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Elucidating Phytochemical Production in *Juniperus* sp.: Seasonality and Response to Stress Situations

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ⓈSupporting Information

ABSTRACT: Phenolic-enriched extracts from Portuguese junipers were described as potential neuroprotective natural products. However, evaluation of plants for pharma/nutraceutical industry is based on secondary metabolite content, which is influenced by the environmental conditions. Therefore, it became important to elucidate the metabolic response of the junipers to seasonality and to stress conditions with regard to polyphenol production. Seasonal conditions modulated total phenolic and flavonoids contents of the four juniper species. Higher levels of phytochemicals were obtained when plants were not actively growing. However, only a few differences were registered in the relative contents of psydrin and a procyanidin dimer, according to species. Salt stress and methyl jasmonate elicitation promote different responses within the species. They were effective in enhancing phenolic accumulation, with just a few alterations in the relative contents of procyanidins, epicatechin, and quercetin derivatives. These differences were also reflected in gene expression of important enzymes from biosynthetic pathways.

KEYWORDS: *methyl jasmonate, phenolic content, polyphenols biosynthesis, salinity, season variability*

■ INTRODUCTION

Natural products are important sources of novel leads for therapeutic drugs.[1] Between 1981 and 2002, of the 877 novel medicines developed, 49% were natural products, their derivatives, or synthetic products developed with the natural product as a basis.[2]

Among the diverse phytochemicals produced by plants, polyphenols are an important class. These compounds have many diverse functions in plants, such as color of leaves, flowers, and fruits, antimicrobial, antifungal, insect feeding deterrence, screening from damage by solar UV radiation, chelation of toxic heavy metals, and antioxidant protection from free radicals generated during the photosynthetic process.[3] Some have also been recognized as beneficial for human health because they exhibit anti-inflammatory, antioxidant, insulin-potentiating, anticarcinogenic, antiviral, antiulcer, antiapoptotic, and cardiovascular and neurodegenerative protectant activities among others.[4]

Junipers (*Juniperus* sp.) are plants rich in secondary compounds, particularly terpenoids and phenolics.[5−8] Common juniper extract is an important natural product used widely in many pharmaceutical and technical preparations, in cosmetic products, and as a food additive. Some studies have emerged reporting on diverse biological activities, such as anti-inflammatory,[9] diuretic and antiseptic,[10] hypoglycemic,[11] hypotensive, analgesic, abortifacient, and antinociceptive,[12] antiviral,[13] anticancer,[14] antioxidant,[15,16] and anticholinester-

ase.[15,16] Recently phenolic-enriched extracts from endemic Portuguese *Juniperus* sp. were described as potential natural products in the treatment of neurodegenerative diseases.[17]

The quality of plants intended for processing by the pharmaceutical industry is usually judged on the basis of secondary metabolite content.[18] However, environmental conditions have an important effect on the secondary metabolisms.[19] It has been reported that polyphenol levels can change in response to biotic (pest and pathogens) and abiotic (drought, hypoxia, high light intensity, low or high temperature) stresses.[19,20]

Therefore, it is critical to understand the metabolic response of the *Juniperus* sp. to seasonality as well as to stress conditions in terms of polyphenol production for species to accomplish their full potential.

■ MATERIALS AND METHODS

Plant Material and Growth Conditions. *Seasonal Evaluation.* Five-year-old plants of *Juniperus navicularis* Gand., *Juniperus oxycedrus badia* (H. Gay) Debeaux, *Juniperus phoenicea* L., and *Juniperus turbinata* Guss were used for seasonal evaluation. As *J. navicularis* is a Portuguese endemic and threatened plant, a license for capture (11/

Table 1. Enzyme Transcripts from the Phenylpropanoid Biosynthetic Pathway Evaluated in *J. phoenicea* and *J. oxycedrus badia* by Real-Time RT-PCR[a]

gene	species	GenBank accession no.	primers	PCR product length (bp)
PAL	*J. phoenicea*	GQ389685	fwd: ATGGCTTCTTACACCTCTGAG	188
	J. oxycedrus badia	GQ389686	rev: TCCACCGCTTGACAAATCC	
F3H	*J. phoenicea*	GQ389679	fwd: GGATTGGAAGCCGACGCGGT	168
	J. oxycedrus badia	GQ389680	rev: TGGAGGAGCAGAGTGATG	
LAR	*J. phoenicea*	GQ389683	fwd: CCAGTTGAACCTGCTTTGAG	190
	J. oxycedrus badia	GQ389684	rev: TGACAGTGCCATCACCATAG	
ANS	*J. phoenicea*	GQ389673	fwd: TCAGTGCTCTCACATTCC	193
	J. oxycedrus badia	GQ389674	rev: ATCCTTACTTTCTCCTTGTTC	
ANR	*J. phoenicea*	GQ389671	fwd: ACTTGCCCTTAGCCTACTTACAG	117
	J. oxycedrus badia	GQ389672	rev: AGTTGAGCACTACAAACATCATCC	
ACT	*J. phoenicea*	GQ389669	fwd: CGGCGATGTATGTTGCTATTC	157
	J. oxycedrus badia	GQ389670	rev: CCAGCGAGATCCAACCTAAG	
EF1A	*J. phoenicea*	GQ389677	fwd: CTCTCAGGTTGCCACTTCAG	131
	J. oxycedrus badia	GQ389678	rev: TTCAGTTGTCAGCCCAGTTG	
GAPDH	*J. phoenicea*	GQ389681	fwd: AACTAATTGTCTTGCTCCTCTTG	200
	J. oxycedrus badia	GQ389682	rev: CCTTTCCAACTGCCTTTGC	

[a]For each gene are shown the GenBank accession numbers, gene sequence of primers used in real-time RT-PCR, and respective product length.

2008/CAPT and 12/2008/CAPT) was required for sampling. For all species, voucher samples were authenticated and deposited at the herbarium João de Carvalho e Vasconcelos, Instituto Superior de Agronomia, Lisbon, Portugal. The herbarium numbers are *Juniperus navicularis*, LISI 1064/2007; *J. oxycedrus* subsp. *badia*, LISI 1/2008; *J. phoenicea*, LISI 3/2008; and *J. turbinata*, 1067/2007. All plants were grown in pots, exposed to the same local (Lisbon area) environmental conditions of light, temperature, and humidity. Plants were watered when necessary. Leaf samples were collected monthly through the year 2008, freeze-dried, ground to fine powder, and stored at −80 °C.

Stress Conditions. For stress evaluation, 2-year-old plants of *J. oxycedrus badia*, *J. phoenicea*, and *J. turbinata* were used. Plants were maintained in 1 L pots, inside a growth chamber (Fitoclima 700 EDTU, Aralab, Lisbon, Portugal) with standard conditions: 30 °C temperature, 60% relative humidity, 800 μmol m^{-2} s^{-1} and 16 h/8 h (light/dark) photoperiod. All of the plants, except the ones submitted to drought stress, were watered frequently with 300 mL of water. Plants were acclimatized for 1 week prior to stress initiation. For each condition tested, five plants were used.

(1) Drought Stress. Plants were maintained in standard conditions but without watering for 20 days. Control plants were maintained under standard conditions for 20 days.

(2) Salt Stress. Plants were kept in standard conditions but watered with 300 mL of 250 mM NaCl for 15 days. Control plants were maintained in standard conditions for 15 days.

(3) Methyl Jasmonate (MeJa) Treatment. Plant leaves were sprayed until complete coating with an aqueous solution containing 5 mM MeJa (Sigma-Aldrich) and 0.1% (v/v) Tween 20 (Sigma-Aldrich) at day 0 and effects followed for 120 h. Control plants were sprayed with an aqueous solution of 0.1% (v/v) Tween 20.

Phenolic Extract Preparation. Phenolic compounds were extracted from leaves using a 50% ethanol solution (v/v) in a ratio of 12 mL g^{-1} of freeze-dried leaf powder, as previously described.[21]

Chemical Characterization. *Total Phenolic Content (TPC).* Determination of total phenolic compounds was performed according to the Folin−Ciocalteu method adapted to a microplate reader.[22] Gallic acid was used as the standard, and the results were expressed as milligrams gallic acid equivalents (GAE).

Total Flavonoid Content (TFC). Measurement of total flavonoids was performed according to a modification of the AlCl$_3$ complexation method as described before[22] and expressed as milligram catechin equivalents (CE).

Phenolic Composition. Samples containing 20 μg of GAE were applied to a C18 column (Synergi Hydro C18 column with polar end-capping, 4.6 mm × 150 mm, Phenomonex Ltd.) and analyzed on an LCQ-DECA system controlled by XCALIBUR software (2.0, Thermo Finnigan), as reported by Tavares et al.[23] The LCQ-Deca system comprised a Surveyor autosampler, a pump, a photodiode array (PDA) detector, and a Thermo Finnigan ion trap mass spectrometer. Samples were analyzed in triplicate and in positive and negative modes to aid identification. Peak areas were assigned and quantified using the XCALIBUR software.

Relative Accumulation of Enzyme Transcripts from Phenylpropanoid Biosynthesis Pathway. *Achievement of Specific RNA Sequences from Juniperus sp. (1) RNA Isolation and cDNA Synthesis.* Total RNA was isolated using an adaptation of the method described by Chang et al.[24] The extraction buffer contained 2% (w/v) CTAB, 2% (v/v) 2-mercaptoethanol, 3 M NaCl, 25 mM EDTA, 100 mM Tris-HCl (pH 8.0), 2% (w/v) PVP-40, and 0.04% (w/v) spermidine. Briefly, leaves were ground with liquid nitrogen, and each 1 g of leaf powder was incubated with 20 mL of preheated extraction buffer for 30 min at 65 °C with periodic shaking. Protein extraction was carried out using chloroform/isoamyl alcohol (24:1), and then RNA was precipitated with LiCl overnight at 4 °C.[24] The RNA obtained was dissolved in SSTE (1 M NaCl, 0.5% (w/v) SDS, 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA, pH 8.0), and a second protein removal was performed followed by nucleic acid precipitation with absolute ethanol at −20 °C. The pellet was dried and resuspended in sterile water. RNA quantity and quality were measured spectrophotometrically (ND 1000, Thermo Scientific) and checked by agarose gel electrophoresis. Afterward, RNA was treated with a Turbo DNA-free kit from Applied Biosystems.

Reverse transcription (RT)-PCR used 2 μg of total RNA in a 20 μL volume reaction, 25 μg mL^{-1} anchored oligo(dT)12−18 primer (Invitrogen), and a SuperScript II first-strand synthesis system for RT-PCR (Invitrogen).

Table 2. Total Phenol Content and Total Flavonoid Content for the Four Juniper Species (*J. navicularis*, *J. oxycedrus badia*, *J. turbinata*, and *J. phoenicea*) across Different Months[a]

species	month	total phenolic content (mg GAE g^{-1} DW)		total flavonoid content (mg CE g^{-1} DW)	
J. navicularis	Jan	80.84 ± 1.32	a	37.50 ± 0.98	a
	Feb	45.66 ± 1.30	f	20.52 ± 0.38	g
	March	57.91 ± 0.23	de	24.80 ± 0.47	f
	April	56.75 ± 1.67	d	24.92 ± 0.65	ef
	May	69.48 ± 2.41	bc	33.14 ± 0.89	b
	June	63.07 ± 2.56	cd	29.83 ± 1.06	d
	July	54.26 ± 0.97	e	27.20 ± 0.33	e
	Aug	65.52 ± 4.05	b	30.81 ± 0.87	cd
	Sept	65.30 ± 5.59	bcd	30.24 ± 1.25	d
	Oct	68.43 ± 1.35	b	31.88 ± 0.68	bcd
	Nov	71.61 ± 0.36	b	32.68 ± 0.66	bc
	Dec	81.24 ± 2.77	a	36.10 ± 0.67	a
J. oxycedrus badia	Jan	94.37 ± 7.68	ab	54.18 ± 1.02	b
	Feb	72.91 ± 2.73	d	43.96 ± 0.99	f
	March	57.06 ± 2.20	f	35.71 ± 0.51	g
	April	55.86 ± 0.89	e	37.71 ± 0.38	g
	May	56.30 ± 2.81	f	38.32 ± 0.58	g
	June	80.61 ± 2.35	cd	50.91 ± 0.79	cd
	July	60.89 ± 2.94	f	37.60 ± 2.01	g
	Aug	77.68 ± 3.80	d	48.44 ± 1.55	de
	Sept	74.65 ± 5.78	d	47.23 ± 1.21	ef
	Oct	83.52 ± 2.22	bcd	52.07 ± 0.73	bc
	Nov	90.86 ± 0.62	bc	54.70 ± 0.49	b
	Dec	102.55 ± 6.29	a	62.01 ± 2.26	a
J. phoenicea	Jan	61.42 ± 0.69	d	25.94 ± 1.57	bc
	Feb	41.30 ± 0.43	e	16.90 ± 1.32	e
	March	26.56 ± 1.34	f	9.95 ± 0.46	f
	April	44.68 ± 1.75	e	16.95 ± 0.09	e
	May	31.32 ± 0.67	f	10.67 ± 0.18	f
	June	60.18 ± 0.61	d	23.89 ± 0.74	cd
	July	43.52 ± 0.81	e	15.30 ± 0.92	e
	Aug	69.75 ± 1.14	c	25.75 ± 0.83	bc
	Sept	71.65 ± 3.69	bc	23.17 ± 0.15	d
	Oct	77.65 ± 2.81	b	27.27 ± 1.10	ab
	Nov	74.61 ± 2.64	b	26.92 ± 0.47	b
	Dec	89.07 ± 3.85	a	29.73 ± 0.67	a
J. turbinata	Jan	90.90 ± 1.84	b	34.50 ± 1.05	b
	Feb	62.89 ± 2.15	de	23.20 ± 0.84	d
	March	51.94 ± 1.57	e	19.88 ± 0.11	e
	April	91.66 ± 4.03	b	34.25 ± 0.70	b
	May	75.67 ± 0.23	cd	29.83 ± 0.80	c
	June	73.05 ± 1.27	d	28.60 ± 0.73	c
	July	86.67 ± 2.25	bc	33.40 ± 0.71	b
	Aug	94.11 ± 2.55	b	33.88 ± 1.98	b
	Sept	95.07 ± 3.73	b	34.63 ± 1.47	b
	Oct	70.31 ± 2.39	d	25.00 ± 0.50	d
	Nov	113.52 ± 12.66	a	38.44 ± 0.90	a
	Dec	95.74 ± 10.15	b	33.69 ± 0.76	b

[a]Different letters (a−f) denote significantly different samples at $p < 0.05$.

(2) Gene Amplification, Cloning, and Sequencing. To determine the accumulation of transcripts that encode some of the most important phenylpropanoid biosynthesis pathway enzymes, specific sequences of phenylalanine ammonia-lyase (PAL), flavanone 3-hydroxylase (F3H), anthocyanidin synthase (ANS), leucoanthocyanidin reductase (LAR), and anthocyanidin reductase (ANR) for *J. oxycedrus badia* and *J. phoenicea* were amplified. For real-time RT-PCR

normalization purposes, the sequences of actin (ACT), elongation factor 1α (EF1A), and glyceraldeyde 3-phosphate dehydrogenase (GAPDH) were also determined.

For each gene of interest, amino acid and nucleic acid sequences from diverse species, giving special attention to gymnosperm species, were aligned, and primers were designed for the most conserved

regions. Primers were chosen to obtain PCR products with approximately 200−500 bp size.

Genes of interest were amplified in PCR reactions containing 0.1−2 μL of cDNA, 0.625 U *Taq* DNA polymerase (Fermentas), 0.5 μM of each primer, 2.5 mM MgCl$_2$, 0.2 mM of each dNTP, 75 mM Tris-HCl (pH 8.8), and 20 mM (NH$_4$)$_2$SO$_4$. The quality of PCR products was visually inspected by electrophoresis, and the generation of only a single band of the expected size was the criterion for specificity. The PCR products were then directly cloned using TA Cloning Kit Dual Promoter (pCRII) from Invitrogen, according to the manufacturer's instructions. One Shot INVαF′ Chemically Competent *E. coli* (Invitrogen) was transformed using the vector previously prepared. Transformed colonies grown in selective medium and the plasmids were purified using an Illustra Plasmid Prep Mini Spin Kit (GE Healthcare).

The fragments were sequenced using the plasmid internal primers and their identities confirmed by comparison with sequences in the database. Obtained sequences were also deposited in the database with GenBank Accession numbers GQ389669−GQ389686 (Table 1).

Relative Quantification of *Juniperus* sp. Transcripts by Quantitative Real-Time RT-PCR. Primers for real-time RT-PCR were designed using the conserved regions of sequences previously obtained for both species. Beacon Designer 7.5 (PREMIER Biosoft International) software was used, and primers were designed to obtain products with sizes between 80 and 200 bp (Table 1).

Total RNA isolation, DNase treatment, and cDNA synthesis were performed as described above.

Real-time RT-PCR was performed using an iCycler iQ system (Bio-Rad). Each reaction contained 10 μL of 2× iQ SYBR Green Supermix (Bio-Rad), 1.0 μL of diluted cDNA sample, and 0.5 mM of each gene-specific primer in a final volume of 20 μL. The following program was applied: 95 °C, 3 min; then 45 cycles at 94 °C, 10 s; 60 °C, 20 s; 72 °C, 30 s. All reactions were performed in triplicate. To check the specificity of the PCR reaction, melting curves were analyzed for each data point. Efficiency was determined by comparison of experimentally determined and theoretically expected threshold values, in cDNA mixtures using serial dilutions. ACT, EF1A, and GAPDH were used for internal normalization. The expression rates were determined using Gene Expression Analysis for iCycler iQ Real-Time PCR Detection System v1.10-2004 (Bio-Rad). The calculation method took into account the different amplification efficiencies determined for each gene and also calculated a normalization factor, which is the geometric mean of the relative quantities for all reference genes.

Statistical Analysis. The results reported in this work are the averages of at least three independent experiments and are represented as the mean ± SD. Differences among treatments were detected by analysis of variance with Tukey's Honestly Significant Difference multiple-comparison test (α = 0.05) using SigmaStat 3.10 (Systat).

■ RESULTS

Seasonal Evaluation of Phytochemicals. To understand the seasonal dynamics of phytochemical compounds in the four different juniper species, plants were grown in the same environmental conditions to avoid differences in key growth drivers such as soil, moisture, temperature, and solar exposure among other parameters.

The highest value of TPC was obtained for *J. turbinata* and the lowest for *J. phoenicea* (Table 2). These were also the two species for which the highest differences between the minimum and maximum values attained through the year were observed. These differences were ∼235, 118, 82, and 78% for *J. phoenicea*, *J. turbinata*, *J. oxycedrus badia*, and *J. navicularis*, respectively. The highest TPC values were usually registered in the winter (months of October−January) and for *J. navicularis* and *J. turbinata* also in August and September. The lowest values were noted at the beginning of spring (February/March to April/May) depending on the species and also in July, except for *J. turbinata*. A similar pattern was observed across the species for

TFC (Table 2). However, the highest TFC was exhibited by *J. oxycedrus badia* and the lowest in *J. phoenicea*.

Selected samples were analyzed for phytochemical composition using LC-MS. The months chosen were April, May, July, and December. The rationale was to analyze junipers at the stage of active growth (April), the stage when they stop growing (May), and the months when most junipers register low levels of TPC and TFC (July) and high levels of TPC and TFC (December).

Phytochemicals were then identified (Figure S1 in the Supporting Information) and their levels quantified relative to each other (Figure 1; Table S1 in the Supporting Information).
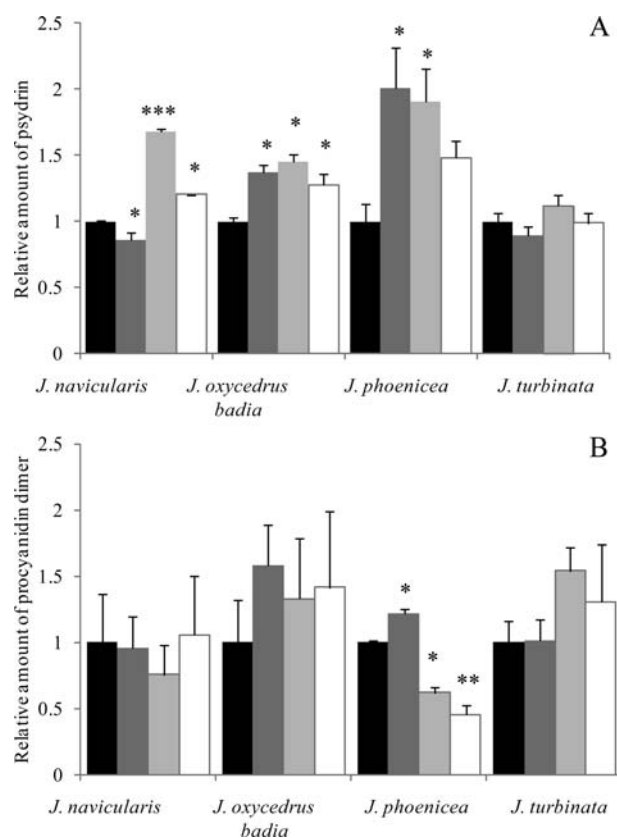

Figure 1. Relative quantification of psydrin (A) and procyanidin dimer (B) for the four *Juniperus* sp. in April (black bars), May (dark gray bars), July (light gray bars), and December (white bars). Phytochemicals were quantified by LC-MS and normalized against the April values, which was the month generally presenting the lowest TPC contents. Statistical differences relative to April values are indicated by ∗ ($p < 0.05$), ∗∗ ($p < 0.01$), and ∗∗∗ ($p < 0.001$).

Analyzed on the basis of equivalent phenol content, LC-MS identified only two compounds as showing differentially significant quantitative changes across the seasons: psydrin (5-methyl-4-*O*-(β-D-glucopyranosyl)-3(2*H*)-furanone, peak 1, RT 8.22 (Figure S1 and Table S1 in the Supporting Information; Figure 1A); and a procyanidin dimer, peak 7, RT 15.87 (Figure S1 and Table S1 in the Supporting Information; Figure 1B). Psydrin exhibited significant changes in *J. navicularis*, *J. oxycedrus badia*, and *J. phoenicea* but not in *J. turbinata*. Levels of psydrin increased in May, July, and December in comparison with the levels in April. This increase was ∼50% for *J. navicularis* (July) and *J. phoenicea* (May and July). The exception was in May for *J. navicularis*, when psydrin levels slightly decreased. Only *J. phoenicea* showed statistically

significant alterations in the levels of the procyanidin dimer, its levels increasing in May and decreasing in July and December.

Changes in Polyphenol Composition in Response to Stress. Young plants of *J. oxycedrus badia*, *J. phoenicea*, and *J. turbinata* were submitted to drought and salinity stresses as well as MeJa elicitation. These stresses are reported to induce changes in phenolic content in plants.[25−27] Young plants were selected because they are normally more responsive to stress treatment. However, drought stress did not significantly alter TPC and TFC (results not shown). Different *Juniperus* species responded differently to salt stress and MeJa treatment. *J. oxycedrus badia* was the only species that responded to salt stress (Figure 2) and *J. phoenicea* the only species that responded to MeJa treatment (Figure 3).
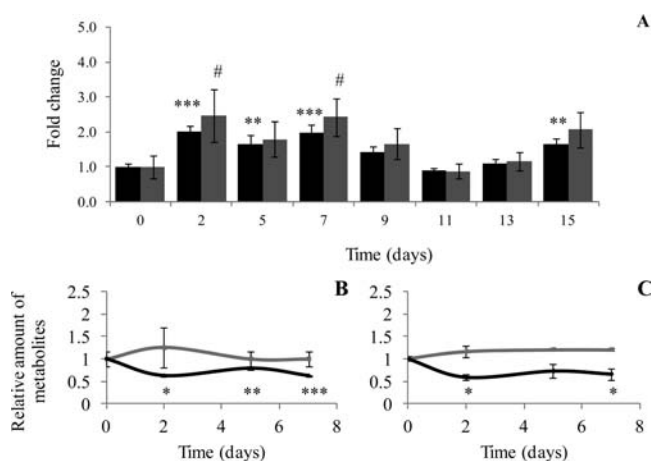

Figure 2. Changes in total phenol content and total flavonoid content in *J. oxycedrus badia* subjected to salt stress. TPC (black bars) and TFC (gray bars) were evaluated (A). Significant statistical changes in total phenolic content in comparison with plants at 0 days are indicated by ∗ ($p < 0.05$), ∗∗ ($p < 0.01$), and ∗∗∗ ($p < 0.001$); significant statistical changes in total flavonoid content in comparison with control plants are indicated by # ($p < 0.05$). LC-MS also noted that the contents of epicatechin (B) and procyanidin trimer (C) were the only two compounds that changed due to salt stress. Values of control plants are represented by the gray line and as black values for the salt-stressed plants. Significant statistical changes in metabolite levels in comparison with control plants are indicatted by ∗ ($p < 0.05$), ∗∗ ($p < 0.01$), and ∗∗∗ ($p < 0.001$).

In response to salt stress, *J. oxycedrus badia* showed increased TPC and TFC (Figure 2A). These increases were noted 2 days after stress application and persisted until day 7. Then, TPC and TFC returned to initial levels, with only a slight increase in TPC on day 15. To assess if changes in total phenol content were accompanied by qualitative changes in composition, the samples were analyzed by LC-MS. For the stress experiments, 11 major peaks that were found in most of the juniper samples were picked for quantification. These made up the major peaks/components present. However, other peaks present in specific species did not appear to vary upon treatments. On the basis of an equivalent amount of phenols, the profiles were very similar, and only the levels of epicatechin (Figure 2B; Table S2 in the Supporting Information) and a procyanidin trimer were altered significantly by the salt treatment (Figure 2C; Table S2 in the Supporting Information). In comparison with control plants, both epicatechin and the procyanidin dimer decreased between the second and seventh days of salt stress.
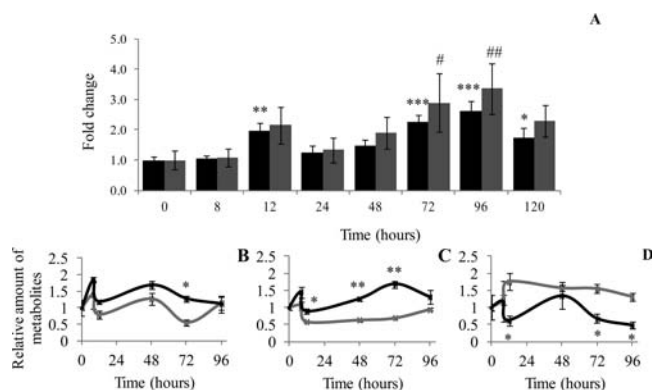

Figure 3. Changes in total phenol content and total flavonoid content in *J. phoenicea* subjected to MeJa treatment. TPC (black bars) and TFC (gray bars) were evaluated (A). Significant statistical changes in total phenolic content in comparison with plants at 0 h are indicated by ∗ ($p < 0.05$), ∗∗ ($p < 0.01$), and ∗∗∗ ($p < 0.001$); significant statistical changes in total flavonoid content in comparison with control plants are indicated by # ($p < 0.05$) and ## ($p < 0.01$). LC-MS also noted that the contents of procyanidin dimer (B), epicatechin (C), and quercetin-rhamnoside (D) were the only three compounds that changed due to MeJa treatment. Values of control plants are represented by the gray line and as black values for the elicited plants. Significant statistical changes in metabolite levels in comparison with control plants are indicated by ∗ ($p < 0.05$), ∗∗ ($p < 0.01$), and ∗∗∗ ($p < 0.001$).

Following MeJa application, TPC and TFC changed significantly (Figure 3A). The response was biphasic, with an initial increase at 12 h after treatment, which was accompanied by a nonsignificant increase in TFC, followed by a decrease. At 72 h, both parameters (TPC and TFC) showed significant increases that persisted until 96 h. LC-MS analysis revealed that the only compounds that were differentially altered in response to the treatment were a procyanidin dimer, epicatechin, and quercetin-rhamnoside (Figure 3, panels B, C, and D, respectively). In comparison with control plants that were sprayed only with Tween 20, MeJa induced increases in a procyanidin dimer 72 h after treatment (Figure 3B; Table S3 in the Supporting Information). Epicatechin also clearly increased from 12 to 72 h after treatment (Figure 3C; Table S3 in the Supporting Information). Quercetin-rhamnoside levels decreased compared to control at 12 h after MeJa application, recovered to control levels at 48 h, and then were reduced at 72−96 h (Figure 3C; Table S3 in the Supporting Information).

Transcription Regulation of Enzymes from Phenylpropanoid Biosynthetic Pathway as Response to Stresses. To verify the transcriptional regulation of the synthesis of metabolites affected by stresses, the mRNA levels of some crucial enzymes of the phenylpropanoid biosynthetic pathway were determined by real-time RT-PCR. For that, specific nucleotide sequences of *J. oxycedrus badia* and *J. phoenicea* were determined. Relative quantification of transcripts was determined for each enzyme using a normalization factor calculated using the relative quantities of the reference genes ACT, EF1A, and GAPDH. Transcript levels of reference genes were monitored for all conditions tested, and it was verified that their levels did not vary significantly.

In response to salt stress, the levels of PAL and ANR transcripts increased in *J. oxycedrus badia* (Figure 4A). For both enzymes, their transcript accumulation was enhanced 2 days after stress initiation and persisted until day 5. The relative
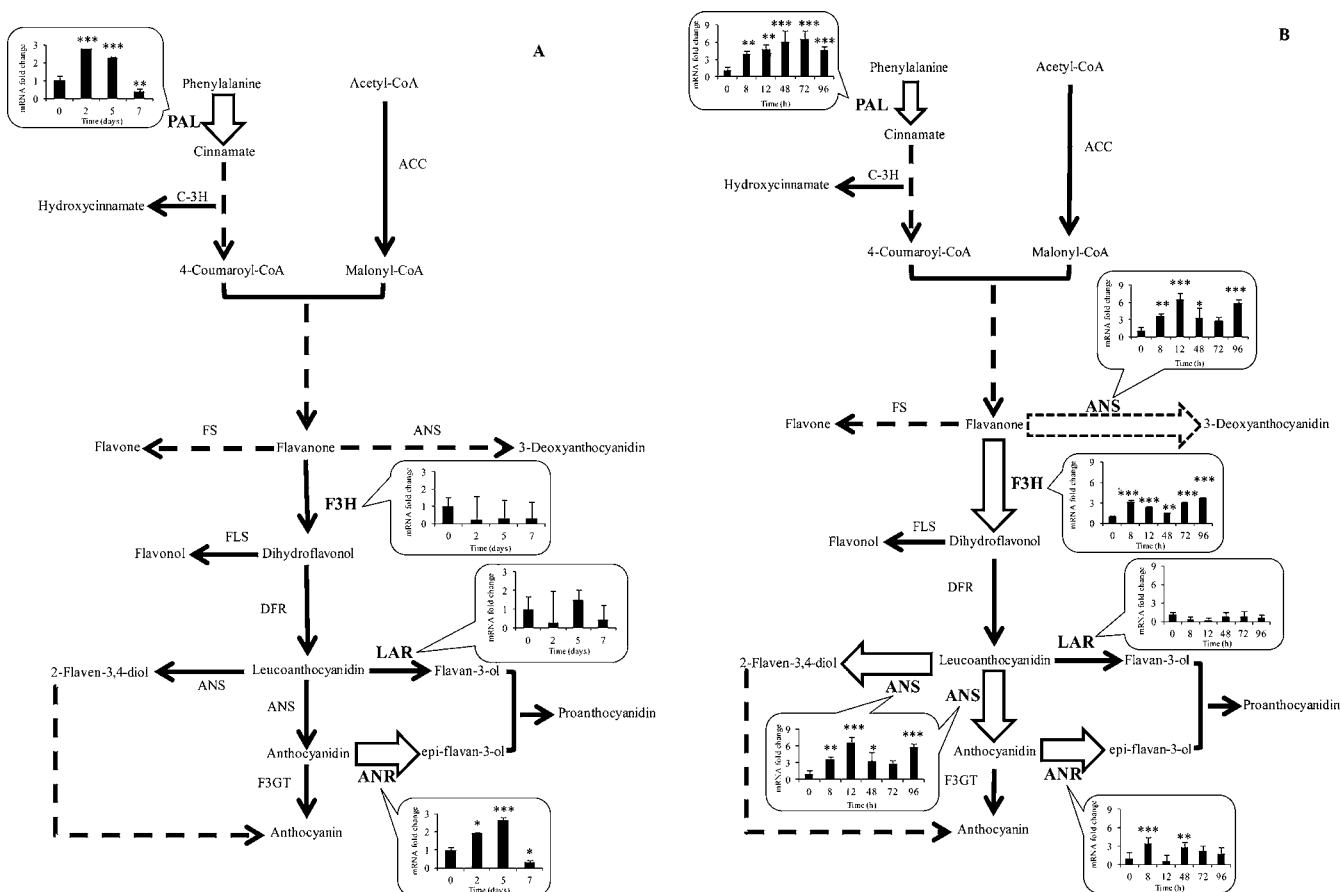
Figure 4. Schematic representation of the main alteration in expression of enzymes of the phenylpropanoid biosynthetic pathway under stress conditions: *J. oxycedrus badia* subjected to salt stress (A) and *J. phoenicea* subjected to MeJa treatment (B). Direct reactions are denoted with solid arrows and indirect reactions with dashed arrows. Enzymes whose mRNA levels were analyzed are written in bold. Reactions mediated by enzymes whose mRNA showed significant differences due to stress are highlighted with block arrows. Graphical representation of mRNA fold change are shown in the respective balloons. Results were obtained from three independent replicates, and significant statistical changes are indicated by ∗ ($p <$ 0.05), ∗∗ ($p < 0.01$), and ∗∗∗ ($p < 0.001$). ACC, acetyl-CoA carboxylase (EC 6.4.1.2); ANR, anthocyanidin reductase (EC 1.3.1.77); ANS, anthocyanidin synthase or leucocyanidin oxygenase (EC 1.14.11.19); C-3H, *p*-coumarate 3-hydroxylase (EC 1.14.x.x); DFR, dihydroflavonol-4-reductase (EC 1.1.1.219); F3GT, flavonoid-3-glucosyltransferase or anthocyanidin/flavonol 3-*O*-glucosyltransferase (EC 2.4.1.115); F3H, flavanone 3-hydroxylase or flavanone 3-dioxygenase (EC 1.14.11.9); FLS, flavonol synthase (EC 1.14.11.23); FS, flavone synthase (EC 1.14.11.22); LAR, leucoanthocyanidin reductase (EC 1.17.1.3); PAL, phenylalanine ammonia-lyase (EC 4.3.1.5).

accumulations of F3H and LAR transcripts were not significantly changed in response to salt stress.

On the other hand, MeJa treatment induced changes in the accumulation of PAL, ANS, F3H, and ANR transcripts in *J. phoenicea* (Figure 4B) but no change in transcript level for LAR. The level of PAL transcripts increased 8 h after treatment and stayed high until 96 h. ANS and F3H also showed a biphasic response with increases within 12 h and a second peak at 96 h. The expression of ANR was slightly different as it was increased at 8 h compared to control, reduced at 12 h, and increased at 48 h and then was maintained to 96 h.

■ DISCUSSION

Seasonal Evaluation of Phytochemicals. Phytochemicals from some Portuguese *Juniperus* sp. have recently been described as exhibiting potential in the development of therapies for the prevention of neurodegenerative diseases.[17] Therefore, to translate this to practical application it is important to understand the seasonal dynamics of phytochemical compound production. Here, four Portuguese juniper species were evaluated and grown under the same environ-

mental conditions to eliminate any growth condition differences. Although some differences in TPC and TFC among the different species were found (Table 2), some general trends were observed. The highest TPC was obtained in the winter months of October–January, and the lowest values were seen in the beginning of spring (February/March–April/May) and in July. A similar tendency was verified for TFC. In the literature, the seasonal dynamics of polyphenolic compounds have been reported. For example, in *Salix* species,[28] phenolic glycoside levels decreased over the growing season, with the highest contents detected during plant dormancy (winter). A similar trend was also seen for *Apocynum venetum* and *Poacynum* species,[29] for which the influence of environment (geolocation and climate) was also shown to be significant with respect to polyphenol content.

To identify and quantify phytochemicals that may be preferentially affected due to environmental factors varying during the seasons, extracts were evaluated by LC-MS based on the same amount of phenol content. The phytochemical profile of *Juniperus* sp. gives a characteristic LC-MS profile and is relatively similar within species.[17] Consequently, our focus was in the relative quantification of compounds that changed

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significantly with respect to the different conditions. The chemical profile of juniper species presented only small differences. The compounds that were significantly altered among species (Figure 1) were psydrin (peak 1, RT 8.22, in Figure S1 and Table S1 in the Supporting Information) and a procyanidin dimer (peak 7, RT 15.87, in Figure S1 and Table S1 in the Supporting Information). Psydrin exhibited seasonal changes in *J. navicularis*, *J. oxycedrus badia*, and *J. phoenicea*. For all of these species psydrin levels are higher after the phase of active growth (April). The content of the procyanidin dimer changed only in *J. phoenicea* in May, the month after active growth, when its levels were highest.

Resource allocation hypotheses such as the carbon-nutrient balance[30] and growth differentiation balance[31] have proposed that changes in carbon source–sink relationships may be a consequence of carbon availability. That availability determines variations in the relative partitioning of carbon to growth and production of carbon-based secondary metabolites. Secondary metabolite production has been found in diverse species (such as *Zingiber officinale*,[32] *Digitalis lanata*,[33] and *Hypericum perforatum*[34]) to increase as a result of primary metabolism enhancement, for instance, through CO_2 modulation. The shikimic acid pathway is able to convert simple carbohydrate precursors derived from glycolysis and the pentose phosphate pathway into aromatic amino acids,[32] such as phenylalanine, the precursor compound of the phenylpropanoid biosynthetic pathway.

In Mediterranean conditions, temperatures and water availability in winter and summer are more extreme for plants, which may decrease photosynthetic efficiency.[35,36] In spring and autumn, conditions that allow a high photosynthetic yield are exploited and active growth occurs. Thus, it is reasonable to hypothesize that during periods of growth inactivity (winter and summer), available carbon may be reallocated to secondary metabolites. Under conditions favorable to growth, the opposite may occur. Our results fit this hypothesis as maximum TPC and TFC were found in winter and minimum values in spring. The production of secondary metabolites was modulated across the seasons, but the main differences were in the total levels of phenolics, not in their composition. Any enrichment in a few metabolites (psydrin and a procyanidin dimer) was mostly observed after the period of active growth.

Influence of Stress on Polyphenol Production. To assess the influence of stresses on secondary metabolite production, young plants from *J. oxycedrus badia*, *J. phoenicea*, and *J. turbinata* were submitted to drought, salinity, and MeJa elicitation. However, only *J. oxycedrus badia* changed its phenolic content as a response to salt stress and *J. phoenicea* as a consequence of MeJa elicitation. The fact that *J. oxycedrus badia* was the only species that exhibited enhanced TPC and TFC contents in response to salt stress (Figure 2A) is likely to be associated with the adaptation of this species to mountainous environments rather than the saline coastal regions, favored to the other species. In the literature, contradictory effects of salinity on phenolic contents can be found, depending on the plant and salinity levels applied.[25,37] Although there was a general trend of increasing TPC and TFC with salt treatment, there were also changes in the relative amounts of specific compounds (Figure 2B,C; Table S2 in the Supporting Information) as determined by LC-MS. However, changes in the relative amounts were significant only for epicatechin (peak 8, RT 16.74, in Figure S1 in the Supporting Information) and a procyanidin trimer (peak 9, RT 17.32, in Figure S1 in the

Supporting Information). The relative contents of these two particular metabolites were decreased by salt treatment. Other phenolics showing increases in their relative amounts (without reaching significance) were quercetin rutinoside (peak 13, RT 21.86, in Figure S1 in the Supporting Information) and luteolin/isoscutellarein rhamnoside (peak 21, RT 27.88, in Figure S1 in the Supporting Information). It appears that the salt stress produced a general increase in polyphenol content per se and that a differential response in specific polyphenolics is minor. These alterations in metabolites were accompanied by increases in transcripts of PAL and ANR enzymes (Figure 4A). The significant enhancement of PAL transcripts corroborates the hypothesis of a general enrichment in phenolic compounds, without specific enhancement/reduction of any particular class of compounds. Induction of PAL transcripts and activity by salt stress is well reported in the literature for other plants[38,39] as well as by other stresses such as water stress.[40] However, enhancements in ANR transcript levels were accompanied by decreases in epicatechin and procyanidin trimer levels. These results may suggest that post-transcriptional/translational regulation may occur, limiting the translation of those transcripts and/or the activity of those enzymes. Alternatively, reductions in epicatechin or the procyanidin dimers may be associated with enhanced levels of higher molecular weight oligomers and therefore unextractable procyanidins.[41] That regulation should be the theme of future studies to elucidate the specific mechanisms of stress responses in junipers.

Endogenous MeJa is a signaling compound that modulates various physiological processes in plants.[42] Under stresses such as wounding and pathogen attack, volatile MeJa can be released into the air from the wounded plants[43] and can induce plants to increase secondary (defense) metabolite production and consequently enhance their defensive systems. Therefore, exogenous MeJa has been used to usefully induce secondary metabolites such as alkaloids, terpenoids, and phenolics in some plants and plant cell cultures.[44−48] In mushrooms, elicitation using MeJa, besides increasing polyphenols and flavonoids contents, also enhances antioxidant enzyme activity, such as catalase and superoxide dismutase, and decreases polyphenol oxidase transcription and activity.[49] In the present work *J. phoenicea* was the only species that responded to MeJa by increasing TPC and TFC levels (Figure 3A). The TPC response showed a biphasic pattern that was reflected in the increase of the relative amounts of the procyanidin dimer (peak 7, RT 15.87, in Figure S1 in the Supporting Information) and epicatechin (peak 8, RT 16.74, in Figure S1 in the Supporting Information) (Figure 3B,C; Table S3 in the Supporting Information). Conversely, the relative amount of quercetin-rhamnoside (peak 19, RT 25.43, in Figure S1 in the Supporting Information) was reduced, also in a biphasic manner (Figure 3D; Table S3 in the Supporting Information). These results are corroborated by the transcriptomic analysis (Figure 4B). MeJa induced the gene expression of PAL from 8 to 96 h, which would drive a generalized augmentation of phenolic compounds. Induction of PAL transcripts by MeJa is well reported in the literature[47,50] as well as enhanced PAL activity.[48] Increases in F3H, ANS, and ANR transcripts were also registered, and this was reflected in the levels of some of their associated metabolic end product points (procyanidin dimer, epicatechin). The increase in transcripts of this branch could lead to metabolic flux deviation to the synthesis of proanthocyanidin and epiflavan-3-ol, thus disfavoring flavonol biosynthesis.

Overall, the four juniper species presented a common pattern of phytochemical production with higher levels in periods when plants were not actively growing and lower levels in the remaining periods. Therefore, seasonal conditions modulated the total content of phenolics and flavonoids. However, qualitatively, phytochemical composition was not greatly changed, with only the relative contents of psydrin and a procyanidin dimer enhanced in some months, and this varied according to species. Salt stress and MeJa elicitation were effective in enhancing phenolic accumulation; however, different susceptibilities were exhibited by the different species. In addition to the increase of total phenolic and flavonoid contents, both stresses caused alterations in the relative phytochemical composition. Procyanidins, epicatechin, and quercetin derivatives were the most responsive to stresses, being altered their relative amounts in both stresses. Salt stress reduced procyanidin and epicatechin contents in *J. oxycedrus badia* extracts and enriched quercetin derivatives, whereas the opposite effect was noted after MeJa elicitation in *J. phoenicea*. This suggests differential metabolic regulation in response to those stresses between species. The mechanisms underlying these responses are important in considering juniper a viable and sustainable source of compounds/extracts for end-user industries such as pharmaceuticals and nutraceuticals.

◼ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figure S1 and Tables S1−S3. This material is available free of charge via the Internet at http://pubs.acs.org.

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◼ ABBREVIATIONS USED

ACT, actin; ANR, anthocyanidin reductase; ANS, anthocyanidin synthase; CE, catechin equivalents; EF1A, elongation factor 1α; F3H, flavanone 3-hydroxylase; GAE, gallic acid equivalents; GAPDH, glyceraldeyde 3-phosphate dehydrogenase; LAR, leucoanthocyanidin reductase; MeJa, methyl jasmonate; PAL, phenylalanine ammonia-lyase; TE, trolox equivalents; TFC, total flavonoids content; TPC, total phenolic content

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