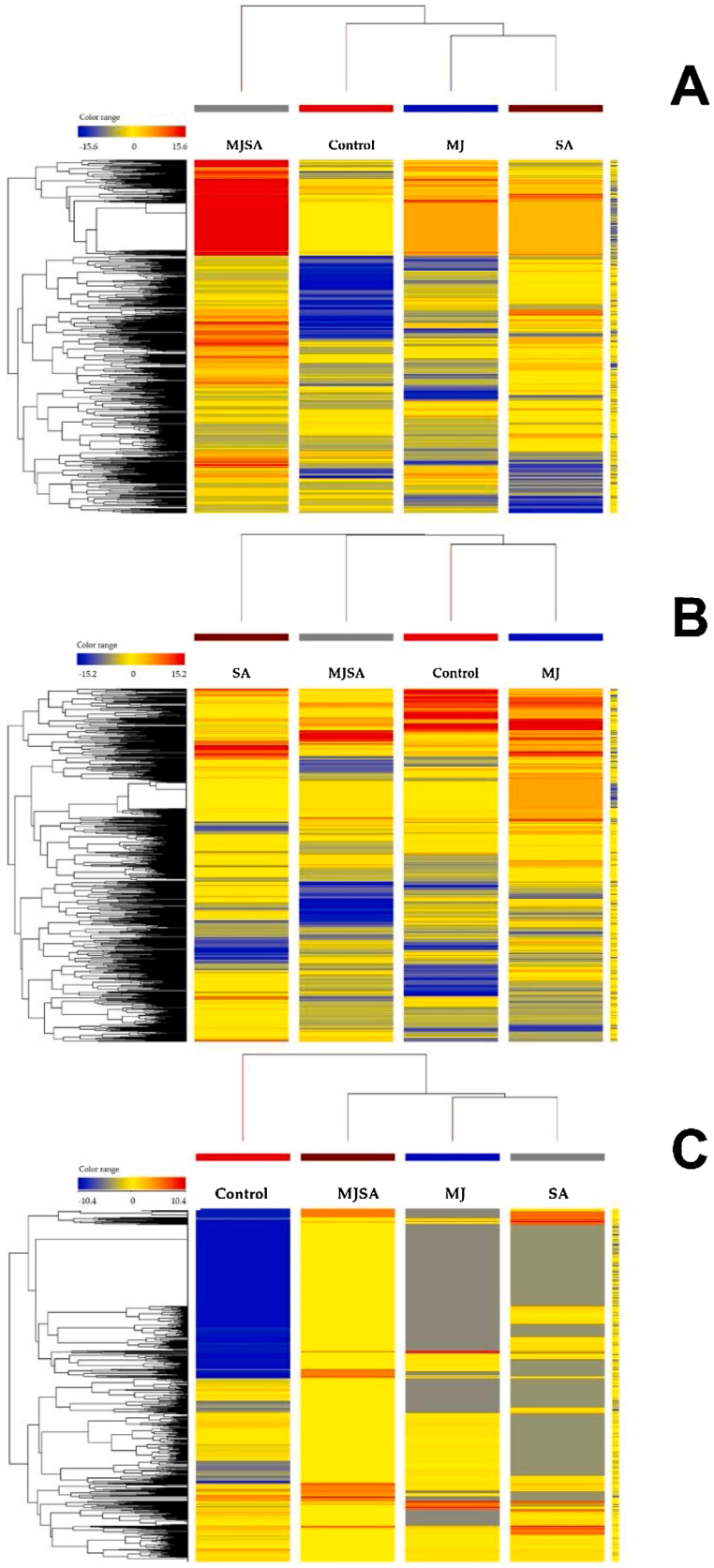

Fig. 2. Unsupervised HCA performed for **A.** BD, **B.** BH, and **C.** BT PSCCs elicited with MJ and SA and their combination. A fold-change based heatmap was built and samples were clustered according to Ward's algorithm, based on Euclidean distances.

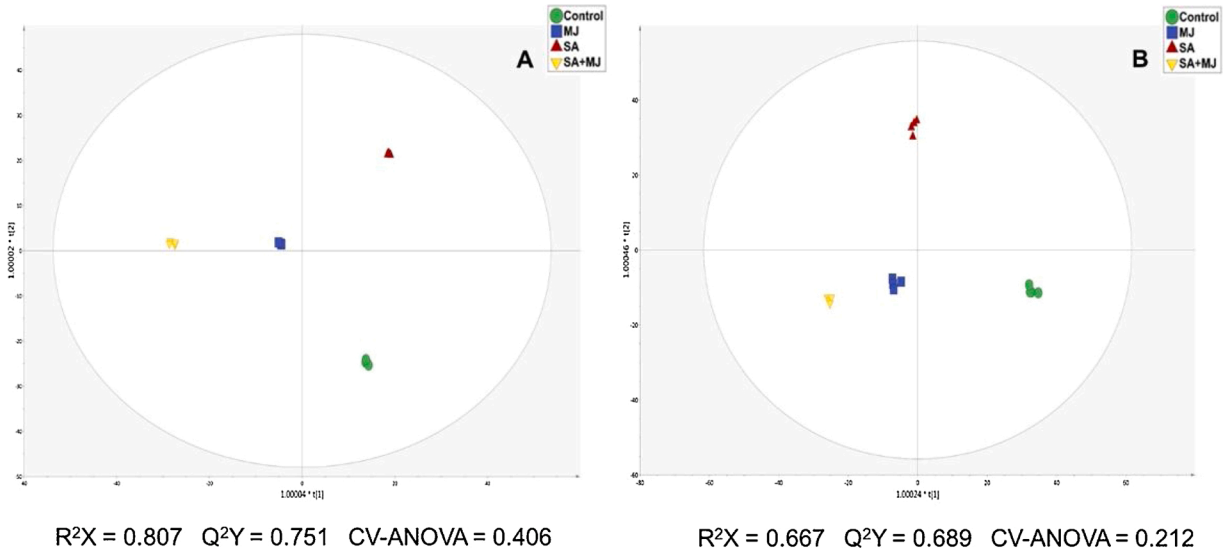

Fig. 3. OPLS-DA on the metabolomic profile of elicited Bryophyllum PSCCs (● - Control; ■- MJ; ▲- SA; ▼- MJSA). **A.** PSCCs from BD. **B.** PSCCs from BH.

for BH (Fig. 3B), whose different treatments were predicted as independent among them and the untreated control, together with a built model with high linearity and predictability values ($R^2X = 0.667$ and $Q^2Y = 0.689$, respectively). In both cases, statistical differences between experimental and predicted results were evaluated by CV-ANOVA, revealing that no significant differences were observed ($p > 0.05$). Overall, the results from both supervised and unsupervised analyses indicate that MJ and SA play an effective role as elicitors on PSCCs from BD and BH, which is differential among them on every species. Furthermore, the combination of both elicitors showed a species-dependent effect, whereas no significant elicitation effects were observed on BT PSCCs at the selected concentrations (Figs. 2 and 3).

Table 1
Discriminant metabolites identified by VIP (Variable Importance in Projection) selection method from multivariate supervised OPLS-DA for BD elicited PSCC.

Class	Compounds[a]	VIP Score	LogFC MJ vs control	LogFC SA vs control	LogFC MJSA vs control
Phenolic compounds					
Phenolic acids	1-*O*-sinapoyl-β-D-glucose	1.52 ± 0.90	7.70	0.37	−3.30
Flavonols	myricetin 3-*O*-gentiobioside	1.44 ± 0.42	8.24	1.61	−1.61
Flavones	wogonin 7-*O*-β-*D*-glucoside	1.30 ± 1.21	16.21	4.08	11.06
Isoflavones	dalcochinin glucoside	1.46 ± 1.00	7.82	−13.87	−2.42
	ononin	1.42 ± 1.24	6.91	−1.72	−0.10
	(-)-maackiain-3-O-glucoside	1.33 ± 0.79	7.66	0.59	−2.72
	calycosin 7-*O*-glucoside	1.30 ± 1.21	16.21	4.08	11.06
Pterocarpans	(-)-phaseollidin	1.47 ± 0.42	8.42	1.32	11.29
Stilbenes	resveratrol monomethyl ether	1.33 ± 0.49	−2.11	4.07	0.39
Lignans	(7S)-*trans*-hinokiresinol	1.36 ± 1.13	4.01	−6.46	0.61
	(+)-secoisolariciresinol diglucoside	1.31 ± 0.54	−7.87	−4.37	−8.88
Alkaloids					
Indole alkaloids	5α-carboxystrictosidine	1.49 ± 0.64	12.01	10.14	0.65
Isoquinoline alkaloids	(S)-stylopine	1.63 ± 0.52	8.44	4.24	6.81
	hemanthamine	1.32 ± 0.32	−15.17	−2.32	−4.93
Glucosinolates					
Aliphatic glucosinolates	gluconapin	1.35 ± 0.70	5.60	23.74	15.83
	(*E*)-7-(methyltsulfanyl)heptanal oxime	1.34 ± 0.57	1.29	0.64	20.01
	(+)-*trans*-isoalliin	1.32 ± 1.09	8.13	0.99	11.86
	(E)-9-(methylthio)nonylhydroximoyl-glutathione	1.30 ± 0.74	−6.62	−3.25	−2.58
Glucosinolate activation	indol-3-ylmethyl-glutathione	1.46 ± 0.56	11.65	4.85	5.76
Terpenoids					
Sesquiterpenoids	artemisinic aldehyde	1.31 ± 0.54	−5.96	−2.48	−5.78
Saponins	hederagenin	1.36 ± 0.90	1.31	−17.52	3.35
Carotenoids	10′,6-diapocarotene-10′,6-dial	1.34 ± 0.69	6.41	−9.87	1.58
Phytoalexins					
	furcatin	1.66 ± 0.66	10.76	4.53	5.45
	uniconazole-P	1.54 ± 0.91	7.64	−1.02	10.55
	juvenile hormone III	1.49 ± 0.52	21.01	4.67	11.18
	gossypol	1.40 ± 0.37	26.64	20.29	16.18
Amino acids and derivatives					
	N-benzoyl-L-glutamate	1.48 ± 0.57	7.44	−0.16	3.81
	L-α-amino-ε-keto-pimelate	1.40 ± 1.27	24.88	4.61	20.37
	Se-methyl-Se-L-methionine	1.34 ± 0.38	0.54	12.50	14.26
	D-erythro-1-(imidazol-4-yl)-glycerol 3-phosphate	1.33 ± 0.36	0.95	12.57	23.05

[a] Compounds present their corresponding VIP score values (> 1.30) and the logarithm of fold change (logFC) for each treatment with respect to control.

Additionally, with the aim of identifying the metabolites showing the highest contribution to discrimination among treatments, their significant VIP scores were obtained for BD (Table 1) and BH (Table 2), together with their fold change values of each treatment with respect to control. According to the results, different secondary metabolite families were represented for the discrimination caused by elicitation on bryophyllum PSCCs: phenolic compounds, alkaloids, glucosinolates, terpenoids, phytoalexins and amino acid derivatives (Tables 1 and 2). Phenolic compounds accounted for the most part of discriminant compounds that were mainly defined by phenolic acids, flavonoids and anthocyanins glycosides. Concerning N-containing compounds, alkaloids, glucosinolates, and amines were mainly selected. Regarding terpenoids, different subfamilies were involved in the discrimination, including sesquiterpenoids, saponins, and carotenoids. Finally, different phytoalexins and amino acid derivatives were also highlighted as discriminant compounds in both cases. In addition, in the case of BH, the lipid and hormone profiles were also affected by elicitation (Table 2).

Finally, in order to provide insight into the effect of elicitation on the metabolite pool of bryophyllum PSCCs, a Volcano analysis was performed with the aim of achieving the metabolic fingerprint caused by each elicitor and their combination, throughout the construction of biosynthesis plots, where the effect of every treatment with respect to control was depicted (Figs. 4 and 5). The entire list of compounds derived from Volcano analysis is provided as Supplementary Table S2 and Table S3 for BD and BH, respectively.

The Volcano analysis performed for BD elicited PSCCs revealed that secondary metabolism is strongly stimulated under elicitor treatments, following an increasing effect: MJSA > SA > MJ, suggesting that the combined MJSA treatment caused the maximum effect driven by the synergistic effect between the two elicitors (Fig. 4A). It is noteworthy that all treatments caused a positive effect on secondary metabolism with respect to control, by inducing the biosynthesis of mainly 3 families of secondary metabolites, starting by the most induced: phenylpropanoid derivatives, N-containing metabolites (including alkaloids, glucosinolates, and polyamines) and terpenes (Fig. 4B).

Among the phenylpropanoid derivatives that showed an elicited accumulation, the ones showing the highest induction were flavonoids and quinones, maintaining the same synergistic effect found for MJSA. Flavonoids showing the strongest increase were flavones, such as baicalin, and apigenin, chrysin, and isovitexin glycosides, meanwhile the most elicited quinones were lawsone and juglone (Table S2). In addition, SA independently boosted the biosynthesis of another flavonoid subfamilies, including anthocyanidins, mainly pelargonidin and peonidin derivatives (Table S2).

N-containing compounds were the second group of secondary metabolites showing the strongest up-regulation caused by elicitation. In this group, SA and the combined MJSA treatment (Fig. 4B), showed a similar effect on the biosynthesis of these compounds. The synergistic effect found between MJ and SA for the accumulation of alkaloids from different biosynthetic origins, including indoleterpenic alkaloids and precursors, such as tabersonine and 16-epivellosimine, and benzylisoquinoline alkaloid precursors, such as N-formyldemecolcine and (*S*)-N-methylcoclaurine was reported (Fig. 4B; Table S2). On the other hand, SA itself caused the up-regulation of either benzylisoquinoline alkaloid precursors, such as (*S*)-nandinine, and non-common alkaloid precursors. In contrast, all steps related to glucosinolate metabolism were also up-regulated by both SA and MJSA, including the accumulation of precursors for aliphatic glucosinolates: 1-(methylsulfanyl)-7-aci-nitro-heptane, 3-[(3′-methylsulfanyl)propyl]malate, and (*S*)-3-methyl-2-oxopentanoate; true aliphatic glucosinolates: glucoiberverine and gluconapin; and products derived from glucosinolate activation, such as dhurrin, taxiphyllin, and p-hydroxymandelonitrile. Finally, amines were mainly represented by spermidine and folic acid precursors, such as 5-[[4-methoxy-3-(phenylmethoxy)phenyl]methyl]-2,4-pyrimidinediamine (Table S2).

Terpene biosynthesis was also elicited following the same increasing pattern MJSA > SA > MJ. However, elicitation was performed differently by these treatments on this family. Thus, MJ was responsible for the up-regulation of terpenoid biosynthesis with a low number of isoprene subunits, mainly monoterpenoids, such as geranyl acetate, and sesquiterpenoids, such as gossypol (Table S2). In turn, both SA and MJSA affected terpenoid biosynthesis with high number of isoprene subunits: diterpenoids, mainly phytoalexins, such as kauralexin B2 and their precursors; and triterpenoid precursors, as squalene (Table S2).

The biosynthesis plots obtained from the results for the elicitation of BH PSCCs are shown in Fig. 5. As it can be easily observed, a different metabolic fingerprint was obtained for BH PSCCs, being MJ the only elicitor causing a positive effect on secondary metabolism (Fig. 5A). In addition, MJ caused a positive induction of fatty acid and lipid biosynthesis, thus indicating that elicitation by MJ had a more integrative effect on this species, as stated above. Lipid metabolism was predominantly focused on glycolipid and phospholipid biosynthesis, which can be related to the higher growth rate found for BH PSCCs (Fig. 1A). Secondarily, the modulation of lipid metabolism also conferred the production of relevant volatile organic compounds (VOCs), represented by (*Z*)-3-hexanal and (*E*)-2-hexenol (Table S3; Fig. 5A).

Focusing on secondary metabolism, the same compound families as in BD PSCCs were the most affected by elicitation: phenylpropanoid derivatives, N-containing compounds, and terpenes, following also the same order than for BD (Fig. 5B). Among them, the metabolism of phenylpropanoids and N-containing metabolites was repressed by SA and a synergistic negative effect was found for the elicitor combination, MJSA. This inhibitory effect was also reported to polyketide biosynthesis.

Concerning phenylpropanoids, anthocyanidin, flavone, and flavonol glycosides, and coumarins were up-regulated by MJ (Table S3). Anthocyanidins were mainly represented by cyanidin and delphinidin glycosides. Isovitexin and kaempferol glycosides were the representative metabolites for flavones and flavonols, respectively, whereas bergaptol was the most significant up-regulated coumarin. To a lower extent, SA also boosted anthocyanidin and flavonol glycosides biosynthesis, together with lignans. On the contrary, both SA and MJSA strongly inhibited flavanol and flavone biosynthesis, since (+)-gallocatechin and (-)-epigallocatechin biosynthesis (both flavanols) were harshly repressed (Table S3).

Alkaloids were the family of N-containing compounds most positively affected by MJ elicitation, including those of indole terpenic, benzylisoquinoline, and purine origins, as it was observed for 16-epivellosimine, (*S*)-cheilanthifoline, and caffeine, respectively, that were also up-regulated in lower amounts by SA and MJSA (Table S3). In addition, MJ secondarily modulated aliphatic glucosinolate biosynthesis, as seen for 6-(methylsulfanyl)hexyl-desulfoglucosinolate and glucoiberverine, whereas it was repressed by SA and MJSA (Table S3). In this case, amines were mostly represented by a putrescine derivative: N-carbamoylputrescine.

Concerning terpenoids, all subfamilies with different isoprene subunit numbers were positively affected by elicitation, from monoterpenoids to carotenoids, following the contribution: MJ > MJSA > SA (Fig. 5B). Specifically, monoterpenoids were represented by geranyl acetate, sesquiterpenoids included phytoalexins metabolites, such as (+)-*δ*-cadinene and heliespirone C, triterpenoids, as betulinic aldehyde, and 4′-hydroxyadonixanthin as a carotenoid. Additionally, indole terpenic alkaloids were also included in this family (Table S3).

4. Discussion

Although several bryophyllum species have been largely applied for multiple ethnomedicinal purposes, as it is the case of *B. daigremontianum* (BD), *B.* × *houghtonii* (BH), and *B. tubiflorum* (BT), scarce attention has been paid to the characterization of their secondary metabolism, since the most part of phytochemical reports has been conducted on

Table 2
Discriminant metabolites identified by VIP (Variable Importance in Projection) selection method from multivariate supervised OPLS-DA for BH elicited PSCC.

Class	Compounds[a]	VIP Score	LogFC MJ vs control	LogFC SA vs control	LogFC MJSA vs control
Phenolic compounds					
Phenolic acids	4-hydroxybenzoate	1.37 ± 0.43	5.06	1.60	1.69
Anthocyanins	pelargonidin-3-O-β-D-glucoside	1.32 ± 0.27	−5.64	−18.59	−17.38
Flavonols	myricetin 3-O-β-D-glucoside	1.33 ± 0.91	4.63	5.12	7.92
	gossypetin 7-O-glucoside	1.31 ± 1.10	0.05	−0.11	8.15
	3,7,4′-trimethylquercetin 2′-O-β-D-glucoside	1.31 ± 0.82	−4.28	−9.62	5.89
	quercetagetin 7-O-glucoside	1.30 ± 1.01	10.07	−5.45	18.18
Flavones	luteolin triglucuronide	1.40 ± 0.34	15.42	4.18	16.75
	6,7-dehydrobaicalein	1.32 ± 0.59	−1.83	−2.36	−1.30
	vitexin	1.32 ± 0.27	−5.64	−18.59	−17.38
	isovitexin 2′'-O-rhamnoside	1.30 ± 1.57	8.53	−2.33	−1.12
	isovitexin 2′'-O-arabinoside	1.30 ± 0.39	5.51	9.72	−6.47
Isoflavones	coumestrol	1.32 ± 0.53	−6.66	−2.36	−11.09
Flavanol	(-)-epicatechin	1.37 ± 0.29	6.31	0.53	2.93
Lignans	(+)-sesaminol gentiotrioside	1.32 ± 0.43	0.75	13.32	−3.69
Coumarins	4-methylumbelliferyl glucoside	1.35 ± 0.83	4.95	0.43	3.47
Alkaloids					
Indole alkaloids	raucaffricine	1.46 ± 0.39	0.88	6.83	9.99
	16-epivellosimine	1.33 ± 0.63	39.21	19.12	18.93
Isoquinoline alkaloids	(*R*)-norcoclaurine	1.45 ± 0.98	5.16	−0.48	0.73
	pumiloside	1.36 ± 0.72	5.16	−0.48	17.61
Other	7-*O*-acetylsalutaridinol	1.43 ± 0.59	−0.41	−10.24	−3.45
	dopaquinone	1.42 ± 0.60	8.74	8.58	11.39
	miraxanthin V	1.39 ± 0.45	−5.78	4.35	6.62
	4-(1-methyl-5-hydroxy-2-pyrrolidinyl)-3-oxobutanoate methyl ester	1.34 ± 0.45	4.69	−0.79	−0.99
	(S)-autumnaline	1.34 ± 1.17	−14.09	−0.44	0.50
	(S)-tetrahydropalmatine	1.31 ± 0.23	0.67	0.21	−8.46
	β-chaconine	1.31 ± 0.84	5.16	−0.48	16.69
Glucosinolates					
Aliphatic glucosinolates	glucomalcommin	1.48 ± 0.38	5.38	3.88	16.77
	4-benzoyloxybutylglucosinolate	1.40 ± 0.34	5.16	17.75	1.06
	6-(methylsulfanyl)hexyl-glucosinolate	1.40 ± 0.46	5.16	17.08	0.73
	4-(methylsulfanyl)butyl-glucosinolate	1.40 ± 0.36	5.16	20.10	15.54
	(E)-1-(glutathion-S-yl)-N-hydroxy-ω-(methylsulfanyl)octan-1-imine	1.38 ± 0.50	5.44	15.20	18.81
	gluconapin	1.38 ± 0.31	1.00	14.64	10.72
	2-[(2′-methylsulfanyl)pentyl]maleate	1.36 ± 0.31	0.78	2.80	−1.75
	1-(methylsulfanyl)-5-aci-nitropentane	1.35 ± 0.76	0.60	−0.43	2.77
	(E)-6-(methylsulfanyl)hexanal oxime	1.33 ± 0.22	6.96	−7.96	2.90
	7-(methylsulfanyl)-2-oxoheptanoate	1.31 ± 0.31	−0.54	5.28	0.79
Indole glucosinolates	(Z)-indol-3-ylacetaldoxime	1.40 ± 0.31	0.00	19.09	0.00
	1-(1H-indol-3-yl)-2-aci-nitroethane	1.30 ± 0.27	−1.26	−12.54	−4.86
Aromatic glucosinolates	(Z)-1-(L-cysteinyglycin-S-yl)-N-hydroxy-2-phenylethan-1-imine	1.32 ± 0.71	10.91	5.584	0.21
Terpenoids					
Monoterpenoids	hemigossypolone	1.38 ± 0.79	5.72	−0.05	−4.55
Sesquiterpenoids	2,7-dihydroxycadalene	1.37 ± 0.42	−6.92	−1.19	−6.30
Triterpenoids	(20S)-ginsenoside Rh2	1.35 ± 0.73	5.16	−0.48	16.96
	showacene	1.35 ± 0.77	5.16	−0.48	16.06
	20-a-22-b-dihydroxycholesterol	1.34 ± 0.75	6.81	−0.67	13.75
	soyasapogenol A	1.30 ± 0.69	15.80	4.81	13.73
Polyketides					
Quinones	(2Z)-3-(3,4-dioxocyclohexa-1,5-dien-1-yl)prop-2-enoate	1.40 ± 0.33	3.90	2.09	−4.49
	ubiquinone-1	1.39 ± 0.46	16.99	11.21	9.33
	1,8-dihydroxy-3-methylnaphthalene	1.39 ± 0.45	14.55	16.70	−4.01
	6-methoxy-3-methyl-2-all-trans-nonaprenyl-1,4-benzoquinol	1.34 ± 0.74	5.16	−0.48	15.30
	3-demethylubiquinol-9	1.33 ± 0.82	5.16	−0.48	13.92
	plastoquinol-9	1.33 ± 0.81	5.16	−0.48	15.68
Phytoalexins					
	ancymidol	1.44 ± 0.46	6.00	0.71	−6.15
	furcatin	1.39 ± 0.42	6.41	−2.34	1.27
	3-hydroxy-5-methoxybiphenyl	1.35 ± 0.38	1.32	3.24	−2.70
	allosamidin	1.35 ± 0.77	−5.28	−6.80	−2.43
Hormones					
Indoleacetic acid derivatives	(indol-3-yl)acetyl-glycine	1.44 ± 0.48	−4.45	1.51	4.80
	(indol-3-yl)acetyl-myo-inositol L-arabinoside	1.37 ± 0.79	−4.53	−5.49	1.87
Abscisic acid derivatives	Abscisic acid	1.30 ± 0.31	−2.38	−2.06	−6.57
Gibberellin derivatives	gibberellin A_8	1.43 ± 0.72	11.58	4.17	−0.80
	gibberellin A_9	1.30 ± 0.47	−0.33	5.16	−0.29
Jasmonate derivatives	(+)-jasmonate	1.55 ± 0.61	12.06	3.63	6.42
Brassinosteriod derivatives	24-epicathasterone-22-O-sulfate	1.36 ± 0.24	−0.42	6.23	1.53

(*continued on next page*)

Table 2 (*continued*)

Class	Compounds[a]	VIP Score	LogFC MJ vs control	LogFC SA vs control	LogFC MJSA vs control
	(22R,23R)-28-homobrassinolide 22-sulfate	1.31 ± 0.39	5.16	17.71	9.61
Salicylate derivatives	salicylate	1.36 ± 0.31	5.06	−19.30	−13.42
Lipids					
Sphingolipids	phytosphingosine (C18)	1.58 ± 0.61	3.93	0.06	1.80
Glycerolipids	1−18:2−2-18:2-monogalactosyldiacylglycerol	1.37 ± 0.27	18.91	18.71	0.73
	1−18:3−2-18:2-monogalactosyldiacylglycerol	1.34 ± 0.39	0.21	1.48	0.08
	1−18:3−2-16:0-digalactosyldiacylglycerol	1.31 ± 0.35	16.24	15.95	0.00
Amino acids and derivatives					
	N-(4-hydroxybenzoyl)-L-glutamate	1.40 ± 0.30	−10.40	6.98	6.22
	L-aspartate	1.37 ± 1.01	2.13	−4.53	−4.28
	D-octopine	1.37 ± 0.26	5.87	5.63	−13.01
	L-homomethionine	1.35 ± 0.76	0.60	−0.43	2.77
	cycloglutamate	1.34 ± 1.10	6.09	−5.80	0.47
	N^8-acetylspermidine	1.32 ± 0.27	0.27	0.56	−16.73
	L-arginin	1.31 ± 0.39	2.90	−2.32	−1.81
	L-leucine/ L-isoleucine	1.31 ± 0.44	7.91	8.61	2.76
	L-lysine	1.31 ± 0.31	−6.29	−1.78	−5.77
	L-ethionine	1.31 ± 0.82	5.74	−0.43	2.77
	hypoglycine A	1.30 ± 0.60	−6.01	−2.40	−19.50

[a] Compounds present their corresponding VIP score values (> 1.30) and the logarithm of fold change (logFC) for each treatment with respect to control.

B. pinnatum (Andrade et al., 2020; García-Pérez et al., 2020c). Thus, these three underexplored species were selected for this study. To date, restricted analyses have been performed for the identification of bufadienolides and flavonoids from *Bryophyllum* sp., focusing exclusively on *ex vitro* grown plants (Katrucha et al., 2020; Stefanowicz-Hajduk et al., 2020b). Consequently, in order to unravel the biosynthetic potential of these species, a combinatorial approach concerning elicited PSCCs and untargeted metabolomics is required, leading to their phytochemical valorization and making possible their biotechnological exploitation.

The PSCC technology offers a set of promising advantages, including fast biomass accumulation and the capability of easily enhancing secondary metabolism throughout the addition of elicitors (Lozano-Milo et al., 2020; Nadeem and Ahmad, 2019). The reliability and ease of developing downstream applications associated with PSCCs, in combination with untargeted metabolomics stands out as a novel and powerful strategy to unravel the metabolic fingerprinting attributed to elicitation, thus conferring a broad metabolic representation of *Bryophyllum* sp. for their consideration as a valuable source of bioactive compounds. Due to the exhaustive results obtained for *Bryophyllum* sp., this combinatorial approach concerning elicitation and untargeted metabolomics, presents an unexplored two-folded potential: on one hand, its application to poorly described medicinal plants, assessing their phytochemical valorization; and, on the other hand, its effectiveness to provide insight about the mechanism of action of elicitors and their combinations on plant secondary metabolism.

In this case, two well-known abiotic elicitors were applied to bryophyllum PSCCs: methyl jasmonate (MJ) and salicylic acid (SA), along with their combined treatment (MJSA). According to the results, a controversial relationship between MJ and SA was shown to present a species-dependent behavior, as differential effects were reported for cell lines from different bryophyllum species, associated with cell biomass accumulation and secondary metabolism induction. Although these three bryophyllum species are genetically closely-related, they exhibit a differential developmental behavior, as widely reported for their specific requirements related to organogenesis (García-Pérez et al., 2020d), mineral nutrition (García-Pérez et al., 2020c), and production of phenolic compounds (García-Pérez et al., 2020b). The hypothesis on the species-dependent effect of both elicitors is reinforced by the results obtained for PSCCs from other species, such as *Hypericum perforatum* (Wang et al., 2015), and *Vitis vinifera* (Xu et al., 2015) where 50 μM MJ did not alter plant cell growth with respect to untreated cells, whereas a significant decrease in plant growth was observed under the same treatments in other species, such as *Mentha piperita* (Krzyzanowska et al., 2012) and *Ophiorrhiza mungos* (Deepthi and Satheeshkumar, 2017). The same species-dependent behavior on plant cell growth was observed for 100 μM SA, showing an inhibiting effect in *O. mungos* (Deepthi and Satheeshkumar, 2017) and *Gardenia jasminoides* (Liu et al., 2018), an enhancing effect in *Momordica dioica* (Chung et al., 2017), and no effect on *V. vinifera* (Xu et al., 2015). The highest values for cell growth reported to BH in all treatments in comparison with other species can be explained on the basis of the induction of a series of metabolites related to primary metabolism, since phytohormones, amino acid, and lipid metabolism was enhanced under elicitation on this species (Table S3).

The robustness and suitability of untargeted metabolomics for unravelling the shift in metabolic fingerprints associated to elicitation of bryophyllum PSCCs was reported by the high number of compounds annotated, ranging from 1500 to almost 3,000. This approach, not requiring *a priori* hypotheses and allowing to profile such a large number of compounds, contrasts with the restricted results previously found on bryophyllum plants. Indeed, previous information focused on the identification of phenolic compounds and bufadienolides, with limited compounds reported (Casanova et al., 2020; Omojokun et al., 2020; Stefanowicz-Hajduk et al., 2020b). Regarding the effect of elicitation on secondary metabolism, three major groups of secondary metabolites were affected by this process on bryophyllum PSCCs: phenylpropanoids, N-containing compounds, including mainly alkaloids, glucosinolates, and amines, and terpenoids. Thus, elicitation caused an integrative effect on BD and BH plant cell secondary metabolism, since the major biosynthetic pathways were affected by the treatment with MJ and SA and their combination, depending on the species. This fact provides insight about the integrative crosstalk between both elicitors, devoted to the induction of protective mechanisms leading to stress tolerance induction (Ho et al., 2020; Li et al., 2019). A synergistic effect was found for MJSA treatment on BD; however, in the case of BH, MJSA combination caused a negative effect on plant biosynthesis. This observation may probably be due to the MJ-mediated SA biosynthesis in BH PSCCs, thus promoting an imbalance on the biosynthetic activity when SA was exogenously incorporated to PSCCs, leading to an antagonistic effect between them, as previously reported by different authors in other species (Lee et al., 2016; Yang et al., 2019).

Focusing on the mode of action of elicitors, MJ constitutes the most applied elicitor in PSCCs, due to their central role in plant cell signaling in response to different stresses (Giri and Zaheer, 2016). Consequently, it acts as a master regulator of secondary metabolism, with the ability to enhance bioactive compound production via biosynthetic genes overexpression, and it is responsible for the induction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) overproduction driven by stress, thus causing severe impairment of plant cell

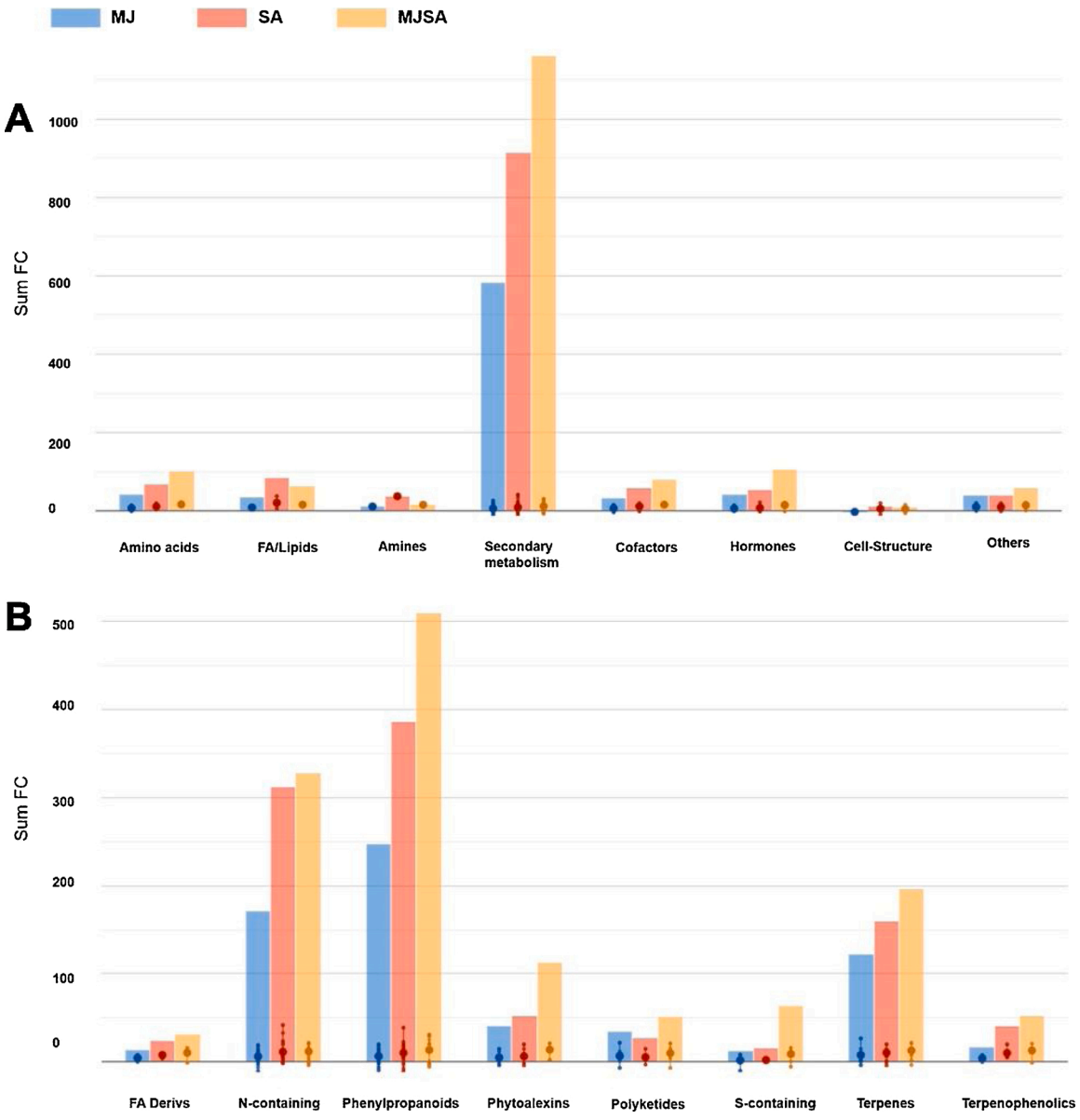

Fig. 4. Biosynthesis plots obtained for BD PSCCs elicited with MJ and SA and their combination, MJSA. **A.** Full biosynthesis. **B.** Secondary metabolite biosynthesis. The large dots represent the average (mean) of all log Fold-change (FC) for metabolites and the small dots represent the individual log FC for each metabolite. FA/Lipids: fatty acids and lipids; Amines: amines and polyamines; Cofactors: cofactors, prosthetic groups, electron carriers and vitamins; FA Derivs: fatty acids derivatives; N-containing: nitrogen-containing secondary compounds; S-containing: sulfur-containing secondary compounds.

homeostasis (Li et al., 2018). As a signaling molecule, MJ induces an array of intracellular transduction pathways that may interfere with other signaling pathways in order to create synergistic or antagonistic effects, as it occurs with SA and other hormones, such as auxins and ethylene (Giri and Zaheer, 2016; Ho et al., 2020; Li et al., 2019). As a result, it is assumed that SA is responsible for the early response against stress, followed by the late response developed by MJ (Yang et al., 2019). Nevertheless, some authors have reported that MJ inhibits SA signaling and function through their common cell targets, which include mitogen-activated protein kinases (MAPKs) (Mine et al., 2017) and other molecular targets related to redox homeostasis (González-Bosch, 2018), thus suggesting an antagonistic effect on cell physiology. Such antagonism may be observed in elicited PSCCs from BH, as suggested by the negative effect on secondary metabolism reported for the combination of MJ and SA (MJSA; Fig. 5B).

The oxidative stress associated with elicitation may be responsible for the response observed at a metabolic level, in which elicited bryophyllum PSCCs showed an accumulation of secondary metabolites with antioxidant activity, mainly represented by phenolic compounds (García-Pérez et al., 2018, 2020a, 2020b, 2020e, 2021; Murthy et al., 2014). In particular, different flavonoid subfamilies, stilbenes, and lignans were pointed out as the major compounds affected by elicitation on BD and BH.

In addition, MJ and SA have been reported to induce oxidative stress (Poór, 2020; Wang et al., 2020), accompanied by the modulation of biosynthetic genes from the phenylpropanoid pathway (Mendoza et al., 2019). Different key enzymes from the initial steps of the phenylpropanoid pathway, such as phenylalanine ammonia-lyase (PAL) and tyrosine ammonia lyase (TAL), were induced under MJ and SA elicitation as determined for different PSCCs, including *Scrophularia striata* (Sadeghnezhad et al., 2020), *M. piperita* (Cappellari et al., 2020), and *Ocimum basilicum* (Vyas and Mukhopadhyay, 2018; Złotek et al., 2016). In contrast, MJ and SA have been combined for the elicitation of phenolic compounds with differential results, showing an increase in

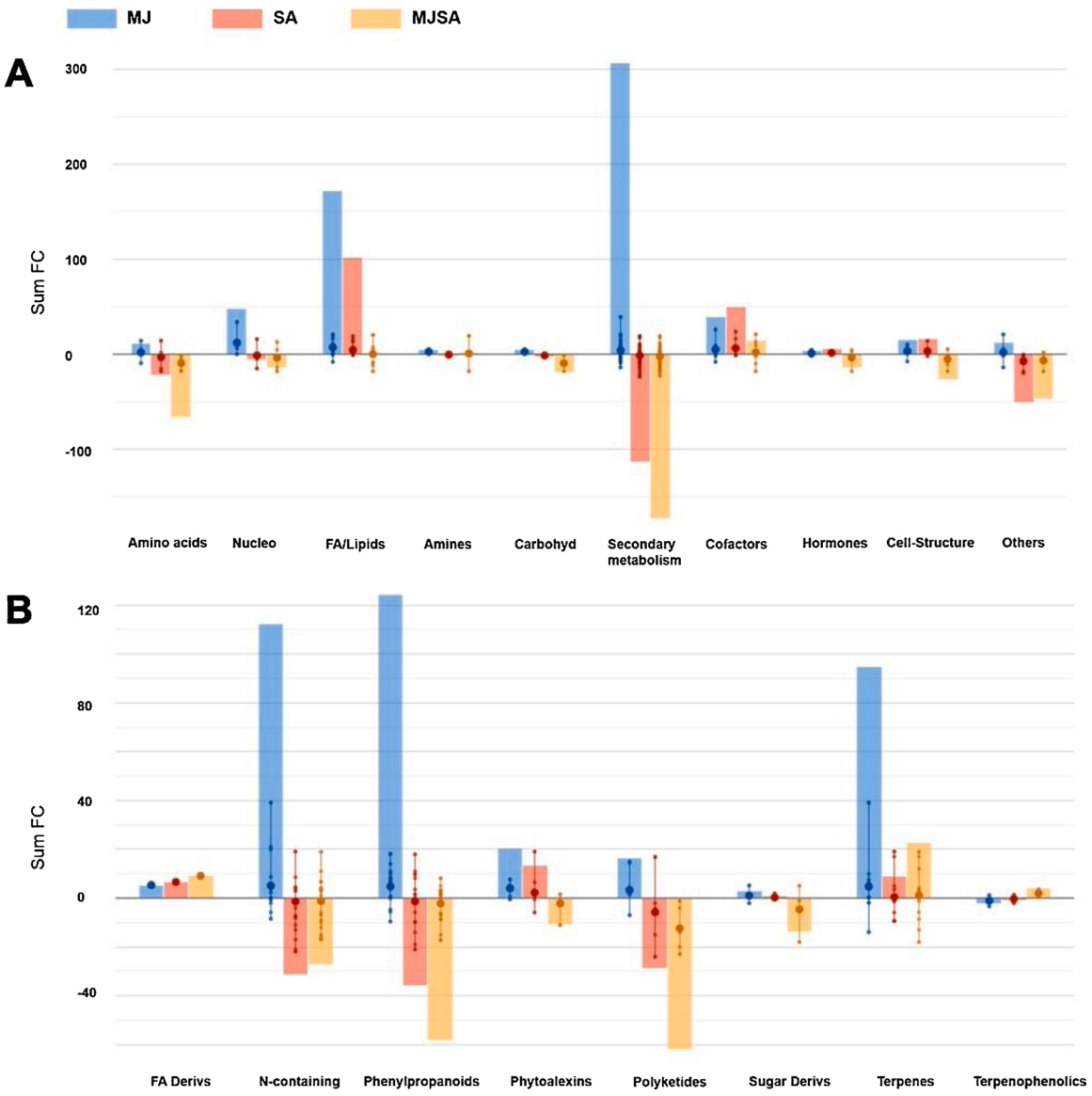

Fig. 5. Biosynthesis plots obtained for BH PSCCs elicited with MJ and SA and their combination, MJSA. **A.** Full biosynthesis. **B.** Secondary metabolite biosynthesis. The large dots represent the average (mean) of all log Fold-change (FC) for metabolites and the small dots represent the individual log FC for each metabolite. FA/Lipids: fatty acids and lipids; Amines: amines and polyamines; Cofactors: cofactors, prosthetic groups, electron carriers and vitamins; FA Derivs: fatty acids derivatives; N-containing: nitrogen-containing secondary compounds; S-containing: sulfur-containing secondary compounds.

Rehmannia glutinosa hairy root cultures (Piątczak et al., 2016), although no effect was reported on phenolic compounds biosynthesis on *Thevetia peruviana* (Mendoza et al., 2019). According to phenolic compounds previously reported on *Bryophyllum* sp., most part of phytochemical analyses performed on leaves from *ex vitro*-grown plants have highlighted that these species contain mainly phenolic acids, such as ferulic acid, caffeic acid, and protocatechuic acid (Bogucka-Kocka et al., 2016; Chernetskyy et al., 2018), and flavonoids. Different subfamilies of flavonoids were reported to bryophyllum leaves, present as glycosides, such as flavonols, including quercetin, myricetin, and kaempferol, and flavone glycosides, such as acacetin and luteolin (Fürer et al., 2016; García-Pérez et al., 2021; Stefanowicz-Hajduk et al., 2020a). These subfamilies were identified under the elicitation of bryophyllum PSCCs, but other subfamilies were reported for the first time associated with this subgenus, according to the results: isoflavones, stilbenes, and lignans in the case of BD (Table 1); and anthocyanidins, isoflavones, flavanols, and lignans in the case of BH (Table 2).

Besides the induction of oxidative stress, the application of elicitors on PSCCs promotes the induction of plant systemic resistance, leading to the activation of defense mechanisms against different stresses (Siddaiah et al., 2017). In this sense, the metabolic profiling of elicited bryophyllum PSCCs provides shreds of evidence about the induction of this defensive response throughout the accumulation of stress-related metabolites, as in the case of glucosinolates and phytoalexins. Glucosinolates were firstly described as secondary metabolites mainly found in cruciferous vegetables (Barba et al., 2016; Malhotra and Bisht, 2020), but subsequent phytochemical analyses have pointed that they are involved in the acquisition of plant resistance against different stresses, as it is the case of elicitation (Sánchez-Pujante et al., 2017). Aliphatic glucosinolates, deriving from methionine, were mainly accumulated in both species, BD and BH under elicitation. In addition, in the case of elicited BD PSCCs, products from glucosinolate activation were reported (Table 1). These derived by-products are formed after glucosinolate enzymatic hydrolysis, deriving into bioactive molecules, such as isothiocyanates (Blažević et al., 2020).

The application of elicitors to PSCCs has already been implemented as a successful strategy for the production of glucosinolates, showing higher yields in these systems than in whole plants (Sánchez-Pujante

et al., 2017). In this sense, glucosinolate biosynthesis has been enhanced by the exogenous application of MJ and SA in other plant species, including *A. thaliana* (Kuo et al., 2020), *Raphanus sativus* (Chen et al., 2019), and *Brassica* sp. (Chiu et al., 2019). This enhancement was mainly driven by elicitor activity on the expression of glucosinolate biosynthetic genes in charge of core glucosinolate structure formation, such as *CYP83 A1*, $FMO_{GS\text{-}OX1}$, and *AOP2*, throughout the induction of different *MYB* transcription factors (Chen et al., 2019; Sánchez-Pujante et al., 2019; Wasternack and Strnad, 2019), being AOP2 one of the enzymes responsible for aliphatic glucosinolate biosynthesis (Petersen et al., 2018), found in elicited BD and BH PSCCs. To the best of authors' knowledge, this is the first time that this family of metabolites was reported in *Bryophyllum* sp.

Phytoalexins were another family of compounds closely related to the induction of plant defense mechanisms against herbivores and abiotic stresses (Ahmed and Kovinich, 2020). Phytoalexins were mainly represented by terpenoid-indole alkaloids (TIAs) and terpenes in the case of *Bryophyllum* sp. As it occurred for other species, alkaloid and terpenoid biosynthesis was up-regulated by MJ and SA (Buraphaka and Putalun, 2020; Sharifzadeh Naeini et al., 2020). Indole alkaloids are further condensed to form terpenoid indole alkaloids (TIAs), by merging two different biosynthetic pathways: the shikimate pathway, that confers the indole moiety, and the mevalonate pathway, providing the terpenoid moiety (Abouzeid et al., 2019; Ratnadewi, 2017). TIA biosynthesis was preferentially studied using the medicinal plant *Catharanthus roseus*, in which MJ was shown to induce key biosynthetic gene activity (including *N*-putrescine methyltransferase, *PMT*, and *N*-methylputrescine oxidase, *MPO*) throughout the activation of *bHLH* transcription factors (Wasternack and Strnad, 2019). In addition, MJ-elicited *C. roseus* PSCCs showed an overexpression of strictosidine synthase (*STR*) via Octadecanoic Responsive Catharanthus Alkaloid (*ORCA*) transcription factors modulation (Singh et al., 2020). There are multiple evidences supporting the accumulation of TIAs by MJ and SA elicitation, as it was the case of MJ and SA-elicited *Argemone mexicana* (Trujillo-Villanueva et al., 2012) and *C. roseus* (Vázquez-Flota et al., 2020). In the case of terpenoids, monoterpenoids and sesquiterpenoids have been mainly characterized as phytoalexin compounds (Reim et al., 2020), such as artemisinin derivatives (Kariya et al., 2020), gossypol (Tian et al., 2016), and juvenile hormone III (Zachariah and Leela, 2018), as well as saponins and carotenoids (Sun et al., 2019). These phytoalexins are described also for the first time on elicited bryophyllum PSCCs.

In addition to glucosinolates and terpenoids, different metabolites related to plant growth and stress tolerance were also affected by elicitation on bryophyllum PSCCs. In the case of BH, the phytohormonal profile was altered under elicitation, thus suggesting that hormones, besides their roles on primary plant processes, may present additional functions related to stress tolerance, as it is the case of brassinosteroids, which play an efficient role on plant detoxification (Sharma et al., 2018). In the same way, amines, such as putrescine (in the case of BH) and spermidine (in BD) also play relevant roles as phytohormone-like compounds in charge of abiotic plant stress tolerance, coping with the protection against oxidative stress and interfering with different stress-related signaling pathways (Alcázar et al., 2020). Additionally, volatile organic compounds (VOCs), found on elicited BD PSCCs, play an important role in plant communication and provide an active contribution to stress modulation, interfering with elicitor signaling pathways (Brilli et al., 2019).

Besides its role in plant defense and systemic resistance, elicitation contributed to the enhancement of bioactive compounds biosynthesis with phytopharmacological properties of bryophyllum plants. In this sense, phenolic compounds constitute a large family of compounds, well-known for their associated antioxidants, antimicrobial, and cytotoxic activities (Leri et al., 2020; Wang et al., 2018). Interestingly, besides the antioxidant activity associated with these compounds, the combined accumulation of isoflavones and lignans reported for BD and BH, acting as phytoestrogens, may indicate that elicitation enhanced the medicinal properties associated with bryophyllum since both compounds have been seen to exert an exceptional potential on the prevention of a series of complex diseases, as it is the case of breast cancer and other hormonal diseases (Basu and Maier, 2018; Boucher et al., 2018). In the same way, the interest for glucosinolates has risen exponentially in the last years because of their health-enhancing properties, being classified as efficient antimicrobial and cancer chemopreventive agents, according to different epidemiological studies (Maina et al., 2020; Zhang et al., 2017). Similarly, alkaloids have been widely exploited thanks to their functions as anticancer, antiarrhythmic, and antimalarial agents (Kukula-Koch and Widelski, 2017; Shen et al., 2020). Thus, the elicitation of bryophyllum PSCCs, together with the application of untargeted metabolomics, emerged as a successful strategy to depict the biosynthetic and phytochemical potential of these unexplored medicinal plants. At the same time, they provided a robust system to decipher the effect of abiotic stress on plant cells (Barrales-Cureño et al., 2021; Danaraj et al., 2020), by characterizing the integrative role of two well-known elicitors, MJ and SA, that caused a significant metabolic reprogramming on bryophyllum PSCCs.

5. Conclusions

In this work, PSCCs conferred a successful biotechnological system to enhance the rate of plant secondary metabolites biosynthesis throughout the inclusion of abiotic elicitors, such as MJ and SA. The combination of elicited PSCCs with untargeted metabolomics emerged as a powerful approach to unravel the biosynthetic potential of little-studied plants with medicinal potential, as it is the case of *Bryophyllum* sp. In this work, MJ and SA were used alone and combined to bryophyllum PSCCs, showing differential effects, depending on the plant species, on primary metabolism, affecting cell growth, and secondary metabolism.

MJ and SA showed a synergistic effect on BD PSCCs, whereas they played an antagonistic role on BH PSCCs, being MJ alone the only elicitor causing a positive effect on plant secondary metabolism. In turn, BT PSCCs did not significantly alter their secondary metabolism rate at the elicitor concentrations tested. In the cases of BD and BH, the biosynthesis of three major families of secondary metabolites was up-regulated under elicitation, following the order: phenylpropanoids, N-containing compounds, and terpenoids. Phenylpropanoids were mainly represented by phenolic compounds, suggesting that the biosynthesis of these compounds with associated antioxidant activity was driven by the oxidative stress induced by elicitors. N-containing compounds were essentially divided into glucosinolates and alkaloids. Glucosinolates have gained much attention in the last years, thanks to their associated health-enhancing properties, and they have been closely related to the plant response against different stresses. Glucosinolates, as well as alkaloids, were reported to bryophyllum plants for the first time, thus suggesting that PSCCs elicitation combined with untargeted metabolomics suppose a convenient strategy to unravel the full phytochemical and biosynthetic potential of medicinal plants. Finally, terpenoids biosynthesis were also elicited on bryophyllum PSCCs, being part of terpenoid indole alkaloids, and providing the first reports of phytoalexins and saponins biosynthesized by these species.

Overall, the application of untargeted metabolomics led to an integrative analysis of the cell metabolic reprogramming on bryophyllum PSCCs under abiotic stress, mediated by elicitors. Thus, untargeted metabolomics successfully reflected the results of the combination of gene expression and protein regulation, driven by abiotic stress, at a phenotypical level. This way, the combination of both cutting-edge technologies led to the consideration of bryophyllum plants as a valuable source of bioactive compounds, beyond the previously available limited knowledge about these species, thus suggesting the use of their elicited PSCCs as a robust biotechnological system for the large-scale production of secondary metabolites with high interest for food, cosmetic, and pharmacological industries.

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CRediT authorship contribution statement

Pascual García-Pérez: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Writing - original draft, Writing - review & editing. **Begoña Miras-Moreno:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Writing - original draft, Writing - review & editing. **Luigi Lucini:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing - review & editing. **Pedro P. Gallego:** Conceptualization, Formal analysis, Investigation, Validation, Writing - review & editing.

Declaration of Competing Interest

The authors declare no competing interests.

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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.indcrop.2021.113322.

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