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Investigation of the Expression Pattern of Key Genes in the Biosynthetic Pathway of Pinene and Limonene Metabolites in Different *Juniperus* Species from North Khorasan, Iran

Running Title: Expression of Key Genes in the Biosynthetic Pathway of Pinene and Limonene in *Juniperus*

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ABSTRACT

Medicinal plants play a crucial role in the biodiversity of many countries and are rich sources of secondary metabolites, forming the basis for numerous valuable pharmaceuticals. Although the biosynthesis of these compounds is under genetic control, it is profoundly influenced by environmental factors. In this study, the expression of key genes involved in the monoterpene biosynthetic pathway, including *limonene synthase (lim)*, *pinene synthase (pin)*, and *ARM repeat proteins (arm)*, was investigated in three *Juniperus* species (*Juniperus sabina*, *J. polycarpus*, and *J. communis*) across two tissues (leaf and fruit). Field samples were collected from the Salouk region in North Khorasan province. Genes involved in the biosynthesis of the main metabolites were identified, and specific primers were designed. Subsequently, RNA extraction, cDNA synthesis, and PCR optimization were performed, and finally, gene expression was evaluated using the qRT-PCR method. The results showed that gene expression was significantly affected by both tissue type and plant species. Specifically, *pin* gene expression in fruit ranged from 0.0399 in *J. communis* to 0.152 in *J. sabina*, and in leaf from 0.0023 to 0.0093, indicating a substantial increase in expression in fruit compared to leaf. Furthermore, *lim* gene expression in fruit ranged from 0.0149 in *J. communis* to 0.0285 in *J. sabina*, and in leaf from 0.0019 to 0.0202, demonstrating increased expression in fruit across all species. Regarding the *arm* gene, expression in fruit was higher in *J. communis* and *J. sabina* compared to *J. polycarpus*, with expression values of approximately 0.0334 in *J. communis* fruit and 0.0324 in *J. sabina* fruit, while in leaf, values were similar to or lower than these amounts in other species. These results indicate that *pin* and *lim* genes exhibit the highest expression levels in fruit, and the *arm* gene displays differential expression patterns depending on the species and plant tissue.

Keywords: Alpha-pinene, *arm* gene, *lim* gene, Limonene, *pin* gene, qRT-PCR analysis

INTRODUCTION

The use of medicinal plants as one of the oldest therapeutic methods has evolved alongside human civilization and has played a fundamental role in providing healthcare for centuries. Despite significant advancements in the pharmaceutical industry in recent decades, the side effects associated with chemical drugs have led to renewed interest in natural sources and herbal medicines [1]. Statistics indicate that approximately 80% of the world's population relies on medicinal plants for primary healthcare, and nearly half of all prescribed drugs are of plant origin [2].

The active compounds in medicinal plants are primarily secondary metabolites; low-molecular-weight molecules with diverse chemical structures that are produced in response to biological and environmental stimuli [3, 4]. These compounds are not only responsible for the therapeutic properties of plants but also play a significant role in coping with environmental stresses. Their production is governed by complex genetic networks, the expression of which can be influenced by factors such as temperature, light, altitude, soil type, and biotic stresses [5, 6]. Therefore, analyzing the expression patterns of genes involved in the biosynthetic pathways of secondary metabolites is a crucial step in understanding regulatory mechanisms, identifying key genes, and optimizing the production of pharmaceutical compounds [7].

Among these, juniper trees (*Juniperus spp.*) from the Cupressaceae family hold a special position due to their valuable phytochemical compounds, resilience to harsh climatic conditions, and significant ecological role. These native plants, particularly in mountainous and arid regions of Iran such as North Khorasan province, play an effective part in soil conservation, erosion control, and biodiversity sustainability [8]. Out of approximately 60 *Juniperus* species worldwide, five are found in Iran, with three species, *J. communis*, *J. foetidissima*, and *J. polycarpus*, selected as target species in this research.

Phytochemical assessments have shown that the essential oil compounds present in various organs of the juniper plant, particularly monoterpenes such as α-pinene, β-pinene, and limonene, are important secondary metabolites with pharmaceutical, antioxidant, antibacterial, and aromatic properties [9]. The biosynthesis of these compounds begins via the MEP/DOXP pathway in plastids and is catalyzed by enzymes such as Geranyl diphosphate synthase (GPPS) and monoterpene synthases like α-pinene synthase and limonene synthase [10]. Investigating the expression of genes in these pathways, along with regulatory factors such as ERF transcription factors and ABC transporters, can provide new perspectives on genetic modification, sustainable utilization, and conservation of the genetic resources of these species [11].

Previous studies have investigated the phytochemical composition of *Juniperus communis* and reported considerable diversity in major monoterpenes such as alpha-pinene and limonene [12].

Given the biological, pharmaceutical, and ecological importance of species within the genus *Juniperus*, this research was designed to comprehensively investigate the expression of genes involved in the synthesis of secondary metabolites in three selected juniper species in the natural habitats of northeastern Iran. The study focuses on analyzing the expression patterns of key genes in the biosynthetic pathway of terpenoids and other secondary metabolites. The main hypothesis of the research is that the genetic diversity among different juniper species and differences in ecological conditions lead to variations in the expression patterns of biosynthetic pathway genes, resulting in differential production of secondary metabolites. The findings of this study can play a significant role in formulating breeding programs, genetic conservation, and sustainable management of plant resources.

The present study was conducted to investigate the expression of these biosynthetic genes at the molecular level in the leaf and fruit tissues of selected *Juniperus* species, aiming to provide new insights into the genetic basis of monoterpene biosynthesis.

MATERIAL AND METHODS

Field Sampling and Identification of *Juniperus* Species

This study was conducted as an integrated project involving both field (habitat) and laboratory components. In the field section, to investigate the expression pattern of genes responsible for synthesizing secondary metabolites in three selected species of the genus *Juniperus*, namely *J. sabina*, *J. polycarpus*, and *J. communis*, sampling was performed from their natural habitats in North Khorasan province. The study area was located in the Salouk region of North Khorasan province, situated at approximately 37°19.0242′ N and 57°03.7232′ E. These areas were selected due to the wide distribution of the target species in mountainous habitats and their diverse ecological conditions, enabling a more precise assessment of phenotypic diversity. After sample collection, to ensure accurate species identification, the plants were taxonomically identified by experienced botanists. For scientific documentation, herbarium specimens of the collected species were prepared and registered with specific accession numbers at the Herbarium of the Agricultural and Natural Resources Research Center of North Khorasan: Khsh.CU/J. communis 101, Khsh.CU/J. polycarpus 102, and Khsh.CU/J. sabina 103.

The Salouk region in North Khorasan province has a semi-arid climate, characterized by relatively hot summers and cold winters. Annual rainfall is low, and relative humidity fluctuates throughout the year. This region experiences a significant frost period, and the plant growing season typically extends from spring to early autumn. *Juniperus* habitats are mainly distributed in mountainous areas with calcareous and rocky soils at elevations above 2000 meters above sea level. Climatic information for the region was extracted from meteorological data for the period 2018-2024 (Table 1).

Table 1 Summary of climatic conditions at the *Juniperus* sampling site (2018-2024)

Climatic Parameter	Observed Value
Temperature range (°C)	9.7 – 22.8
Mean temperature (°C)	16.1
Relative humidity range (%)	32 – 78
Mean relative humidity (%)	54.3
Mean daily precipitation (mm)	≈ 1.0
Mean wind speed (m/s)	8.9

Soil Sampling and Preparation for Physical and Chemical Analysis

To evaluate the physical and chemical characteristics of the soil and investigate their relationship with the quantity and quality of the studied species, soil samples were collected from each of the three plant collection areas along a 100-meter transect in each habitat. Samples were taken from beneath the plant canopy at a depth of 30 cm, as this layer has the most contact with the roots and directly affects plant growth and nutrition. This depth also provides a suitable reflection of the ecological conditions of the plant growth substrate.

Soil samples were air-dried upon arrival at the laboratory. They were then manually ground in a mortar, milled, homogenized, and passed through a 2 mm mesh sieve. This step aimed to homogenize the samples and increase the accuracy and reproducibility of the results.

Subsequently, various physical and chemical parameters of the soil were measured, including total nitrogen (N), absorbable phosphorus (P), potassium (K), electrical conductivity (EC), lime content, organic carbon, soil texture (percentage of sand, silt, and clay), and trace elements (micronutrients). Analysis of these indices was conducted to better understand the environmental conditions of the habitats and to evaluate the role of soil characteristics in the distribution and growth of *Juniperus* species.

Identification of Genes Involved in the Synthesis of the Plant's Main Metabolites

To identify genes and enzymes related to the synthesis of the main metabolites present in the essential oils of the studied *Juniperus* species, the predominant chemical compounds of this plant were first extracted using reliable scientific sources and literature review. Based on previous findings, the three predominant compounds in the essential oils of these species are α-pinene, β-pinene, and limonene, all classified as monoterpenoids.

In the next step, for a more detailed analysis of the biological pathways associated with the synthesis of these compounds, the KEGG (Kyoto Encyclopedia of Genes and Genomes) database was utilized. By searching for the aforementioned metabolites in KEGG, related biosynthetic pathways, along with the enzymes and genes involved in each step of the synthesis process, were identified. These pathways are primarily subsets of terpene biosynthesis pathways, especially monoterpene biosynthesis.

Key enzymes identified in these pathways include monoterpene synthases, which have a catalytic role in converting isoprenoid precursors to monoterpenic compounds such as α-pinene, β-pinene, and limonene. The genes encoding these enzymes are primarily classified within the TPS (terpene synthase) family.

Identifying these pathways and related genes provides a suitable foundation for subsequent molecular studies, including gene expression analysis, investigation of genetic diversity, or genetic modification to enhance the production of active pharmaceutical compounds in different juniper species.

Continuing the process of identifying biosynthetic pathways, genes responsible for synthesizing key enzymes were also extracted using the KEGG database. These genes are recognized as key molecular elements in regulating and controlling the production of target metabolites and play a direct role in the biosynthesis of important compounds such as α-pinene, β-pinene, and limonene.

Through a detailed examination of biological pathway maps in the KEGG database, genes encoding enzymes such as α-pinene synthase, β-pinene synthase, and limonene synthase were identified, and their position in the terpenoid biosynthetic chain was analyzed. These genes are located in distinct but related pathways of monoterpene synthesis and are considered control points in regulating metabolic pathways.

The information obtained from this stage will serve as a valuable foundation for future studies in molecular fields, such as gene expression analysis, investigation of functional gene diversity, or planning for genetic modification aimed at increasing the yield of secondary compounds (Figure 1).

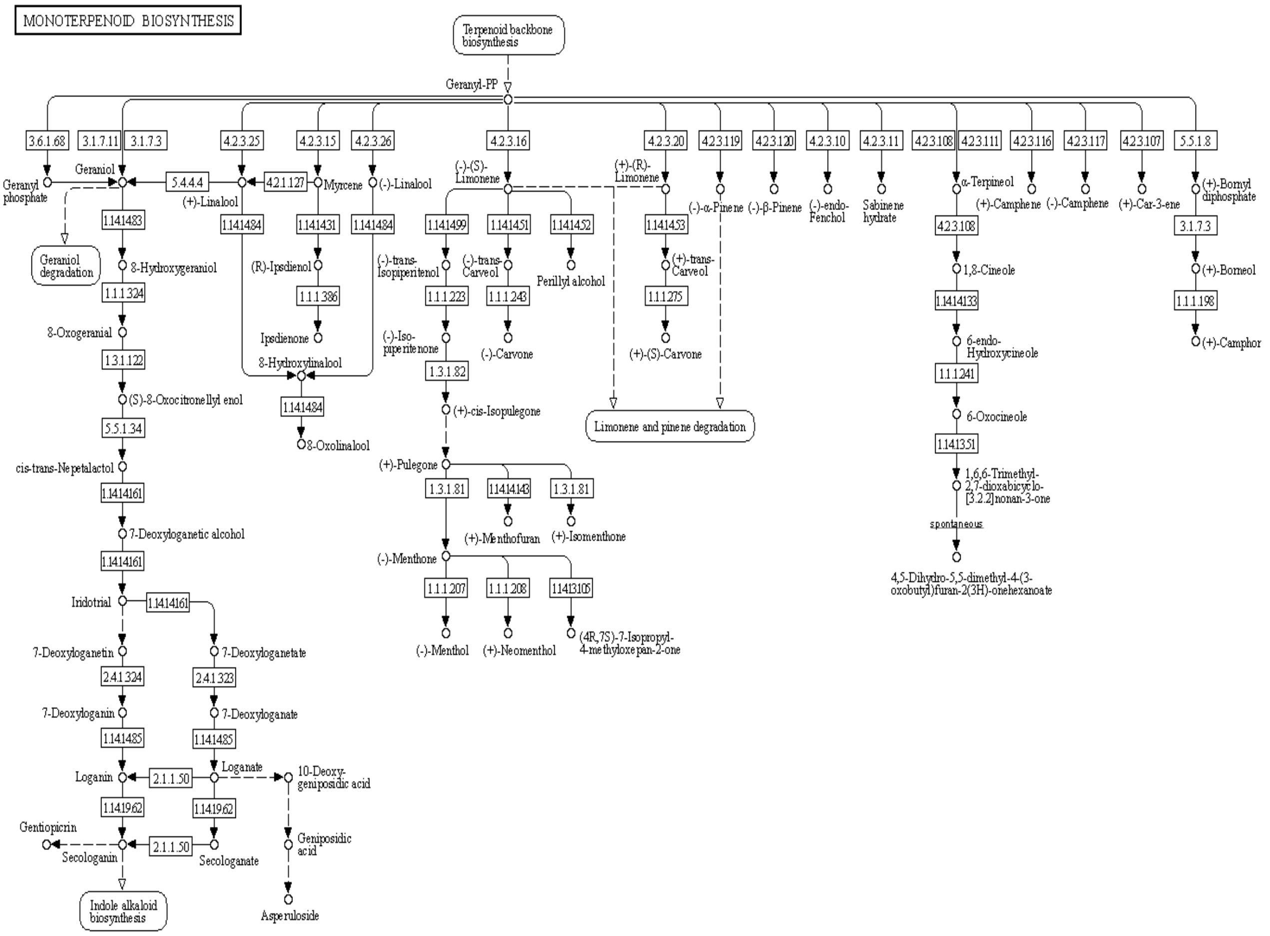

Fig. 1 Biosynthetic pathways and genes responsible for the synthesis of key enzymes were identified using the KEGG database [13].

Sequence Selection and Primer Design

To design specific primers for genes involved in the synthesis of target metabolites, nucleotide sequences of the relevant genes from related species such as *Abies grandis* and *Pinus taeda* were first extracted from the NCBI database. These sequences were comparatively analyzed using the BLAST tool on NCBI to identify homologous genes in related plant species. Sequences with the highest identity were then selected and aligned using the T-Coffee software to determine conserved regions suitable for primer design.

For designing Real-Time qPCR primers, conserved regions near the poly-A tail were chosen as design targets, with an amplicon product length between 150 and 250 base pairs. Technical characteristics of the primers, including homodimer and heterodimer formation tendencies, stem-loop structures, GC percentage, and melting temperature (Tm), were carefully evaluated using Oligo 7 and Vector NTI Express Designer software.

For normalization of gene expression data, the *Elongation factor* gene was selected as the housekeeping gene. Designed primers were synthesized by Bioneer Company (South Korea) and used in the experiments. Complete primer specifications, including nucleotide sequence, Tm, length, and other features, are provided in Table 2.

Table 2 Nucleotide sequences of selected primers and their characteristics.

Gene	Primer Sequence (5' → 3')	PCR Product Length (bp)	TM (°C)	PCR Amplification Efficiency
PIN	F: GGCTCAAGGAAGAGACACGCTC R: TCACTGATCATGGCGTTG	195	57 61	1.83
ARM	F: GCAAAGACAAGCCCTCC R: CCCTCTAATACCATGTCAT	207	66 65	1.97
LIM	F: TTCAACATTGTGCAGGC R: CCATAGACATCGTAAATATCATC	236	63 61	1.99

RNA Extraction and Quality Assessment

To prevent RNA degradation by RNase, all used equipment, including tubes, mortars, falcons, and other laboratory tools, was sterilized by autoclaving twice for 20 minutes at 121°C and 1.2 atmospheres of pressure.

During the extraction step, 100 mg of plant leaf tissue was first completely powdered in liquid nitrogen and transferred to 2 ml tubes. RNA extraction was performed using the RNeasy Plant Mini Kit (Qiagen, UK) according to the manufacturer's instructions.

400 µL of buffer AP1 and 4 µL of DNase were added to each tube. After vortexing, the mixture was incubated at 65°C for 10 minutes, during which it was gently inverted 2-3 times. Then, 130 µL of buffer P3 was added, and the mixture was kept on ice for 5 minutes. Centrifugation was performed at 20,000 g for 5 minutes at room temperature.

The resulting extract was transferred to a QIAShredder column, and after re-centrifugation, the supernatant was collected. To this solution, 1.5 times the volume of buffer AW1 was added, and after mixing, 650 µL of it was transferred to an RNeasy Mini Kit column and centrifuged. Washing was performed with two aliquots of buffer AW2 for 1 and 2 minutes, respectively (at speeds of 6,000 and 20,000 g). Finally, the column was transferred to a new tube, and 100 µL of buffer AE was added for RNA elution. After 5 minutes of incubation at room temperature and a final centrifugation, the extracted RNA was stored at -20°C.

To assess RNA quality, 1% agarose gel electrophoresis was performed in TAE buffer. Samples were mixed with loading buffer and 5 µL was loaded into each well. Electrophoresis was conducted at 70 V for 40 minutes. The gel was then imaged under UV light using a Gel Doc device, and RNA bands were visualized. Additionally, a NanoDrop device was used to quantify RNA concentration.

cDNA Synthesis and Quality Check

To remove potential genomic DNA contamination, extracted RNA was treated with DNase I enzyme (Code RB125A, RNA Company, Iran). Subsequently, first-strand cDNA synthesis was performed in a 20 µL volume using the corresponding kit according to the manufacturer's instructions. The accuracy of cDNA synthesis and primer performance was confirmed by performing PCR for the target genes and examining the products on agarose gel.

qRT-PCR Analysis

qRT-PCR amplification was performed using a Rotor-Gene 2000 instrument (Corbett Life Science, Sydney, Australia). The PCR reaction was set up in a 20 µL volume using the SYBR® Green Real-Time PCR Master Mix (Code RB120, RNA Company, Iran). The reaction mixture contained 10 µL of 2× SYBR solution, 1 µL of cDNA, 1 µL of each primer (20 nmol concentration), and 7 µL of RNase-free water.

The thermal cycling conditions included an initial denaturation step at 95°C for 5 minutes, followed by 35 cycles of: 1 minute denaturation at 95°C, 1 minute annealing at 50-60°C (according to the primer melting temperatures), and 15 seconds extension at 72°C.

After amplification, a melting curve from 60 to 95°C was recorded and analyzed to confirm the specificity of the amplicons and the absence of non-specific products. Cycle threshold (Ct) values and reaction efficiencies were calculated using LinRegPCR software. Relative gene expression was analyzed and compared based on Ct data and PCR efficiency using REST software. All experiments were performed with three biological and three technical replicates to ensure accuracy.

To determine the expression levels of the studied genes using qPCR, Ct (cycle threshold) values were first obtained for each sample. The Ct value represents the number of cycles at which the fluorescence signal generated from PCR product amplification crosses the threshold limit; a lower Ct value indicates a higher initial template amount and consequently higher gene expression.

Since Ct values can be influenced by technical factors such as initial RNA quantity, RNA quality, cDNA synthesis efficiency, and minor variations in reaction conditions, it is necessary to normalize the data relative to a housekeeping/reference gene. Normalization was performed using the ΔCt method.

In the ΔCt method, for each sample, the difference between the Ct of the target gene and the Ct of the reference gene is calculated as shown in Equation 1:

$$\Delta Ct = Ct_{\text{target}} - Ct_{\text{reference}} \quad (1)$$

To present the results in a quantitative and more interpretable format, ΔCt values were converted to a "normalized expression" scale (Equation 2):

$$\text{Normalized Expression} = 2^{-\Delta Ct} \quad (2)$$

RESULTS

Soil Test Results

The results of soil tests from the growth sites of the different *Juniperus* species showed a soil pH of 7.7, which is slightly higher than the optimal range of 6.5 to 7, indicating relatively alkaline soil conditions. This pH level is generally suitable for nutrient uptake by plants and does not have a significant negative impact on tree growth. The soil electrical conductivity (EC) was measured at 1.3 dS/m, which falls within the desirable range of less than 3, indicating no excessive soil salinity; thus, the conditions are considered suitable for the growth of *Juniperus* species. The soil saturation percentage (SP) was reported as 41%, which is slightly above the optimal range of 35-40%. This may indicate relatively high moisture retention in the soil, which, if excessive, could lead to issues such as reduced root aeration [14]. The neutralizing materials or lime content (TNV) of the soil is 2%, which is within the optimal range and plays a stabilizing role in soil pH. The organic carbon (O.C) content of the soil was reported as 3.8%, higher than the optimal range of 1.5-2%, indicating soil rich in organic matter. This improves soil structure and provides nutrient sources for beneficial microorganisms. The total nitrogen (N) content of the soil is 0.34%, which is higher than the optimal range (0.1-0.2%), indicating soil richness in this vital element for plant growth. On the other hand, the absorbable phosphorus (P_A.V) content is very low, at 2 ppm, which is significantly lower than the optimal range of 10-12 ppm. This deficiency can pose a serious limitation for tree growth, as phosphorus plays a key role in plant energy processes and cell division. In contrast, the absorbable potassium (K_A.V) content is very high, at 962 ppm, exceeding the optimal range of 300-350 ppm, indicating soil richness in this important element. In terms of texture, the soil from the sampling site consists of 36% sand, 60% silt, and 4% clay, classifying it as a silt loam according to soil taxonomy. This soil texture typically has a suitable capacity for water and nutrient retention, but the high silt percentage may lead to reduced soil aeration and permeability. Finally, micronutrients such as iron, copper, manganese, boron, and zinc were not reported or their values are unknown in the soil samples. Given the importance of these elements in plant physiological functions, they need to be evaluated and possibly amended in future experiments. Overall, the soil at the *Juniperus* growth sites is in good condition regarding pH, salinity, organic matter, and potassium content, but phosphorus deficiency and possibly micronutrient deficiencies could hinder optimal tree growth. Therefore, it is recommended to manage soil nutrition with emphasis on correcting phosphorus deficiency and investigating micronutrients, and to monitor soil moisture to prevent excessive saturation percentage.

The results of the comprehensive soil analysis are detailed in Table 3. As shown, the key physical and chemical parameters of the soil at the *Juniperus* growth sites were evaluated against optimal ranges. The data indicate that while the soil is rich in organic matter (O.C = 3.8%) and potassium ($K_{A.V}$ = 962 ppm), it suffers from a significant deficiency in absorbable phosphorus ($P_{A.V}$ = 2 ppm). Furthermore, the levels of essential micronutrients such as Iron (Fe), Copper (Cu), Manganese (Mn), Boron (B), and Zinc (Zn) were below the detection limit or not reported, highlighting potential nutritional limitations for optimal plant growth and metabolite production. The soil texture was classified as silt loam, with a high saturation percentage (SP = 41%), which could influence root aeration and water dynamics. This characterization of the edaphic environment is crucial for interpreting the observed patterns of gene expression and secondary metabolite biosynthesis in the studied *Juniperus* species under their natural habitat conditions.

Table 3 Results of soil tests from the growth sites of different *Juniperus* species.

Parameter	Symptoms	Test Result	Desired Limit	Unit
Soil reaction (acidity)	pH	7.7	6.5-7	—
Electrical conductivity (salinity)	EC	1.3	<3	$dS\ m^{-1}$
Soil saturation percentage	SP	41	35-40	%
Neutralizing materials (lime)	TNV	2	<10	%
Organic carbon	O.C	3.8	1.5-2	%
Nitrogen	N	0.34	0.1-0.2	%
Absorbable phosphorus	$P_{A.\ V}$	2	10-12	ppm
Absorbable potassium	$K_{A.\ V}$	962	300-350	ppm
Sand	Sand	36	40	%
Silt	Silt	60	35	%
Clay	Clay	4	25	%
Soil texture class	—	Silt loam	Loam	—
Iron	Fe	—	5-20	ppm
Copper	Cu	—	0.5-2	ppm
Manganese	Mn	—	5-30	ppm
Boron	B	—	22-60	ppm
Zinc	Zn	—	0.5-6	ppm

Determination of Key Genes in the Biosynthetic Pathways of the Predominant Metabolites of the Juniper Plant

Following phytochemical analyses of the juniper plant (*Juniperus spp.*), the predominant and bioactive metabolites of this plant were identified, which play an important role in its medicinal and biological properties. To identify genes involved in the biosynthesis of these compounds, the information obtained from the analyses was cross-referenced with data from authoritative genomic databases such as KEGG and NCBI. This approach led to the identification of key genes encoding major enzymes in the metabolic pathways.

In this study, three important genes were selected as representatives of the biosynthetic pathways: the *pinene synthase (pin)* gene involved in the production of alpha- and beta-pinene, the *limonene synthase (lim)* gene involved in limonene synthesis, and the *ARM repeat proteins (arm)* gene, a protein family with specific repeats called armadillo repeats that play regulatory roles in many cellular processes.

To accurately assess the activity levels of these genes, the Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) method was employed. This method enables the quantitative measurement of gene expression in different samples and is particularly suitable for investigating the effects of various physiological or spatial conditions (such as tissue type) on the regulation of metabolite biosynthesis.

The results of these analyses can be effective in elucidating the molecular mechanisms associated with the production of valuable secondary compounds in juniper and pave the way for future research on enhancing quality, optimizing production, and exploring pharmaceutical and industrial applications of these compounds.

Investigation and Comparison of Candidate Gene Expression in Different Tissues of Three *Juniperus* Species

In this study, the relative expression of *lim*, *arm*, and *pin* genes was measured in three *Juniperus* species (*J. communis*, *J. polycarpos*, and *J. sabina*) across two tissues (leaf and fruit) using qPCR. Following normalization with the *ef* reference gene based on the ΔCt method, the results were reported. The findings revealed that gene expression patterns were simultaneously influenced by both tissue type and plant species. Generally, ΔCt values in fruit tissue were lower than in leaf for most genes, indicating increased normalized expression levels in fruit.

This tissue-specific difference was particularly pronounced for the *pin* gene, with $2^{-\Delta Ct}$ values in fruit approximately 15 to over 20 times higher than in leaf across all three species, a pattern that may indicate the role of this gene in biological processes specific to fruit tissue.

The *lim* gene also showed increased expression in fruit across all three species, although the magnitude of this increase varied among species, with the greatest difference observed in *J. communis*.

In contrast, the *arm* gene did not exhibit a uniform tissue-specific pattern; in *J. communis*, expression in fruit was substantially higher than in leaf, in *J. polycarpos* the difference between the two tissues was minimal, and in *J. sabina*, leaf expression was slightly higher than in fruit, indicating species-dependent regulation of this gene.

Regarding inter-species comparisons, the highest normalized expression levels for *lim* and *pin* genes were predominantly observed in *J. sabina*, while the lowest were in *J. communis*, with *J. polycarpos* generally showing intermediate values. For the *arm* gene, although expression in fruit was similar between *J. communis* and *J. sabina* and higher than in *J. polycarpos*, in leaf tissue the dominant pattern of increased expression in *J. sabina* compared to the other two species was observed. Overall, the results of this study demonstrate that gene expression regulation in different *Juniperus* species is strongly dependent on plant tissue and the specific gene under investigation, with *pin* showing the greatest sensitivity to tissue variations, while *lim* exhibits a more stable species-specific pattern and *arm* displays more complex regulatory behavior. These differences in gene expression may reflect physiological and metabolic distinctions among species as well as the specific functional roles of these genes in different tissues (Table 4).

Table 4 Relative expression analysis of *lim*, *arm*, and *pin* genes in leaf and fruit tissues of three *Juniperus* species (*J. communis*, *J. polycarpos*, and *J. sabina*) using qPCR.

Species	Tissue	Replicate	Gene	Ct	Ct_ref	DeltaCt	NormExpr
J. communis	Fruit	1	*lim*	34.823	28.368	6.455	0.011398596
J. communis	Fruit	2	*lim*	34.384	28.615	5.769	0.018338253
J. polycarpos	Fruit	1	*lim*	34.665	29.303	5.362	0.024315162
J. polycarpos	Fruit	2	*lim*	34.582	29.356	5.226	0.026718818
J. sabina	Fruit	1	*lim*	34.932	29.826	5.106	0.029036271
J. sabina	Fruit	2	*lim*	34.873	29.71	5.163	0.027911433
J. communis	Leaf	1	*lim*	35.347	26.223	9.124	0.001792265
J. communis	Leaf	2	*lim*	35.059	26.176	8.883	0.00211812
J. polycarpos	Leaf	1	*lim*	34.594	27.317	7.277	0.006447699
J. polycarpos	Leaf	2	*lim*	34.357	27.213	7.144	0.007070363
J. sabina	Leaf	1	*lim*	33.256	27.467	5.789	0.018085785
J. sabina	Leaf	2	*lim*	33.451	27.821	5.63	0.020193013
J. communis	Fruit	1	*arm*	33.423	28.368	5.055	0.030081076
J. communis	Fruit	2	*arm*	33.384	28.615	4.769	0.036676506
J. polycarpos	Fruit	1	*arm*	35.21	29.303	5.907	0.016665403
J. polycarpos	Fruit	2	*arm*	35.419	29.356	6.063	0.014957367
J. sabina	Fruit	1	*arm*	34.94	29.826	5.114	0.028875705
J. sabina	Fruit	2	*arm*	34.508	29.71	4.798	0.035946622
J. communis	Leaf	1	*arm*	34.365	26.223	8.142	0.003540086
J. communis	Leaf	2	*arm*	34.54	26.176	8.364	0.003035185
J. polycarpos	Leaf	1	*arm*	33.389	27.317	6.072	0.014864348
J. polycarpos	Leaf	2	*arm*	33.453	27.213	6.24	0.013230396
J. sabina	Leaf	1	*arm*	32.352	27.467	4.885	0.03384297
J. sabina	Leaf	2	*arm*	32.436	27.821	4.615	0.040808118
J. communis	Fruit	1	*pin*	33.013	28.368	4.645	0.039968299
J. communis	Fruit	2	*pin*	33.453	28.615	4.838	0.034963659
J. polycarpos	Fruit	1	*pin*	32.546	29.303	3.243	0.105623298
J. polycarpos	Fruit	2	*pin*	32.845	29.356	3.489	0.089064851
J. sabina	Fruit	1	*pin*	32.543	29.826	2.717	0.152090295
J. sabina	Fruit	2	*pin*	32.682	29.71	2.972	0.12744971
J. communis	Leaf	1	*pin*	35.015	26.223	8.792	0.002256027
J. communis	Leaf	2	*pin*	34.701	26.176	8.525	0.002714684
J. polycarpos	Leaf	1	*pin*	35.006	27.317	7.689	0.004845966
J. polycarpos	Leaf	2	*pin*	34.986	27.213	7.773	0.00457187
J. sabina	Leaf	1	*pin*	34.639	27.467	7.172	0.006934464
J. sabina	Leaf	2	*pin*	34.576	27.821	6.755	0.009258537

Ct: cycle threshold value for the target gene in the qPCR reaction. Ct_ref: Ct value of the reference gene (*ef*) in the same sample. ΔCt: difference between target gene Ct and reference gene Ct (ΔCt = Ct_target − Ct_ref), indicating normalized relative expression. NormExpr: normalized gene expression level calculated as $2^{-\Delta Ct}$, representing the relative abundance of target gene transcripts after normalization with the reference gene; larger values indicate higher gene expression.

Overall Tissue-Dependent Pattern

In all three genes examined, expression levels were higher in fruit tissue compared to leaf, with this difference being particularly pronounced for the *pin* gene, showing several-fold differences between the two tissues. Such a pattern may indicate the role of these genes—especially *pin*—in metabolic pathways or developmental processes specific to fruit tissue. The *lim* gene also showed a similar trend of increased expression in fruit across all three species, although the magnitude of this increase varied among species. In contrast, the *arm* gene did not exhibit a uniform tissue-dependent pattern, with its variation being partially species-dependent, such that in some species the expression difference between leaf and fruit was smaller than that observed for the other two genes.

Species-Specific Differences in Gene Expression

Comparison of species within each tissue showed that *J. sabina* generally exhibited the highest relative expression levels in most cases, while *J. communis* typically showed the lowest expression, with *J. polycarpos* occupying an intermediate position. This trend was consistent across both tissues for the *lim* and *pin* genes, indicating the existence of species-dependent regulatory differentiation for these genes. For the *arm* gene, although this pattern was maintained in leaf tissue, in fruit the expression of *J. communis* and *J. sabina* was closer to each other and higher than in *J. polycarpos*, indicating the complexity of expression regulation for this gene under different tissue conditions.

Magnitude of Expression Variation Among Genes

Comparison of the three genes revealed that *pin* exhibited the widest range of expression variation across species and tissues and could therefore be considered the most sensitive molecular marker for physiological differences in this dataset. The *lim* gene showed moderate variation but a stable species-specific pattern, while *arm* displayed an intermediate pattern dependent on species-tissue interaction.

To visually illustrate the distribution of normalized expression values across different tissues and species, box plots were generated for each gene (Figure 2). As shown in Figure 2, the tissue-dependent expression patterns described above are clearly evident. For the *pin* gene, fruit tissues in all three species show substantially higher normalized expression values compared to leaf tissues, with *J. sabina* exhibiting the highest median expression in fruit. The *lim* gene also demonstrates consistently higher expression in fruit across all species, although the magnitude of the tissue difference varies among species. In contrast, the *arm* gene displays more variable tissue-dependent patterns, with *J. communis* showing higher expression in fruit, *J. sabina* showing slightly higher expression in leaf, and *J. polycarpos* showing minimal tissue differences. The distribution of individual biological replicates (shown as points) indicates good reproducibility within each species-tissue-gene combination, and the box plots effectively capture the central tendency and variability of the data. These visualizations confirm the statistical findings presented in Table 4 and support the conclusion that gene expression in *Juniperus* species is strongly influenced by both tissue type and species identity.

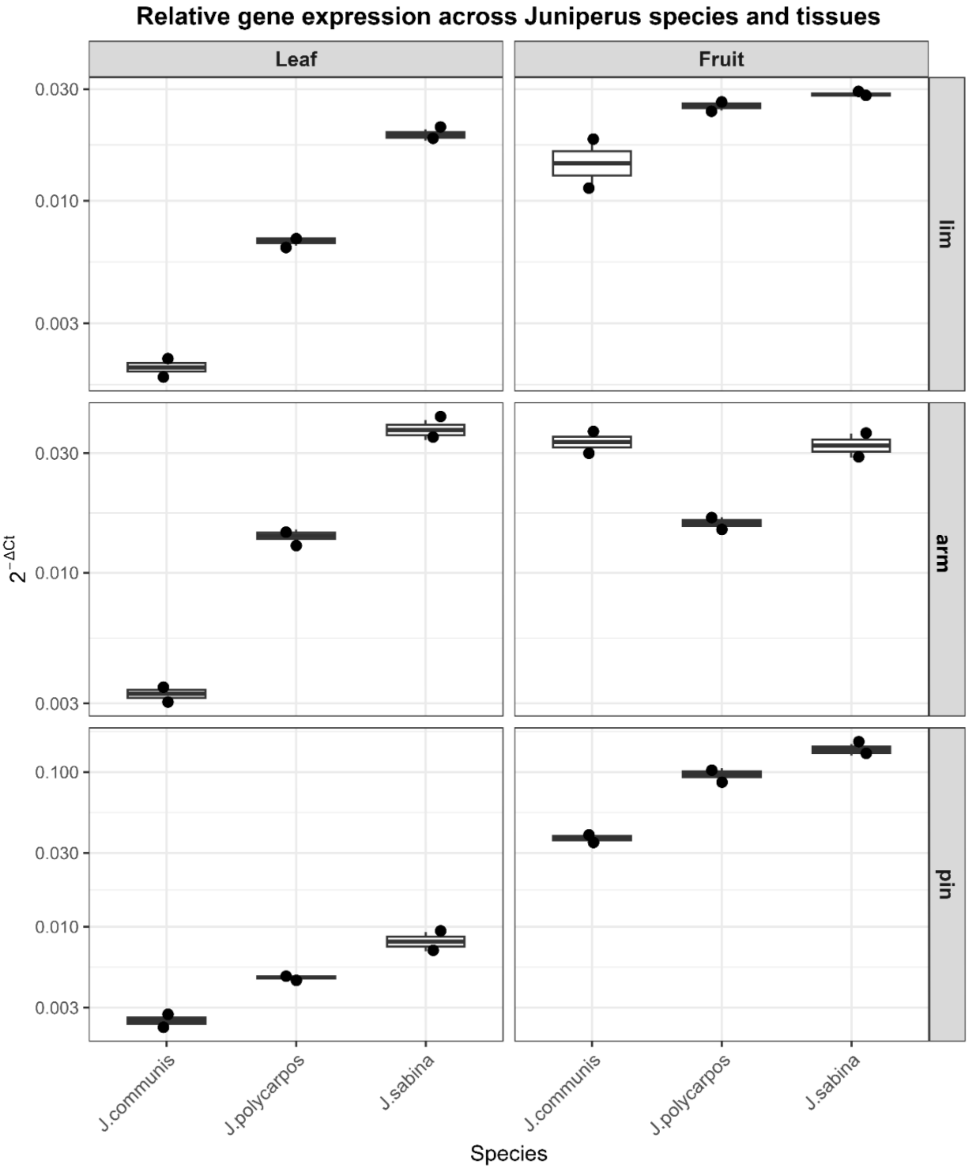

Fig. 2 Box plot representing the normalized relative expression ($2^{-\Delta Ct}$) of *lim*, *arm*, and *pin* genes in leaf and fruit tissues of three *Juniperus* species, including *J. communis*, *J. polycarpos*, and *J. sabina*. Gene expression values were calculated after normalization with the *ef* reference gene based on the ΔCt method. Points represent individual biological replicates, and box plots show the distribution of normalized expression values for each species in each tissue and gene.

Interpretation of Gene Expression Correlation Figure

This figure illustrates the pairwise correlation relationships between the relative expression of *lim*, *arm*, and *pin* genes based on mean $2^{-\Delta Ct}$ values in leaf and fruit tissues of three *Juniperus* species. Each point represents a species, and dashed lines indicate the linear regression trend between two genes in each tissue.

Correlation in Leaf Tissue

In leaf tissue, all three pairwise comparisons (*lim–arm*, *lim–pin*, and *arm–pin*) show positive correlation trends, where increased expression of one gene is associated with increased expression of another gene. This uniform pattern suggests possible co-transcriptional regulation or shared involvement of these genes in active metabolic pathways in the leaf. Furthermore, the relatively wide spacing of species on the graph indicates that species-specific differences in gene expression levels in leaf are considerable, with *J. sabina* typically showing the highest expression and *J. communis* the lowest.

Correlation in Fruit Tissue

In fruit tissue, a different correlation pattern is observed. Although a strong positive relationship between *lim* and *pin* is maintained, in the other two comparisons (*lim–arm* and *arm–pin*), the regression slope is negative, indicating an inverse correlation between these genes in fruit. This shift in correlation direction compared to leaf suggests that gene expression regulation in fruit is more independent and possibly more specialized, and each gene may play a role in different metabolic pathways. Such a pattern is consistent with the physiological and biochemical differences between leaf and fruit.

Comparison of Species Patterns

In both tissues, *J. sabina* is positioned higher on the graphs, indicating higher relative expression levels for most genes, while *J. communis* is typically observed at the lowest expression values, with *J. polycarpos* occupying an intermediate position. This consistent trend indicates species-dependent genetic control in the regulation of gene expression within the genus *Juniperus*.

To further investigate the regulatory relationships among the three studied genes, pairwise correlation analysis was performed between the mean normalized expression values of *lim*, *arm*, and *pin* genes in leaf and fruit tissues (Figure 3). This analysis reveals how the expression of these genes co-varies across different species and tissues, providing insights into potential co-regulation or functional interactions.

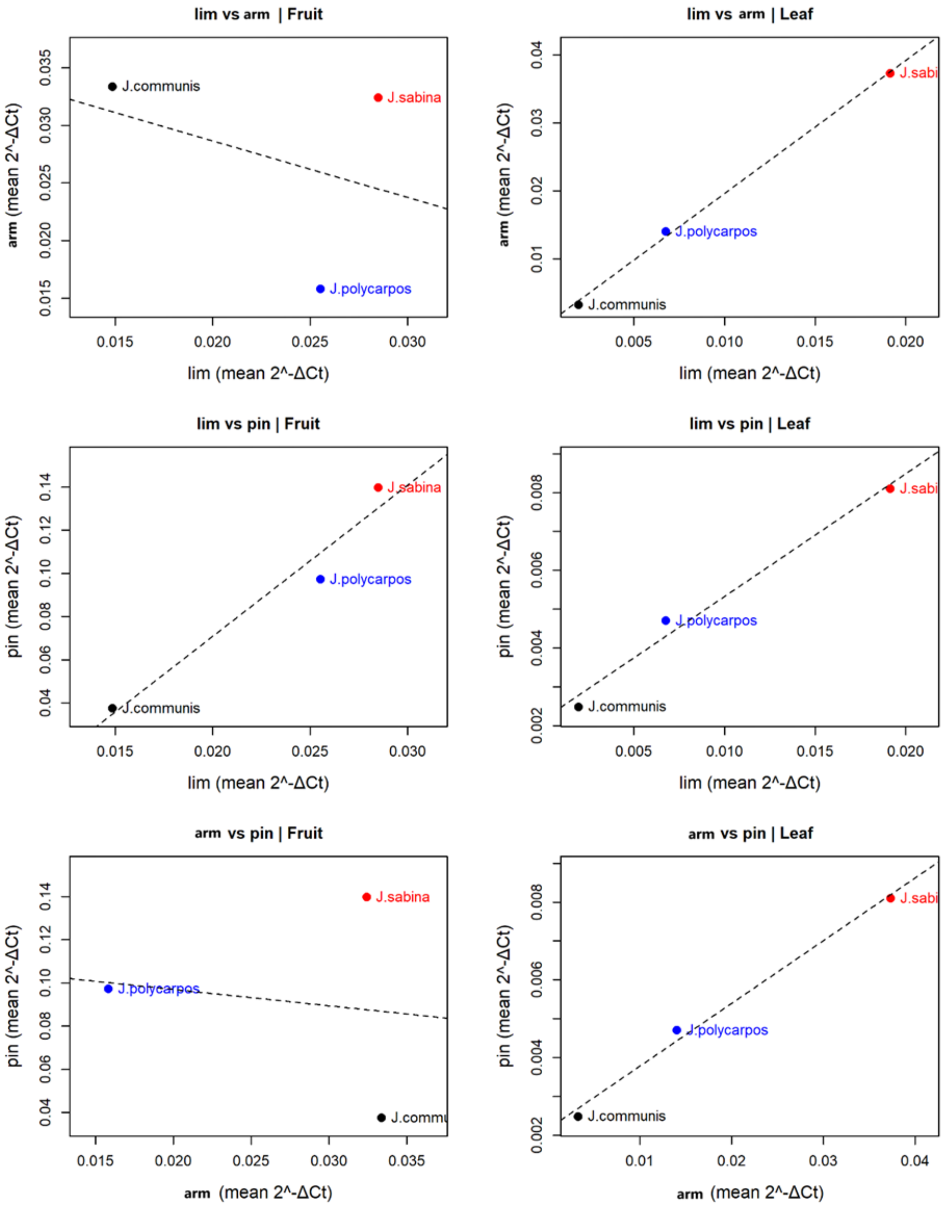

Fig. 3 Pairwise correlation of normalized relative expression (mean $2^{-\Delta Ct}$) between *lim*, *arm*, and *pin* genes in leaf and fruit tissues of three *Juniperus* species, including *J. communis*, *J. polycarpos*, and *J. sabina*. Each point represents the mean expression level for a species in a specific tissue, and dashed lines indicate the linear regression trend between gene pairs.

DISCUSSION

Given that limonene synthase and pinene synthase are key enzymes in the monoterpene biosynthetic pathway, investigating the expression of genes encoding these enzymes, as well as ARM proteins, can provide deeper insights into the regulatory processes leading to monoterpene production in plants. Therefore, in this study, the expression of *limonene synthase*, *pinene synthase*, and *ARM repeat proteins* genes was investigated to determine the role of these proteins in regulating the monoterpene biosynthetic pathway, particularly in plant defense responses. These investigations can contribute to developing strategies for engineering monoterpene metabolism and improving plant defense characteristics.

Similar studies in other plants have confirmed the role of *terpene synthase* genes (such as *limonene synthase* and *pinene synthase*) in the biosynthesis of monoterpene compounds. Additionally, ARM repeat proteins are recognized as complex regulators in many plant metabolic pathways and may play important roles in regulating the production of these compounds [15].

The study by Trindade et al. [16] emphasized the expression of the *α-pinene synthase* gene in two species, *Pinus pinaster* and *Pinus pinea*, showing that the translated amino acid sequences of these two species share over 97% homology. This high sequence homology confirms the likely preservation of biological function of this enzyme across different species. Heterologous expression analysis of full-length and truncated genes lacking 48 amino acids of the transit peptide demonstrated that both versions were capable of producing α-pinene as the main product. These findings indicate that the deleted peptide region does not play a significant regulatory or structural role in enzyme function.

A temperature decrease to 4°C resulted in a two-fold increase in α-pinene production compared to higher temperatures (21°C and 37°C), indicating the functional sensitivity of this enzyme to environmental conditions. This sensitivity may represent a regulatory mechanism for plant adaptation to stressful conditions. Furthermore, gene expression analysis by RT-PCR in axenic cultures and co-cultures with the nematode *Bursaphelenchus xylophilus* showed that in *P. pinea*, *α-pinene synthase* gene expression increased significantly 24 hours after inoculation, while no such change was observed in *P. pinaster*. This difference in response between the two species to nematode infection may result from genetic differences and species-specific regulatory mechanisms, highlighting the importance of species-specific adaptation in conifer defense systems.

Regarding chemical composition, essential oils extracted from *P. pinaster* and *P. pinea* are both rich in monoterpenes, but differences are observed in the abundance of major products; α-pinene and β-pinene predominate in *P. pinaster*, while limonene is more abundant in *P. pinea* [17]. This difference in essential oil composition may directly affect the defensive efficiency of each species and reflects the specific evolution of metabolic pathways associated with resistance in each species.

In the study by Liu et al. [18], transcriptomic analysis identified genes associated with high oleoresin yield in the secondary xylem of *Pinus massoniana*. Candidate genes included *geranylgeranyl diphosphate synthase (GGPS)* and *(-)-α/β-pinene synthase*, which were upregulated in genotypes with high oleoresin production, while the *tricyclene synthase* gene showed lower expression levels, consistent with GC/MS analysis results. Additionally, the potential role of ABC transporters, non-specific lipid transfer proteins, phosphomethylpyrimidine synthase, ERF transcription factors, and pathogen response pathways in regulating oleoresin production was suggested. These findings indicate that oleoresin production in pines is controlled by multiple genetic networks and signaling pathways that include not only terpene synthesis enzymes but also molecular transporters and regulatory factors.

In the study by Liu et al. [19], *d-limonene synthase (FcLS)* genes were first identified from *Fortunella crassifolia* (Rongan kumquat). Sequence analysis of these genes revealed that the open reading frames of 18 *FcLS* genes contained 12 single nucleotide polymorphisms, leading to protein diversity within this large gene family—a phenomenon not previously reported in citrus. The predicted FcLS proteins showed high amino acid sequence identity with other citrus limonene synthases and possessed the typical protein structures of limonene synthase. The function of FcLS1 as an active d-limonene synthase was confirmed through prokaryotic expression, highlighting the key role of this gene family in the production of defensive monoterpenes and plant secondary metabolites.

In the monoterpene biosynthetic pathway, compounds such as limonene and pinene, among the most important members of the monoterpene group, play vital roles in plant metabolism. This biosynthetic pathway involves enzymes such as limonene synthase (LS) and pinene synthase (PS), which convert the main precursor of these compounds, geranyl pyrophosphate (GPP), into various monoterpenes. However, in addition to these enzymes, other proteins also play roles in regulating this pathway, among which ARM repeat superfamily proteins are particularly significant [20].

ARM repeat proteins are a protein family characterized by specific repeats called armadillo repeats, which play regulatory roles in many cellular processes. These proteins are particularly effective in regulating enzyme activity and protein-protein interactions in plant biochemical pathways. In the monoterpene biosynthetic pathway, ARM repeat proteins may act as indirect regulators, modulating the activity of enzymes such as limonene synthase and pinene synthase [21].

One of the prominent features of ARM proteins is their ability to interact with other proteins and form enzymatic complexes that can enhance or reduce biochemical activities. This characteristic is particularly important in regulating monoterpene biosynthetic pathways, as many of these compounds are produced in response to environmental stresses, such as pest attacks or changing environmental conditions. Furthermore, ARM proteins may be involved in plant defense reactions, influencing the regulation and control of defensive monoterpene production [22].

The expression of these genes was quantitatively measured using real-time PCR to examine the extent of expression changes in different plant samples under the influence of environmental conditions and various plant tissues. The results of this study can contribute to a better understanding of the molecular mechanisms underlying the production of active compounds in juniper and pave the way for future research aimed at improving the quality and efficacy of these compounds in pharmaceutical and industrial applications.

Differences in gene expression across different tissues and species of *Juniperus* depend on several factors. One of the main reasons for these differences is tissue-specific regulation related to the particular needs of each tissue. For example, in this study, it was observed that *pin* and *lim* genes were more highly expressed in fruit tissue than in leaf. This difference may be due to the greater need of fruit to produce defensive compounds such as monoterpenes, which are effective in attracting pollinators and defending against pests. In contrast, in leaves, these compounds may be produced at lower levels because the requirements for them in physiological and metabolic processes differ. Additionally, genetic differences between species may also play a role in the production levels of these compounds. For instance, *J. sabina* generally showed the highest gene expression, while *J. communis* exhibited the lowest expression levels. These differences may result from specific genetic adaptations of each species to its environment. Species may respond differently to environmental conditions and produce specific metabolites that aid in defense against environmental stresses. These differences may also be related to ecological variations among species. For example, species growing in drier environments or under greater stress may produce higher levels of defensive compounds such as pinene and limonene. Furthermore, hormonal regulation also influences gene expression differences. Hormones such as abscisic acid (ABA) and cytokinins can help regulate the activity of monoterpene synthesis enzymes. Therefore, differences in hormonal responses and metabolic requirements of different tissues and species can lead to significant variations in the production of active plant compounds. These differences are particularly important in the growth and developmental processes of various plant tissues and may play a role in regulating the production of defensive and bioactive compounds in plants.

This study, by focusing on the investigation of key gene expression involved in secondary metabolite biosynthesis in three different species of the genus *Juniperus*, presents significant innovations to the field of medicinal plant research and bioactive compound biosynthesis. Among the innovations of this research are the simultaneous use of field and laboratory samples, identification and design of specific primers for key genes, and precise evaluation of gene expression using the qRT-PCR method—approaches that had not been comprehensively and simultaneously undertaken in previous studies. Furthermore, emphasizing the comparison of expression levels of *pin*, *lim*, and *arm* genes across different species and different parts (leaf and fruit) provides a fresh perspective on genetic differences and species adaptation in the metabolite biosynthetic pathway. The findings of this research can serve as a valuable foundation for future studies in molecular fields, including gene expression analysis, investigation of functional gene diversity, and planning for genetic modification aimed at increasing secondary metabolite production.

CONCLUSION

The present study investigated the expression of key genes in the monoterpene biosynthetic pathway, including *pinene synthase (pin)*, *limonene synthase (lim)*, and *ARM repeat proteins (arm)*, in three species of the genus *Juniperus* (*J. polycarpos*, *J. communis*, and *J. sabina*). The results of this research demonstrated that the expression of these genes is significantly influenced by both tissue type and plant species. In particular, the *pin* gene exhibited the highest expression levels in fruit, while the *lim* and *arm* genes showed different patterns across the various species. These findings indicate the vital role of these genes in the production of defensive monoterpenes and bioactive metabolites in *Juniperus* plants. Furthermore, the differences in gene expression among different species, such as *J. sabina* and *J. communis*, may be related to genetic adaptations and specific metabolic requirements of each species in response to natural environmental conditions. These results can contribute to a better understanding of the molecular mechanisms underlying the production of active compounds in juniper and provide new opportunities for engineering monoterpene metabolism and improving plant defense characteristics. Finally, this study emphasizes that investigating key gene expression can serve as a powerful and effective tool for identifying and selecting superior *Juniperus* cultivars. These findings can play a significant role in developing management, conservation, and sustainable utilization programs for these valuable species and contribute to enhancing their pharmaceutical and economic applications.

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