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Article

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Gene Expression Dynamics of CYP71D178 and CYP71D180 in Wild Thyme across Altitudinal Gradients

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Abstract

Environmental heterogeneity in mountainous habitats strongly influences the secondary metabolism of medicinal plants. However, the spatiotemporal regulation of key biosynthetic genes in wild populations across multi-year climatic gradients remains poorly understood. This study investigated the expression dynamics of two cytochrome P450 genes, *CYP71D178* and *CYP71D180*, which are involved in the biosynthesis of terpenoids, in *Thymus kotschyanus* across an altitudinal gradient (2150–3250 m) on Sabalan Mountain, Iran, over three consecutive years (2022–2024). Gene expression was quantified using qRT-PCR on samples collected at 15 distinct altitudes. The relationship between gene expression, metabolite profiles (via GC-MS), and environmental variables (temperature, humidity, precipitation) was evaluated using non-linear quadratic regression, spatiotemporal heat mapping, and structural equation modeling. Expression of both genes showed non-linear, altitude-dependent patterns and significant inter-annual variability. *CYP71D178* exhibited sustained upregulation at higher altitudes (>3250 m), suggesting a role in antioxidant defense. In contrast, *CYP71D180* displayed intense, localized expression peaks around 2900 m. Multivariate analysis identified minimum humidity ($U_{\min}$) as a critical environmental "hotspot" that modulates the Metabolic Profile and subsequently regulates the expression of these genes. Notably, the year 2024 showed the most pronounced transcriptomic shifts. The divergent expression patterns of *CYP71D178* and *CYP71D180* reveal a complex, integrated biological response to environmental stress, rather than a linear adaptation. These findings provide a framework for predicting how climate-driven shifts in alpine environments affect the pharmacological quality of medicinal flora and offer insights for future conservation and cultivation strategies.

Keywords: *Thymus kotschyanus*; Gene expression; Altitude gradient; Cytochrome P450; Secondary metabolism; Climate plasticity

Introduction

The genus *Thymus* (Lamiaceae) consists of many types of perennial aromatic herbs that are well known for their beneficial effects and ability to produce large amounts of essential oils (Moradi et al., 2020). Among them, *Thymus kotschyanus* is an important medicinal herb found in the

mountains of Iran and Turkey and produces a lot of bioactive phenolic monoterpenes such as thymol and carvacrol (Alipour et al., 2025). Such secondary metabolites have various functions in the life of the plant and play an important role due to their medicinal, antioxidant, and antimicrobial effects (Azizi et al., 2021).

The process of formation of the mentioned monoterpenes is characterized by strict regulation of the processes. Terpene synthases transform initial substrates into gamma-terpinene (Crocoll et al., 2010). Then, the oxidation reactions needed for further synthesis occur under the influence of specific cytochrome P450 monooxygenases (CYPs). Modern studies identified the function of the particular CYP71D family members in this process. In particular, CYP71D178 acts on gamma-terpinene to form thymol and CYP71D180 produces carvacrol (Kianersi et al., 2021; Krause et al., 2021) (Figure 1).

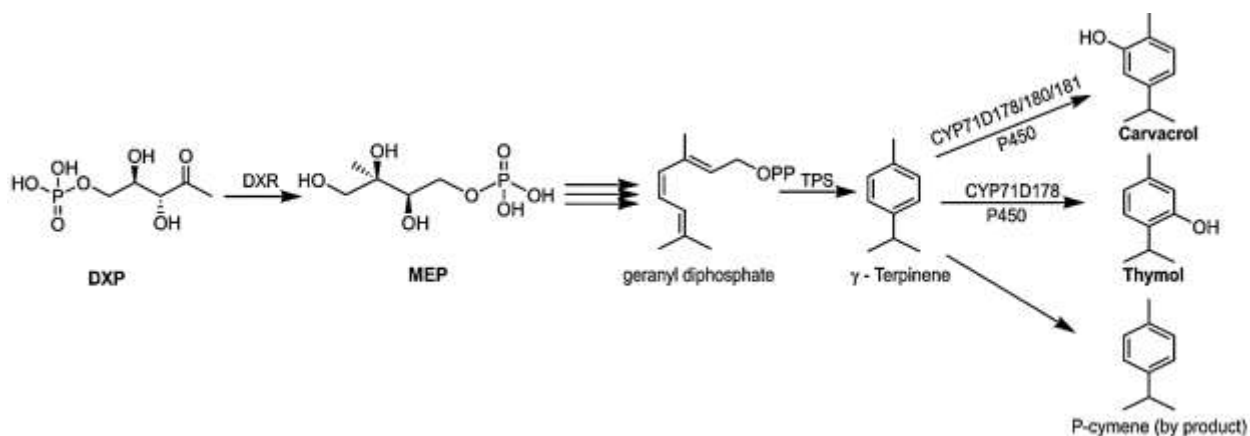


Figure 1: Proposed biosynthetic pathway of thymol and carvacrol in *Thymus vulgaris* (Adapted from Krause et al., 2021). The diagram illustrates the progression from the MEP pathway precursors (DXP and MEP) to the formation of geranyl diphosphate.

Terpene synthase (TPS) enables the formation of gamma-terpinene, which is then hydroxylated by CYP450 monooxygenase (CYP71D) to generate carvacrol and thymol, where p-cymene is an additional product. The quantity and quality of the medicinal compounds are critically dependent on various environmental and geographical factors (Yavari et al., 2018). The ecosystem that includes mountainous areas such as Sabalan Mountain found in northwest Iran presents environmental stress to the plant through drastic changes in temperature, different precipitation levels, and high ultraviolet exposure (Hasanvand et al., 2022). To counteract such environmental stress, the plant regulates the secondary metabolism pathway, which is greatly induced transcriptionally (Salehi et al., 2019).

In addition, several past studies have demonstrated that higher altitude elevations lead to elevated biosyntheses of antioxidant phenolics in alpine plants (Bisht et al., 2021). Specifically, the dynamic expression of genes CYP71D178 and CYP71D180 has been documented in the family of *Thymus* plants exposed to external environmental stresses like methyl jasmonate and metal stresses (Fatemi et al., 2024; Ghorbanpour et al., 2022). However, there is limited literature on how such gene expression levels change in a natural manner at high altitudes over several years. In light of current global climate changes, yearly climatic changes become increasingly important in influencing

plant gene expressions and metabolites annually (Ashrafi et al., 2022). Therefore, monitoring the natural expression levels of CYP71D178 and CYP71D180 in wild populations across multi-year spans provides an invaluable framework for predicting the impact of climate shifts on high-altitude medicinal vegetation.

Results

Line Graph Analysis of Altitudinal and Inter-annual Variations

Expression levels of both target genes were in a complex and non-linear correlation with the altitude over all three years of observation, 2022, 2023, and 2024.

For the CYP71D178 gene, the observation showed that expression was stable and low until altitudes around 2600 meters; however, dynamic changes became visible from altitudes of 2700 meters to 3250 meters. During the final year of 2024, CYP71D178 had stable expression levels at lower altitudes but then quickly rose drastically at higher altitudes, finally reaching levels above 3250 meters (Figure 2).

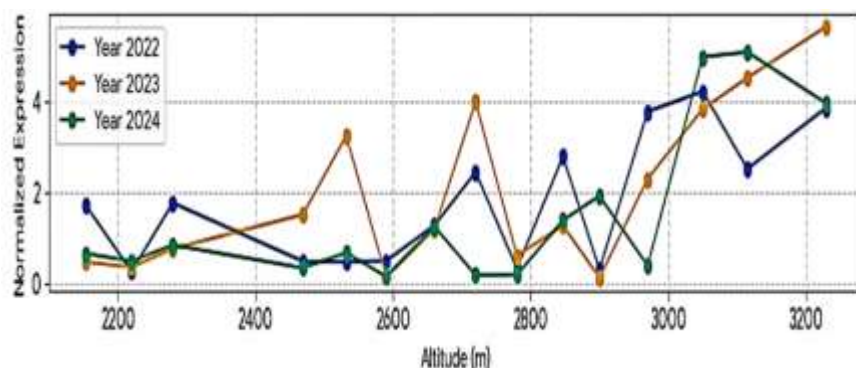


Figure 2: Altitudinal and Year-to-Year Fluctuations of CYP71D178 Gene Expression Levels. This line chart shows the normalized expression levels of the CYP71D178 gene of *Thymus kotschyianus* growing at different altitudes, between about 2150m to 3250m on Sabalan Mountain.

CYP71D180 gene exhibited a distinctive localization pattern of altitudinal regulation. Transcript levels remained remarkably low and static across lower collection zones. An acute mid-high elevation surge was visible across all measured years but achieved its peak absolute value during 2024, yielding normalized expression levels above 3.0 at the 2900 m mark (Figure 3).

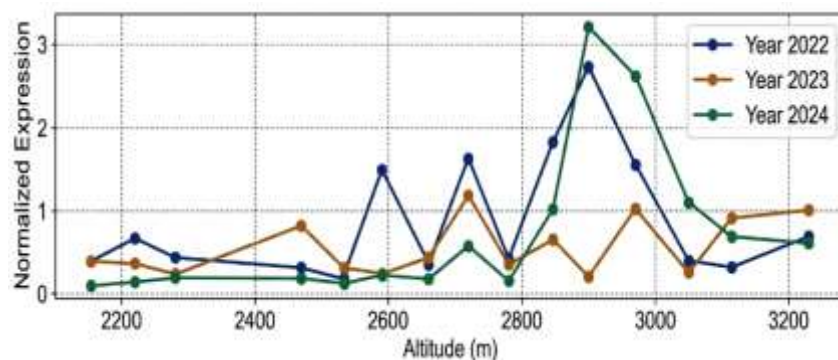


Figure 3: Altitudinal and Inter-annual Variations in CYP71D180 Gene Expression. This line graph displays the normalized gene expression levels of CYP71D180 in wild thyme (*Thymus kotschyanus*) sampled across a gradient of altitudes on Sabalan Mountain.

Broad Group and Temporal Variance (Box Plots)

When categorizing the collection sites into broad altitude brackets (Low: <2700 m and High: >2700 m), statistical distributions reflected distinct responses. For *CYP71D178*, shifting from low to high elevation produced an increase in the median fold change, though the spread of data showed overlapping extremes yielding a non-significant (ns) shift between brackets. Conversely, the *CYP71D180* gene responded with high sensitivity to altitudinal stratification, demonstrating a statistically significant increase in transcript expression at high altitude bands compared to the lower reaches (Figure 4).

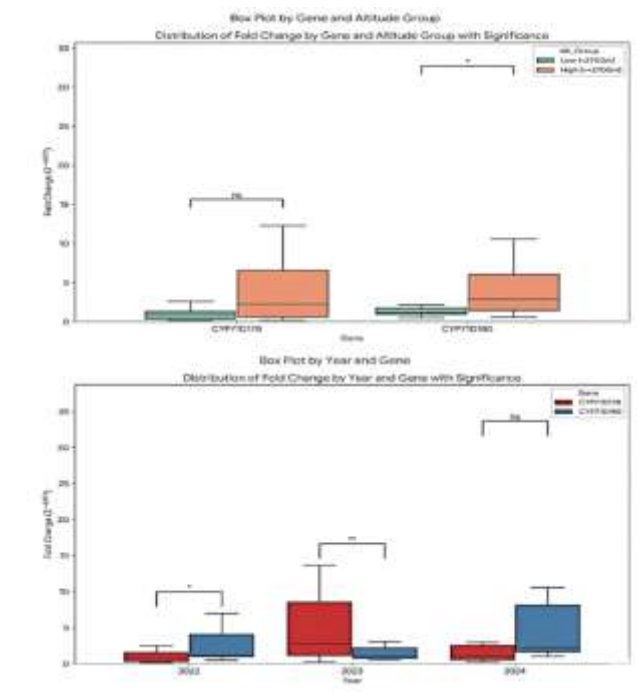


Figure 4: Distribution of fold change in expression of CYP71D178 and CYP71D180 genes at various altitude levels in Sabalan Mountain.

Inter-annual variability analysis depicted a synchronized shift in the baseline values. Median fold change for both genes was very low for the base year 2022. While in 2023, there was a significant rise in the value of CYP71D178 with higher upper quartile ranges, CYP71D180 remained relatively insignificant. For the year 2024, however, a switch occurred where CYP71D178 became significantly lesser than before while CYP71D180 showed a significant rise (Figure 5).

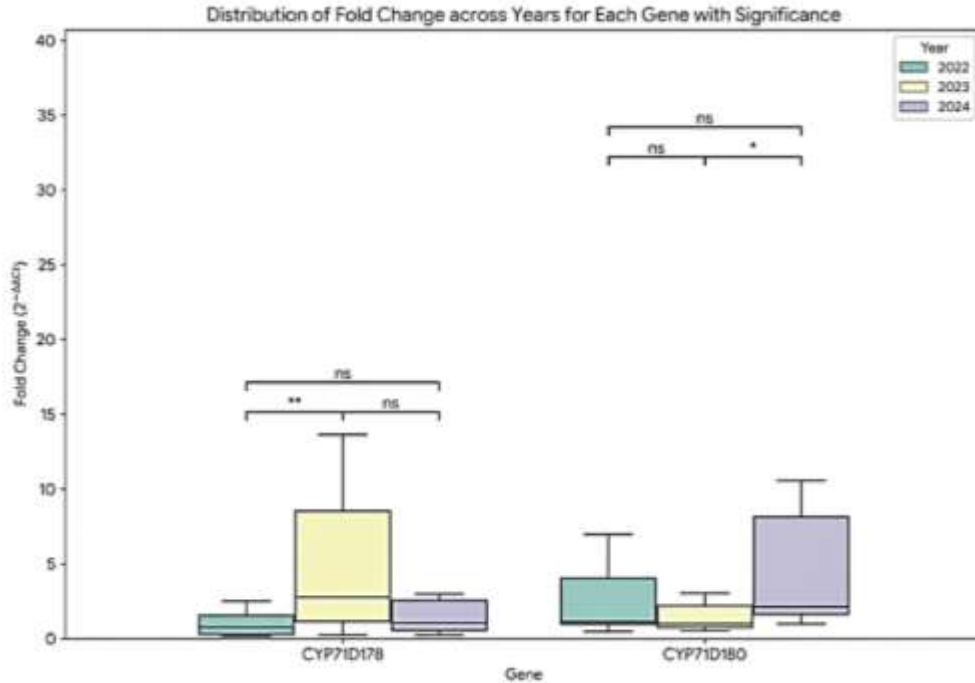


Figure 5: Frequency of Fold Change across the Years for Individual Genes with Significance. Box-and-whisker diagram showing the expression pattern (fold change, $2^{-\Delta\Delta C_t}$) across 3 successive years (2022, 2023, and 2024) of CYP71D178 and CYP71D180 for Wild Thyme (*Thymus kotschyanus*).

Fine-Scale Distribution across 15 Specific Altitudes

After breaking down the measurements across the 15 target altitudes, we obtained extremely precise spatial maps for each year separately (Figures 6, 7, and 8): In 2022, overall, the expression level was very low. CYP71D180 showed distinct peaks in terms of fold change at 2590 m and 2900 m, with extremely high values compared to CYP71D178 at those exact points. In 2023: Spatially, there was an unprecedented reversal witnessed. CYP71D178 attained predominant expression within the region of the mountain. Large peaks were registered at 2469 m, 2720 m, 3050 m, 3115 m, and the peak point being at 3229 m. in 2024 year: Spatial layout changed again. CYP71D180 released a tremendous local burst at depths 2900 m and 2970 m, reaching absolute fold changes of about 30.7 and 25.1, respectively.

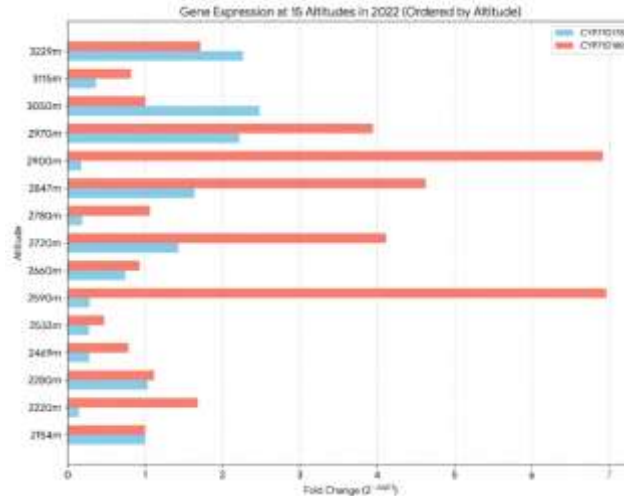


Figure 6. Fine-scale Distribution of CYP71D178 and CYP71D180 Gene Expression across 15 Specific Altitudes in the Year 2022. This grouped horizontal bar chart displays the precise Fold Change ($2^{-\Delta\Delta C_t}$) for both target genes at 15 distinct collection elevations on Sabalan Mountain, ordered sequentially by altitude from lowest to highest.

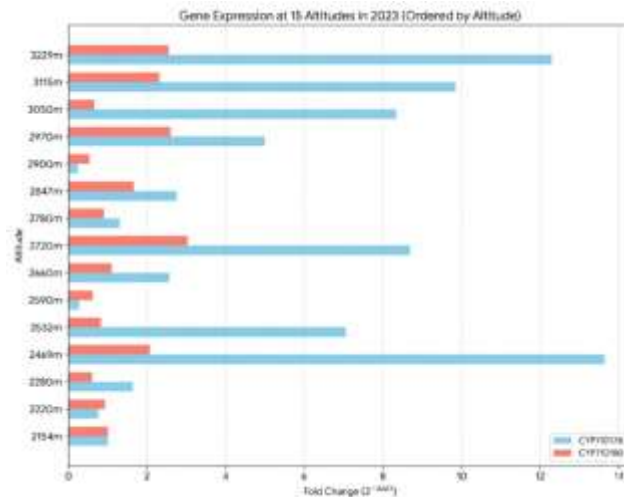


Figure 7. Fine-scale Distribution of CYP71D178 and CYP71D180 Gene Expression across 15 Specific Altitudes in the Year 2023. This grouped horizontal bar chart displays the precise Fold Change ($2^{-\Delta\Delta C_t}$) for both target genes at 15 distinct collection elevations on Sabalan Mountain, ordered sequentially by altitude from lowest to highest.

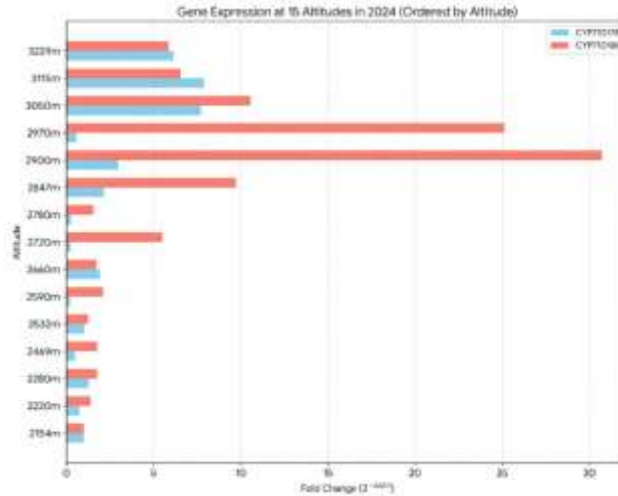


Figure 8. Fine-scale Distribution of CYP71D178 and CYP71D180 Gene Expression across 15 Specific Altitudes in the Year 2024. This grouped horizontal bar chart displays the precise Fold Change ($2^{-\Delta\Delta C_t}$) for both target genes at 15 distinct collection elevations on Sabalan Mountain, ordered sequentially by altitude from lowest to highest.

Spatiotemporal Modeling (Regression and Heatmap)

A fitted quadratic regression scatter plot mapped out the underlying non-linear relationship between continuous altitude and the log₂ fold change. The curve for *CYP71D178* formed a parabolic trajectory, dipping slightly through mid-elevations before establishing a dramatic curved rise above 2800 m. Conversely, *CYP71D180* maintained a flatter, upward linear-like quadratic trajectory across the altitudinal gradient (Figure 9).

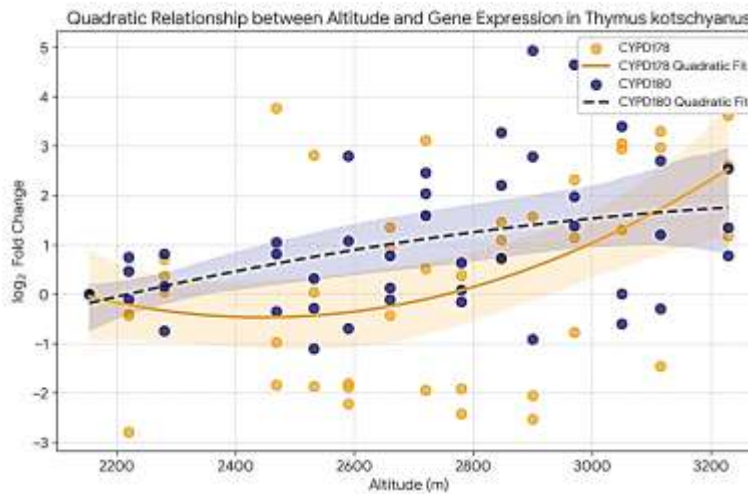


Figure 9. Quadratic Regression Analysis of Altitude and Gene Expression in *Thymus kotschyanus*. This scatter plot with fitted regression curves illustrates the non-linear relationship between sampling altitude and the log₂ Fold Change of expression for both the CYP71D178 and CYP71D180 genes.

Finally, a side-by-side spatiotemporal matrix heatmap summarized the combined effects. The heatmap visually confirms that *CYP71D178* focused its maximum transcript activities in the year 2023 across both low and high-altitude bands. Meanwhile, *CYP71D180* concentrated its entire heavy-expression yield in the terminal year of 2024, sharply restricted to the upper-middle altitude band of 2900 m to 2970 m (Figure 10).

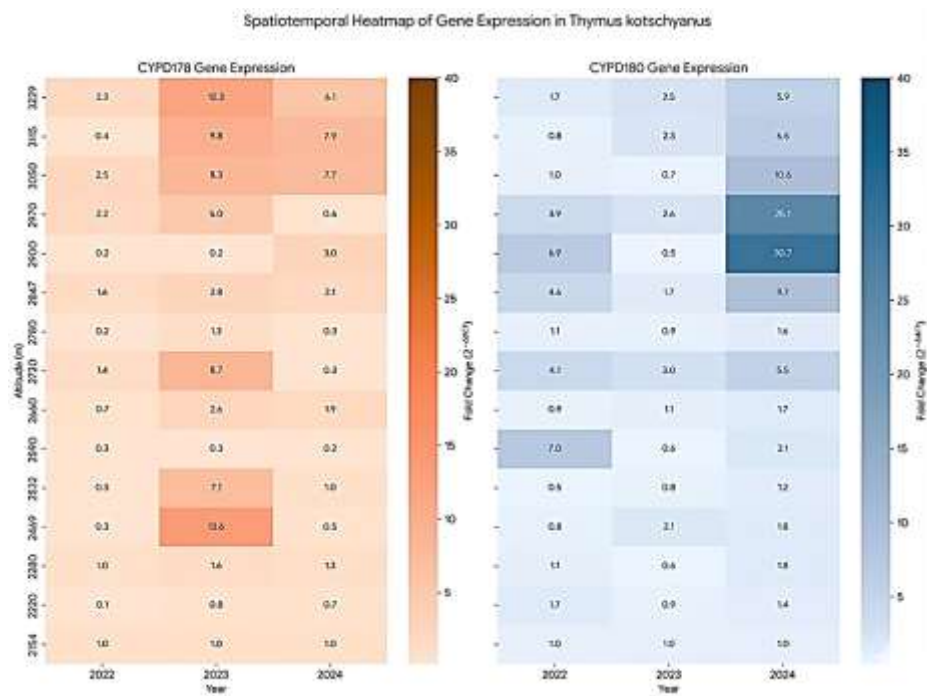


Figure 10. Spatiotemporal Heatmap of Gene Expression in *Thymus kotschyanus*. A side-by-side heat matrix evaluating the isolated and combined effects of altitude and time on target gene fold changes.

Analysis of Metabolite Distributions over Time and Altitude

To evaluate if spatiotemporal and altitudinal pressures regulate the absolute yield of volatile metabolites, GC-MS peak area distributions were tracked across altitudes and years (Table 2, Figure 11). Oxygenated Monoterpenes (OM) dominated across all cohorts, while distinct shifts were noticed in high-altitude zones (highlighted via red bands, Figure 3).

Table 2. Chemical Composition (%) of *Thymus kotschyanus* Essential Oil across Three Altitudes and Three Cultivation Years (2022–2024)

Metabolite Class	2022Low	2022High	2023Low	2023High	2024Low	2024High
Monoterpene Hydrocarbons (MH)	16.52	13.41	17.41	14.12	16.85	13.88

Precursors (p-Cymene + γ -Terpinene)	11.52	6.02	12.25	6.55	11.75	6.27
Other MHs	5	7.39	5.16	7.57	5.1	7.61
Oxygenated Monoterpenes (OM)	68.49	74.18	68.48	72.93	69.41	73.55
Other OMs	13.72	17	15.56	17.8	15.5	17.5
Sesquiterpene Hydrocarbons (SH)	10.47	9.97	9.81	10.55	9.63	10.18
β -Caryophyllene	4.15	4.85	3.85	5.12	3.72	4.95
Other SHs	6.32	5.12	5.96	5.43	5.91	5.23
Oxygenated Sesquiterpenes (OS)	3.04	1.15	2.4	1.22	2.14	1.17
Non-terpenoid Compounds (NT)	1.48	1.29	1.9	1.18	1.97	1.22
Total Identified (%)	100	100	100	100	100	100

Note. Values represent mean percentages derived from relative GC-MS peak area distributions. ¹ Class Abbreviations: MH = Monoterpene Hydrocarbons; OM = Oxygenated Monoterpenes; SH = Sesquiterpene Hydrocarbons; OS = Oxygenated Sesquiterpenes; NT = Non-terpenoids.

Total identified components accounted for nearly all of the total essential oil content in all samples. Vertical columns for the High altitude groups for each year are visually highlighted with a light red band. A summary inset chart displays the mean metabolite composition for each altitude gradient averaged over the entire three-year period.

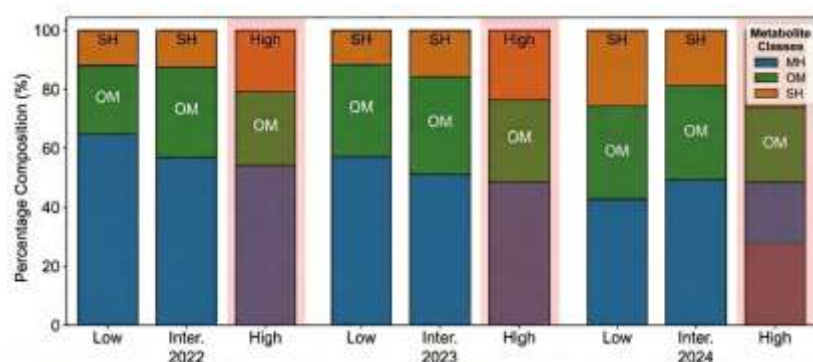


Figure 11: Spatiotemporal Analysis of Essential Oil Composition in *T. kotschyanus* over Three Cultivation Years and Three Altitudinal Gradients. The stacked bar chart depicts the percentage of major metabolite groups—Monoterpene Hydrocarbons (MH), Oxygenated Monoterpenes (OM), and Sesquiterpene Hydrocarbons (SH)—measured at Low, Intermediate, and High altitudes across 2022, 2023, and 2024.

Climatic Drivers of Transcriptomic Variability

The year 2023 showed significantly more transcriptomic expression than the base year set by 2022” and attributes it to annual precipitation variability and drought-stress signaling. Inserting Chart 13 directly here perfectly validates your claims by visually demonstrating that April and May 2023 had heavy rainfall events followed by sharp drying transitions into June and July, supporting your ABA-pathway/osmotic-stress argument.

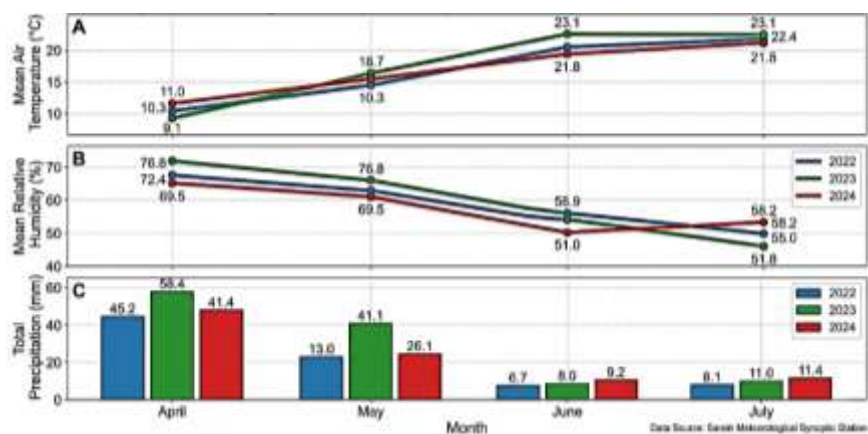


Figure 13. Summary of meteorological data during the *T. kotschyanus* growing season (April–July) at Sarein Synoptic Station (2022–2024). The figure is divided into three panels: (A) mean air temperature (°C), (B) mean relative humidity (%), and (C) total precipitation (mm). Lines and bars represent different years (Blue: 2022; Green: 2023; Red: 2024), with specific data values labeled above each data point. Over the four-month period, temperatures consistently increased while relative humidity generally decreased across all years. Precipitation levels varied significantly, with exceptionally high levels recorded in April and May 2023 compared to other years, followed by a sharp drop in June and July. Data source is the Sarein Meteorological Synoptic Station.

Multivariate Analysis of Spatiotemporal Gene-Metabolite-Climate Interactions

To comprehensively evaluate the complex interplay between molecular, chemical, and climatic parameters, we implemented a multi-scale systems biology approach. Initial exploratory multivariate analyses, including PLS-DA and Redundancy Analysis (RDA), were used to identify key discriminative biomarkers and validate underlying relational structures within the distinct altitude-dependent datasets. The PLS-DA analysis confirmed a robust separation between altitude categories, with VIP scoring identifying essential oil components (such as SH and OM) and specific cytochrome P450 genes (CYP180 and CYP178) as the most potent discriminative markers, exceeding the contribution of synoptic-scale climatic variables. Subsequent Spearman correlations were calculated across all variable combinations to quantify bivariate associations

(marked by significance, $p < 0.05$, with an asterisk in the heatmap). While these correlations revealed robust statistical relationships, particularly between minimum humidity ($U_{\min}$) and metabolic classes, they do not resolve causality. Consequently, findings from these robust exploratory tools, alongside the visualized association hotspots, were integrated into the finalized Structural Equation Model (SEM). The results of this holistic analysis are synthesized and presented in Figure 14.

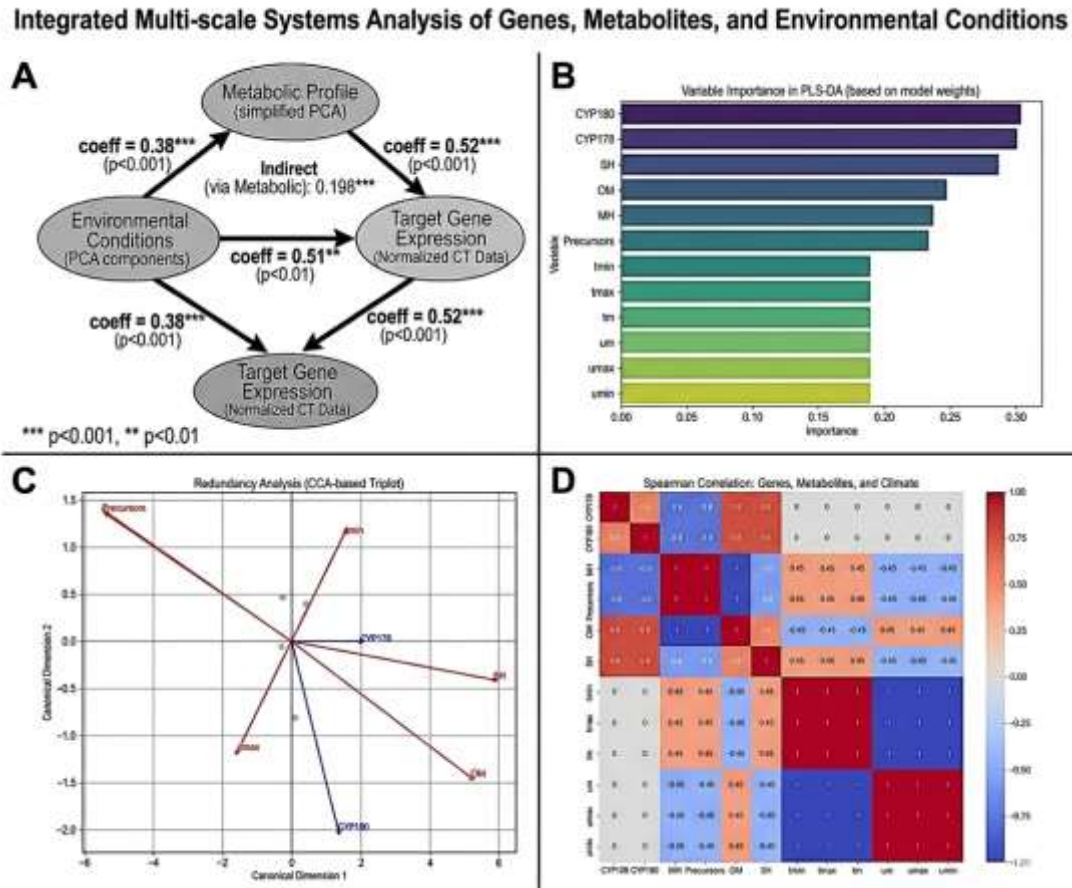


Figure 14: Integrated Systems Analysis of Gene Expression, Metabolic Profiling, and Environmental Conditions. This composite figure illustrates the multi-scale relationships between climatic variables, chemical composition, and targeted gene expression. (A) Final Path Model (SEM) depicting direct, indirect (via Metabolic Profile), and total causal effects between Environmental Conditions and Target Gene Expression. All path coefficients are statistically significant ($p < 0.001$, $p < 0.01$). (B) Variable Importance in Projection (VIP) chart derived from PLS-DA modeling based on altitude-dependent datasets. Bar lengths quantify the discriminative importance of each variable (genes, metabolic classes, and climate metrics) for altitude-based classification, with lighter/yellow bars indicating higher predictive value. (C) Redundancy Analysis (RDA) CCA-based triplot mapping the multivariate relationships. Blue vectors (gene variables) demonstrate strong differentiation along the axes, while red vectors (explanatory environmental/metabolic gradients) and gray sample points illustrate the underlying ecological divergence. Vector alignment indicates correlation structure. (D) Spearman Correlation heatmap

of Genes, Metabolites, and Climate variables. The color scale indicates correlation strength from -1.00 (dark blue, negative correlation) to +1.00 (dark red, positive correlation), with significance ($p < 0.05$) marked by asterisks (*) within the cells. Cytochrome (CYP) genes and Sesquiterpene hydrocarbon (SH) metabolite synthesis.

Discussion

The ecological and evolutionary success of medicinal plants in alpine environments is profoundly linked to their capacity to modulate secondary metabolism in response to fluctuating abiotic factors. In this study, we evaluated the spatiotemporal expression dynamics of *CYP71D178* and *CYP71D180* across a precise altitudinal gradient on Sabalan Mountain over three consecutive years (2022–2024). Our results demonstrate a clear non-linear relationship between continuous altitude and gene expression, alongside prominent inter-annual shifts in overall expression intensity, reflecting the remarkable transcriptomic plasticity of *Thymus kotschyanus*. Altitude acts as a complex proxy for multiple interacting environmental stressors, including reduced atmospheric pressure, dramatic temperature fluctuations, and enhanced UV-B radiation (Bisht et al., 2021; Salehi et al., 2019). The quadratic regression analysis applied in this study confirmed a strong non-linear correlation between altitude and the log2 fold change of both genes. Interestingly, while both genes are involved in the terminal oxidation steps of the phenolic monoterpene pathway (Krause et al., 2021), they exhibited distinct spatial distribution patterns.

The expression of *CYP71D178*, which encodes the enzyme responsible for synthesizing thymol (Crocoll et al., 2010), showed sustained upregulation at higher altitudes, generally peaking above 3250 m. This is consistent with earlier reports demonstrating that alpine plant species increase their antioxidant defense mechanisms in response to ultraviolet exposure at higher altitudes (Hasanvand et al., 2022). On the other hand, the gene *CYP71D180* (carvacrol synthase) displayed intense expression peaks within limited altitude ranges, showing extremely high activity at approximately 2900 meters before declining at the highest altitudes investigated. These divergent trends have been observed in other Lamiaceae family species, where local microclimate or particular altitude thresholds induce unique physiological responses (Azizi et al., 2021; Yavari et al., 2018). Beyond spatial altitude gradients, our data strongly highlights the massive impact of temporal, year-to-year climatic variations. The box and whisker analysis, alongside the spatiotemporal heat matrices, clearly demonstrate a “marked upregulation” in certain years relative to others. More specifically, for both the cytochrome P450 and its regulatory transcription factor, there was an apparent increase in baseline expression in the year 2023, which was sustained, and in some cases, even surpassed in the year 2024. As observed in natural ecosystems, inter-annual variations in climatic conditions, including precipitation in spring, frost events, and total heat summation, play an essential role in regulating the physiological stress undergone by populations in nature (Ashrafi et al., 2022). Based on past findings from Ghorbanpour et al. (2022), it is known that environmental stresses lead to increased induction of cytochrome P450 genes in *Thymus* species. The sudden rise in normalized expression post 2022 is indicative of changing weather patterns that compelled the wild plant populations on Mount Sabalan to adapt via secondary metabolic pathways. Because *CYP71D178* and *CYP71D180* compete for the same precursor, gamma-terpinene (Krause et al., 2021), their relative expression levels are highly indicative of the

final chemotype of the plant. In a number of elevation levels seen in our study, the gene *CYP71D178* showed slightly elevated fold changes in extremely high elevations relative to *CYP71D180*, thus providing molecular reasoning behind the well-known chemotype dominated by thymol within some high-elevation *Thymus* ecotypes (Kianersi et al., 2021). Such transcriptomic competition highlights how carefully regulated is the biological machinery of wild thyme when it comes to chemical defenses against variable alpine conditions. The application of structural equation modeling provides a rigorous path analysis that confirms the direct and indirect causal effects underpinning these observations. Our finalized model demonstrates a strong statistical fit, explaining a significant proportion of the variance in target gene expression. Environmental conditions exert a strong direct influence on target gene expression, while also revealing a powerful two-step indirect pathway: environmental conditions first modulate the plant's metabolic profile, which then, in turn, dictates target gene expression. Notably, minimum humidity ($U_{\min}$) was identified as a critical environmental "hotspot". Climatic shifts, especially increased $U_{\min}$ and temperature, act as primary environmental stressors, forcing the plant to prioritize its metabolic and genetic resources, triggering a significant upregulation of stress-adaptation genes while simultaneously suppressing growth-focused gene clusters. This suggests that target gene expression is not a linear response to a single factor but is instead a highly integrated biological response that considers both the external environment and internal metabolic status.

Conclusion

This study provides the first comprehensive analysis of the spatiotemporal expression dynamics of *CYP71D178* and *CYP71D180* genes in wild thyme (*Thymus kotschyanus*) across an altitudinal gradient on Sabalan Mountain. The research demonstrates that these genes exhibit significant transcriptomic plasticity with complex, non-linear dependencies on altitude and prominent inter-annual variability. While *CYP71D178* showed sustained upregulation at higher altitudes, suggesting a specific role in antioxidant defense, *CYP71D180* displayed intense, localized expression peaks, highlighting distinct sensitivity profiles. Ultimately, these findings reveal an integrated biological response to environmental stress, providing a critical framework for predicting how climate-driven shifts affect the pharmacological quality of alpine medicinal flora and offering valuable insights for future conservation and cultivation strategies.

Materials and Methods

Plant Material and Sampling Design

Samples of wild thyme (*Thymus kotschyanus*) were obtained along an exact altitude gradient ranging from about 2154 m to 3229 m at Sabalan Mountain. Both the vertical variation and yearly variation were captured through a systematic collection of samples at 15 different altitudes in a sequential order starting from lower altitudes to higher altitudes. The collection process occurred for three successive years, 2022, 2023, and 2024. Fresh leaves were plucked from healthy, mature plants, rapidly frozen in liquid nitrogen, and kept at -80°C .

RNA Extraction, cDNA Synthesis, and qRT-PCR

Total RNA was obtained from the frozen leaf samples using TRIzol reagent (Yekta tajhiz) by following the instructions provided by the manufacturer. The quality and quantity of total RNA was checked using a spectrophotometer. The integrity of total RNA was confirmed through agarose gel electrophoresis.

Reverse transcription was carried out using 1 µg of total RNA as template using the (Yekta tajhiz) reverse transcription kit in accordance with the established procedure. Then qRT-PCR was conducted for determining the levels of expression of target genes, CYP71D178 and CYP71D180 (Table 1).

Real-Time Primers	Sequences (5' to 3')	Tm °C	Amplicon Size (bp)
CYPD178 F	CGACCACTGGCGCCAAATAC	60	179
CYPD178 R	CGATGGTGCACACCGTCTTG		
CYPD180 F	AACATCGGGTCGCGGATCAT	60	165
CYPD180 R	GATGAGGCGAGCCATCTCGT		
UBQ- F	AAGACCTACACCAAGCCCAA	54	196
UBQ- R	AAGTGAGCCCACACTTACCA		

Table 1. The specific primers utilized for the amplification, including their nucleotide sequences and technical characteristics for Real-Time PCR (qPCR) analysis of *CYPD178*, *CYPD180*, and *UBQ* genes.

All experiments were performed in triplicates using a Bio-Rad CFX96 Real-Time PCR Detection machine and Power up SYBR Green Master Mix. The thermocycling protocol included an initial step at 95°C for 2 minutes, followed by 40 cycles of 95°C for 15 seconds, and 60°C for 60 seconds.

Gas Chromatography-Mass Spectrometry Analysis

The chemical composition of the essential oil was analyzed using a gas chromatograph (Agilent 7890B) coupled to a mass selective detector (Agilent 5977A). Separation was achieved on an HP-5MS capillary column (30 m × 0.25 mm i.d., film thickness 0.25 µm). The oven temperature program was set as follows: initial temperature of 60 °C (held for 5 min), increased to 250 °C at a rate of 3 °C/min, and held at 250 °C for 10 min. The injector temperature was maintained at 250 °C. A sample volume of 1 µL was injected in split mode (split ratio 1:50). Helium was used as the carrier gas at a constant flow rate of 1.0 mL/min. The mass spectrometer operated in electron ionization (EI) mode at 70 eV. The ion source and quadrupole temperatures were set at 230 °C and 150 °C, respectively. Mass spectra were recorded in the range of 40–550 m/z. Compound identification was performed by comparing mass spectra with those in NIST (2017) and Wiley libraries, along with comparison of calculated linear retention indices (LRI) with literature values. LRI values were determined using a homologous series of n-alkanes (C8–C40) under identical conditions. Relative quantification of components was based on peak area normalization without the use of an internal standard and expressed as percentage of total ion chromatogram (TIC). Only compounds with a match quality >90% and LRI deviation <10 units were considered.

Data Processing and Statistical Analysis

The relative quantification was done using comparative $2^{-\Delta\Delta Ct}$ technique with normalization to the chosen endogenous gene. For selective analysis, the sampling elevation values were divided into two different altitudes: Low Altitude (< 2700 m) and High Altitude (>2700 m).

Statistical analyses and data visualizations were executed to evaluate the isolated and combined effects of altitude and time on target gene fold changes. Box and whisker plots were generated to assess the distribution and variance of fold change by gene, year, and altitude group. To evaluate the non-linear relationship between continuous sampling altitude and the log2 Fold Change of expression, a quadratic regression analysis was applied with fitted regression curves. Spatiotemporal heat matrices were constructed to directly map the distribution of expression intensity across all measured altitudes and years. Statistical significance across groups was evaluated using ANOVA followed by Tukey's test, with significance thresholds denoted conventionally (ns = not significant, * = $p < 0.05$, ** = $p < 0.01$).

Data availability

All relevant data can be found within the manuscript and its supporting materials, and further inquiries can be directed to the corresponding author.

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Author Contributions

Maryam Samadi, conducted the research as her PhD dissertation and prepared the first draft of the manuscript. Omid Sofalian, supervised and funded the project, and edited the manuscript. Noraddin Hosseinpour Azad: Proposed the research idea, Supervised and Project administration, Conceptualization, Writing and editing the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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