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Local Climate and Cultivation Practice Shape Total Protein and Phenolic Content of Mulberry (*Morus* sp.) Leaves in Sub-Mediterranean and Sub-Pannonian Regions of Slovenia

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Abstract

Mulberry (*Morus* sp.) trees, traditionally cultivated for their leaves used in sericulture, have recently gained recognition for their adaptability and valuable ecosystem services. The biochemical composition of mulberry leaves varies both qualitatively and quantitatively, depending on genotype, environmental conditions, and cultivation practices. This study aimed to (1) identify differences in old local white (*M. alba* L.) and black mulberry (*M. nigra* L.) leaves, (2) perform a chemotype analysis of monitored local varieties, and (3) evaluate the influence of selected bioclimatic factors and pruning practices on the biochemical composition of leaves of white mulberry trees across Slovenian mesoregions. Black mulberry exhibited a higher phenolic content, particularly caffeoylquinic acid derivatives (16.05 mg/g dry weight (DW)), while white mulberry contained more quercetin glycosides (6.04 mg/g DW). Ward's clustering identified three chemotypes, two of which had elevated protein and hydroxycinnamic acid levels, making them particularly suitable for silkworm feeding. Considering pruning practices of white mulberries, we determined significantly increased protein contents in yearly pruned trees (187.24 mg/g DW). Principal component analysis revealed interactions between bioclimatic, morphological, and biochemical factors, distinctly separating mulberries from the Sub-Mediterranean and Sub-Pannonian macroregions. White mulberries from Sub-Pannonian regions accumulated more caffeoylquinic acids in leaves under lower precipitation and total insolation, while those from Sub-Mediterranean regions exhibited higher kaempferol derivatives due to photo-thermal stress. These findings highlight the influence of climate and pruning on mulberry biochemical diversity and adaptation.

Keywords: mulberry; *Morus alba*; *Morus nigra*; local genetic resources; phenolics; protein; climatic effect; metabolite screening; pruning

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1. Introduction

White mulberry trees (*Morus alba* L.), particularly abundant in the Sub-Mediterranean and Sub-Pannonian regions of Slovenia, reflect the country's rich silk production heritage [1–4].

Mulberries, once the foundation of sericulture, remain a living testament to Slovenia's sericultural tradition, because their leaves serve as the sole food source for silkworms (*Bombyx mori* L.) [5]. While sericulture thrived in the warmer Sub-Mediterranean and dryer Sub-Pannonian regions of Slovenia, the introduction of synthetic and artificial silk and the dominance of Asian silk production triggered a significant decline in European silk industries. By the early 1960s, Slovenia's traditions of mulberry cultivation and silkworm rearing had nearly vanished [1]. This historical context guided our decision to focus on Sub-Mediterranean and Sub-Pannonian regions for the inventory of mulberry trees, exploring their distinct attributes and the cultural and agricultural legacy they represent.

Despite its modest size of 20,271 km^2, Slovenia exhibits a remarkably diverse climate shaped by its temperate location, high topographic heterogeneity, coastal proximity, and dynamic air circulation patterns. Local factors—including vegetation, water bodies, wind corridors, and urbanisation—further influence these conditions [6]. The country is characterised by three primary climate types: (1) temperate humid deciduous forest zone (zonobiome VI) with hot summers, (2) boreonemoral to montane coniferous-deciduous mixed forests zone (zonobiome VII) including mountain regions of Alps and Dinaric ranges, and (3) Sub-Mediterranean zone (zonobiome IV). Coastal areas experience the highest average annual air temperatures (exceeding 12 °C), while central regions range from 8 to 10 °C, with freezing conditions only in winter months [7–9]. The country's interior endures notable extremes due to winter inversions, spring frosts, and summer droughts. Slovenia benefits from abundant solar energy and an average annual precipitation of 1750 mm [10], with the highest levels in the northwest and the lowest in the northeast [10]. Given the climatic diversity of Slovenia, the adaptive capacity of mulberry trees in different regions warrants detailed investigation.

Chemotypes, also known as chemical varieties, are defined as individuals within the lower-level taxa that differ qualitatively and quantitatively in their metabolite content. Distinct morphological differences can sometimes be observed among chemotypes; however, in many cases they may appear morphologically similar. Variations in chemotypes can be influenced by environmental conditions and seasonal changes [11]. Since leaf metabolites are highly influenced by these factors, chemical typing provides a valuable framework for assessing biochemical diversity and adaptation in mulberries [12].

Through screening of the proteins and phenolic metabolites, this study aimed to determine whether the mulberries from these regions exhibit distinct chemotypes, offering valuable insights for identifying potential germplasm sources. In the past mulberries were predominantly propagated by seeds. Grafting of high-yielding varieties was more common in the warmer regions of Italy and, whereas in Slovenia it was practiced only in the Savinja Valley of Lower Styria [1,13–16]. Based on high variability, we aimed to explore whether these historical germplasm sources influenced the biochemical traits of mulberries in these areas, and to which extent their biochemical profile is affected by bioclimatic parameters and pruning practice.

Mulberries (*Morus* sp.) are woody perennial trees within the Moraceae family, which includes 37 genera and around 1100 species found in temperate, tropical, and subtropical regions [17]. In Europe, the widely distributed species is white mulberry (*M. alba* L.) originating from China and adjusted regions of Central Asia, while black mulberry (*M. nigra* L.), originating from the regions of Iran and Syria, was brought from Middle East to Europe already in the classical antiquity period [18,19].

Both species are best identified by leaf characteristics, bud and shoot morphological descriptors. White mulberry typically has glossy leaves and longer peduncle, while black mulberry has matt leaves, with pubescent abaxial side and short peduncle [20,21]. Black

mulberry is rare in Central Europe, as its leaves are less suitable for silkworm rearing, though the individual trees are more common in the Aegean and Adriatic regions [22,23].

White mulberry leaves have a high protein content reaching levels of up to 280 mg/g DW [3,24,25]. In addition to proteins and their unique composition of amino acids—dominated by aspartic acid, glutamic acid, phenylalanine, lysine, and arginine—the leaves are rich in notable constituents such as carbohydrates, calcium, iron, ascorbic acid, carotenoids, thiamine, folic acid, and phenols [3,26,27]. These compounds make them valuable not only as food for silkworms and other animals but also due to their bioactive properties, which have been recognised in traditional medicine for centuries [28–31].

He et al. [32] analysed the chemical composition of white mulberry leaves grown in different Chinese provinces, reporting a total phenolic content ranging from 11.49 mg GAE/g DW in mature leaves to 30.03 mg GAE/g DW in young leaves. The major phenolic acids identified were chlorogenic acid and its derivatives, along with *p*-coumaric acid, while the predominant flavonol content included rutin, quercetin, and kaempferol glycosides [4,32–34].

Phenolic production in leaves is primarily determined by genotype [35], but is also strongly influenced by climate conditions and environmental factors such as drought, extreme temperature fluctuations, high radiation, and pests [36–38]. These stressors stimulate the synthesis of phenolic acids and flavonoids, which serve as protective antioxidants and ultraviolet (UV) absorbent. UV exposure, temperature extremes, and drought conditions are known to enhance phenolic biosynthesis, aiding the plant's resilience against environmental stress [39–42]. Understanding these adaptive responses to bioclimatic factors is key to explaining phenolic variations in mulberry leaves.

Mulberry trees are traditionally pruned in form of pollarding, where all the branches are cut back to a framework, which promotes a dense head of branches. Pruning acts as a form of abiotic stress, triggering complex molecular and metabolic responses in plants, such asupregulati genes involved in protein processing, nitrogen metabolism, and amino acid biosynthesis, thereby enhancing protein production and growth capacity [43,44]. Stress-induced translational controls, such as selective regulation of tRNA pools, further triggers the synthesis of stress-response proteins [45]. At the same time, pruning downregulates genes of the phenylpropanoid pathway, leading to reduced accumulation of phenolic acids and flavonoids [44,46]. Previously, Šelih et al. [4] observed increased protein levels in pruned white mulberry trees and decreased phenolic content in unpruned mulberry trees.

The objective of this study is to analyse the main metabolites—total proteins as well as total and individual phenolics—in the leaves of old local white and black mulberry trees from the Sub-Mediterranean and Sub-Pannonian regions of Slovenia, taking into account clinal variation associated with geographic position and the impact of environmental factors such as air temperature (°C), total insolation (hours), and precipitation (mm), along with pruning practices. This research aims to enhance the understanding of the adaptation of the local old mulberry trees to their environment and provide potential for future propagation and cultivation strategies.

2. Materials and Methods

2.1. Sampling Method

Field excursions were conducted in the Sub-Mediterranean and Sub-Pannonian regions of Slovenia during the summer season (June to August) of 2023, specifically between 15 June and 10 August. Samplings were conducted on clear days, predominantly between 10:00 and 14:00, in order to minimise the influence of diurnal fluctuations in leaf metabolism and water status. The sampling period—mid-June to mid-August—was deliberately chosen because, at this stage, mulberry leaves are fully developed and physiologically mature.

This ensures that the collected material represents a stable metabolic state, unaffected by the rapid developmental changes that occur earlier in the season. By standardising the sampling window, environmental conditions, and time of day, we aimed to reduce variability linked to phenological stage, microclimatic fluctuations, and circadian rhythms. Such consistency provides reliable and comparable biochemical profiles across sites and genotypes, reflecting peak photosynthetic and metabolic activity of mulberry leaves.

During these excursions, mulberry trees (*Morus* sp.) were systematically inventoried using *Morus*APP (https://morusapp.aracneproject.eu/login, accessed on 7 September 2025) [47]. The application was developed in the framework of European Union's HORIZON-CL2-2022-HERITAGE-01-02 (Grant Agreement 101095188—ARACNE) with the aim of identifying old mulberry (*Morus* spp.) varieties in different European countries. The entered visual observations of individual mulberry trees characterise them in sufficient detail, allowing Aracne partners active in mulberry research to determine morphotypes of the same species through advanced statistical analyses. The application is designed for simple, transparent and at the same time comprehensive way of use. *Morus*APP generates identification numbers for each tree and records global navigation satellite system (GNSS) location coordinates (latitude, longitude) for mapping. In the application, taxonomic and phytogeographical information, accessibility and tree frequency, tree growth habit, tree vigour, pruning practices, trunk shape, morphological characteristics of shoots, leaves and reproductive structures, and observations on diseases and pests were recorded according to mulberry descriptors described in the manual [47].

We classified the sampled mulberry trees into three categories according to pruning frequency and type: (1) unpruned trees, (2) frequently pruned trees, and (3) yearly pruned trees. Frequently pruned trees (2) included those pruned at least once within the last three years for crown maintenance and reduction. Yearly pruned trees (3) were subjected to traditional pollarding, in which all branches are cut back to a framework to promote a dense crown of new shoots. For statistical analysis, pruning categories were treated as categorical variables.

Based on location we classified the inventorised mulberry trees into mesoregions based on Perko [48] to examine clinal variation and the effects of bioclimatic parameters on total protein and phenolic contents. The inventorised mulberries (*Morus* sp.) from the Sub-Pannonian and Sub-Mediterranean regions were grouped into seven mesoregions: Drava Plain and Slovenian Hills (both Sub-Pannonian), and Gorica Hills, Vipava Hills, Karst Plateau, Brkini Hills and Reka Valley, and Koper Hills in the Sub-Mediterranean region, as shown in Figure 1.

In total, 88 mulberry trees from local genetic pool mainly propagated by seeds were analysed, consisting of five black mulberries (*M. nigra*) and 83 white mulberries (*M. alba*). We focused on trees with a circumference greater than 250 cm, which were planted during the sericultural era prior to the Second World War. Due to the scarcity of black mulberries sampled exclusively in the Koper Hills mesoregion, we excluded them from the comparative analysis of bioclimatic effects and instead focused on species-specific differences in biochemical profiles. Our study concentrated on mulberries from the Sub-Mediterranean and Sub-Pannonian regions of Slovenia, areas historically renowned for their significance in sericulture. A total of 83 different white mulberries were inventoried. Eight mulberries were monitored in the Drava Plain mesoregion with the maximum circumference of 347 cm, six were found in Slovenian Hills (max. circumference 401 cm), two in Gorica Hills (max. circumference 358 cm), ten in Vipava Hills (max. circumference 408 cm), 16 in Karst Plateau (max. circumference 752 cm), two in Brkini Hills and Reka Valley (max. circumference 195 cm) and 39 in Koper Hills (max. circumference 400 cm). Monitoring of the pruning frequency revealed that the highest number of pruned trees was found in Brkini Hills

and Reka Valley, whereas in Drava Plain, Slovenian Hills and Gorica Hills most of trees were left unpruned. More detailed information on the sampled mulberries is provided in Supplementary Table S1.

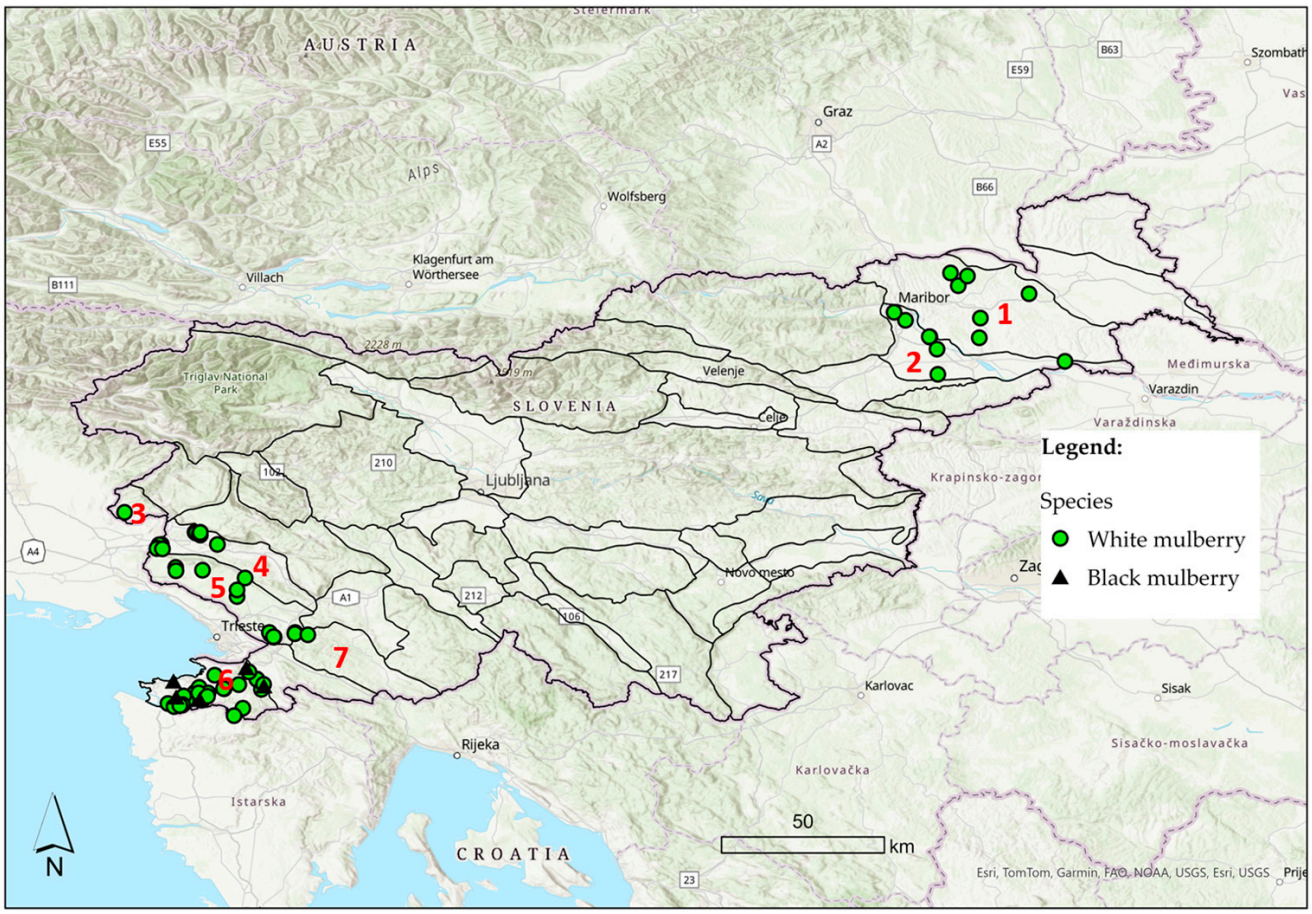

Figure 1. Distribution map of mulberry trees in Slovenia using *Morus*APP. Locations of white mulberry (*Morus alba*) trees are represented by green circles, while black mulberry (*M. nigra*) trees are shown as black triangles. Regionalization of Slovenia into mesoregions according to Perko [48] Sub-Pannonian macroregion: 1: Slovenian Hills (n = 6); 2: Drava Plain (n = 8); Sub-Mediterranean macroregion: 3: Gorica Hills (n = 2); 4: Vipava Hills (n = 10); 5: Karst Plateau (n = 16), 6: Koper Hills (n = 39) and 7: Brkini Hills and Reka Valley (n = 2).

The number of sampled mulberry trees varied considerably among regions, reflecting historical cultivation patterns in relation to traditional land use, sericultural activity, topography, and favourable environmental conditions. A major constraint was that many trees had been cut down after World War II, and certain areas along the World War I battlefront exhibited a low number of large-circumference trees [1]. For this reason, the Goriška Hills and Brkini Hills an Reka Valley regions are represented by only two large trees, which were included in the analysis.

Additionally, logistical limitations—including land ownership, site accessibility, and fieldwork feasibility—further contributed to uneven sampling. In regions with a higher tree density, targeted sampling was conducted to capture broader environmental and genetic variability. Although this resulted in an unbalanced dataset, appropriate statistical methods [e.g., standardisation, non-parametric tests using the median and median absolute deviation (MAD), and principal component analysis (PCA)] were applied to account for these differences and enable robust comparisons across environmental gradients.

For the biochemical analyses, we selected ten fully developed, sun-exposed leaves between the fifth and seventh positions below the shoot apex. The samples were immediately placed in dry ice to preserve their integrity and later transferred to a freezer at −80 °C. Subsequently, the samples were freeze-dried and ground into a fine powder. The prepared samples were stored in airtight vials at −20 °C until further biochemical analyses were conducted. All nutrient concentrations were calculated on a dry weight (DW) basis.

2.2. Chemicals

Sodium carbonate (Na_2CO_3), trichloroacetic acid (TCA), ethanol and HPLC-grade acetonitrile, and methanol were procured from Honeywell International Inc., Charlotte, NC, USA. Folin–Ciocalteu's phenol reagent was supplied by Merck KGaA, Darmstadt, Germany. High-Performance Liquid Chromatography (HPLC) grade formic acid, along with standard compounds including BSA, sodium hydroxide (NaOH), potassium sodium tartrate, gallic acid, chlorogenic acid, *p*-coumaric acid, kaempferol-3-O-glucoside, quercetin-3-O-glucoside, and quercetin-3-O-rutinoside (rutin), were obtained from Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany.

2.3. Determination of Total Protein Content

The total protein content was determined using a modified Lowry method [49], with protein precipitation performed using TCA, as described by Kumar et al. [24].

Briefly, 25 mg of lyophilised leaf powder was homogenised using a Turrax machine at 18,000 rpm for 25 s. During homogenisation, the test tube containing the lyophilised material was slowly lowered and raised to ensure that the Turrax blade remained submerged in the liquid and that the entire content was thoroughly homogenised. The homogenised sample was then centrifuged for 10 min. After discarding the supernatant, the resulting pellet was resuspended in 5 mL of 10% TCA and incubated on ice for 30 min with continuous shaking on an orbital shaker. Following 10 min centrifugation, the supernatant was removed, and the pellet was resuspended in 5% TCA vortexed for 15 s, and incubated with shaking on ice for an additional 20 min. This washing step was repeated to remove low-molecular-weight compounds, including free amino acids, phenolic compounds (notably kaempferol derivatives), and ascorbic acid, which are known to interfere with the Lowry assay. After a final centrifugation (10 min), the purified protein pellet was collected for subsequent analysis.

The protein precipitate was then dissolved in 1 M NaOH, pulse-vortexed, and incubated in a hot water bath at 60 °C for 45 min. The sample was subsequently centrifuged for 10 min, and 50 µL of the supernatant containing proteins was diluted tenfold with distilled water. Subsequently, 500 µL of the diluted protein solution was mixed with 700 µL of Lowry reagent in a plastic cuvette. The mixture was incubated at room temperature for 20 min with continuous shaking. Then, 100 µL of Folin Ciocalteureagent was added, followed by an additional 30 min incubation in the dark at room temperature under continuous shaking. Absorbance was measured spectrophotometrically at 750 nm using a Varian Cary 50 Bio spectrophotometer (Varian Cary®, Cary, NC, USA) within 10 min after the completion of incubation to ensure reliable results.

The total protein content in each sample was calculated using a standard curve based on a BSA standard concentration range of 0.05 to 0.45 mg/mL. Results are therefore expressed as milligrams of BSA-equivalent per gram of dried mulberry leaves (mg BSA/g DW), reflecting relative rather than absolute protein concentrations.

2.4. Extract Preparation for the Analysis of Phenolics

For phenolic analysis, 20 mg of lyophilised mulberry powder was homogenised with 1.5 mL of 3% formic acid in 70% methanol, following Ainsworth and Gillespie's [50] method.

Samples were kept on ice to preserve bioactive compounds, then incubated for 30 min in an ultrasonic bath. After centrifugation at 4 °C for 15 min at 12,000 revolutions per minute (rpm), 100 µL of the supernatant was transferred to dark microcentrifuge tubes for total phenolic content analysis. The remaining samples were centrifuged again for 45 min under the same conditions, and 700 µL of supernatant was stored at −20 °C for individual phenol analysis by HPLC.

2.5. Determination of Total Phenolic Content

For the determination of total phenolics, 100 µL of the sample supernatant was mixed with 200 µL of freshly prepared Folin's reagent (diluted 1:9 with distilled water). After 5 min, 800 µL of 0.87 M Na_2CO_3 solution was added. The mixture was vortexed briefly and incubated in the dark at room temperature with gentle shaking (30 rpm) for two hours. Total phenolic content was quantified using the Folin–Ciocalteu method [50]. Absorbance was measured at 765 nm using a Varian Cary 50 Bio spectrophotometer. A calibration curve was constructed using gallic acid standards in the concentration range of 0.05 to 0.28 mg/mL. Results were expressed as mg gallic acid equivalents per gram of dry weight (mg GAE/g DW). All measurements were performed in duplicate.

2.6. Determination of Individual Phenolics

Individual phenolic compounds were analysed using HPLC with a Waters Alliance 2695 system and a 2996 Photodiode Array detector (PDA) detector at 280 nm for phenolic acids and 350 nm for flavonoids, following the method of Mikulic-Petkovsek [51]. Results were expressed as mg/g of dry weight.

For the gradient HPLC method the following mobile phases were prepared: Mobile Phase A was composed of 97% double deionised water (ddH_2O), 3% acetonitrile, and 0.1% formic acid, while Mobile Phase B consisted of 97% acetonitrile, 3% ddH_2O, and 0.1% formic acid. The gradient elution was programmed as follows: 0–10 min at 95% A and 5% B; 10–15 min at 80% A and 20% B; 15–30 min at 70% A and 30% B; 30–35 min at 10% A and 90% B; 35–45 min at 0% A and 100% B; and finally, at 45 min, returning to 95% A and 5% B.

Chromatographic separation was performed using a Gemini C18 110A column (150 × 4.6 mm, 3 µm) at a constant temperature of 25 °C. The flow rate was maintained at 0.6 mL/min, with an injection volume of 20 µL for both extracts and standards. Each sample was analysed in duplicate, and the extraction was repeated twice.

Phenolic compounds were identified using a mass spectrometer (Thermo Finigan, San Jose, CA, USA) with an electrospray interface (ESI) in negative ion mode. Full scan data-dependent MS2 scanning was performed from m/z 110 to 1500. Chromatographic conditions were identical to those used for HPLC-DAD analysis [51]. The capillary temperature was 250 °C, with sheath and auxiliary gas set at 60 and 15 units, respectively, and a source voltage of 3 kV. Normalised collision energy ranged between 20% and 35%. Spectral data analysis was conducted using Excalibur software version4.1 (Thermo Scientific, Waltham, MA, USA). The limit of detection (LOD) and limit of quantification (LOQ) were determined according to signal-to-noise ratios of 3:1 and 10:1, respectively Compound identification was confirmed by comparing retention times and spectra, supplemented by standard additions and fragmentation as shown in Table 1. Quantification was based on corresponding standards.

For example, quercetin-3-glucoside (Q-3-glu) and quercetin malonyl hexoside (QMH) were quantified in quercetin equivalents, kaempferol acetyl-hexoside (KAH) in kaempferol equivalents, caffeoylquinic acid derivatives in chlorogenic acid equivalents, and *p*-coumaroylquinic acid derivatives in *p*-coumaric acid equivalents.

Table 1. Identification of individual phenolic compounds in mulberry leaves by HPLC-MS: retention time (min), negative molecular ion mode ([M–H]−), MS^2 fragmentation data and relative abundance of each ion in a fragment.

Phenolic Compound	Retention Time [Min]	[MH]-	Characteristic *m*/*z* of Ions in Negative Ion Mode
p-coumaric acid hexoside$_1$	9.89	325	$MS^2 \rightarrow 163$ (100), 119 (40); MS^3 (163) $\rightarrow$ 119 (100), 93 (60)
p-coumaric acid hexoside$_2$	12.40	325	$MS^2 \rightarrow 163$ (100), 119 (40); MS^3 (163) $\rightarrow$ 119 (100), 93 (60)
chlorogenic acid (*trans*-5-caffeoylquinic acid)	12.88	353	$MS^2 \longrightarrow 191$ (100); $MS^3 \longrightarrow 127$ (100), 173 (65), 111 (42), 93 (43), 85 (39)
4-caffeoylquinic acid	13.67	353	$MS^2 \rightarrow 173$ (100), 191 (40), 179 (30); MS^3 (173) $\rightarrow$ 111 (100), 93 (60)
cis-5-caffeoylquinic acid	14.46	353	$MS^2 \longrightarrow 191$ (100), 179 (50), 135 (11); $MS^3 \longrightarrow 85$ (100), 173 (75), 127 (89), 111 (55), 93 (74)
trans-5-*p*-coumaroylquinic acid	15.11	337	$MS^2 \rightarrow 191$ (100), 163 (100), 173 (40); MS^3 (163) $\rightarrow$ 119 (100), 93 (60)
cis-5-*p*-coumaroylquinic acid	15.88	337	$MS^2 \longrightarrow 163$ (100), 191 (5), 119 (6); $MS^3 \longrightarrow 119$ (100)
3-*p*-coumaroylquinic acid	16.69	337	$MS^2 \longrightarrow 163$ (100), 191 (6), 119 (6); $MS^3 \longrightarrow 119$ (100)
quercetin dirhamnosyl-hexoside	18.09	755	$MS^2 \rightarrow 301$ (100); MS^3 (301) $\rightarrow$ 179 (100), 151 (70), 121 (50)
kaempferol dirhamnosyl-hexoside	18.50	739	$MS^2 \rightarrow 285$ (100); MS^3 (285) $\rightarrow$ 151 (100), 133 (70), 117 (50)
quercetin rhamnosyl-hexoside	19.34	609	$MS^2 \rightarrow 301$ (100); MS^3 (301) $\rightarrow$ 179 (100), 151 (70), 121 (50)
rutin	20.51	609	$MS^2 \longrightarrow 301$ (100); $MS^3 \longrightarrow 179$ (100), 273 (20), 257 (14), 151 (94)
quercetin-3-glucoside	21.01	463	$MS^2 \longrightarrow 301$ (100); $MS^3 \longrightarrow 179$ (100), 273 (22), 257 (10), 151 (76)
quercetin acetyl-rhamnosyl-hexoside	21.55	651	$MS^2 \rightarrow 301$ (100); MS^3 (301) $\rightarrow$ 179 (100), 151 (70), 121 (50)
kaempferol rhamnosyl-hexoside	22.20	593	$MS^2 \rightarrow 285$ (100); MS^3 (285) $\rightarrow$ 151 (100), 133 (70), 117 (50)
kaempferol acetyl-rhamnosyl-hexoside	23.07	635	$MS^2 \rightarrow 285$ (100); MS^3 (285) $\rightarrow$ 151 (100), 133 (70), 117 (50)
quercetin malonyl-hexoside	23.27	549	$MS^2 \rightarrow 301$ (100); MS^3 (301) $\rightarrow$ 179 (100), 151 (70), 121 (50)
quercetin acetyl-hexoside	24.33	505	$MS^2 \rightarrow 301$ (100); MS^3 (301) $\rightarrow$ 179 (100), 151 (70), 121 (50)
kaempferol acetyl-hexoside	25.07	489	$MS^2 \rightarrow 285$ (100); MS^3 (285) $\rightarrow$ 151 (100), 133 (70), 117 (50)

2.7. Selected Climatic Parameters

In our study, we incorporated climatic parameters including average, minimum, and maximum air temperature (°C), total insolation (hours), and precipitation (mm) for the sampling season from June to August 2023. Average, minimum, and maximum air temperatures were treated as separate variables and integrated individually into the ArcGIS spatial analysis. These data were provided by the Slovenian Environment Agency which operates under the Ministry of Environment, Climate, and Energy [52]. We additionaly incorporated 30-year averages (June–August 1970–2000) for mean, minimum, and maximum air temperature (in °C), precipitation (in mm), and solar radiation (in kJ m^{-2} day^{-1}) from the WorldClim database [53]. To generate representative summer datasets, all availible raster layers of above mentioned climatic variables for June, July, and August were imported into the ArcGIS environment, where the mean values for the Summer season were calculated. For growing degree days above 10 °C (heat sum of all days above the 10 °C temperature accumulated over 1 year) and growing season length (in number of days), the Chelsa Climate database (1981–2010) [54] was used. Each layer was downloaded at a 30 s horizontal resolution (approximately 1 km) and integrated with our mulberry sampling locations in ArcGIS Pro (version 3.3.0). Using the "Extract Multi Values to Points" tool in ArcGIS Pro, we combined these bioclimatic parameters with our mulberry data points, generating an attribute table for further analysis. The maps were created using ArcGIS Pro,Esri basemaps [55]

2.8. Statistical Analyses

Statistical analyses were conducted using IBM SPSS Statistics version 25 (New York, NY, USA, 2017) and StatSoft, Inc. Statistica version 8.0 (Victoria, Australia, 2007). The assumptions of normal distribution for the measured trait data were assessed using the Kolmogorov–Smirnov test. Non-parametric tests such as Kruskal–Wallis and Man-Whitney U test were employed, because the assumptions of homogeneity of variance and normality were violated. Specifically, we used non-parametric tests to determine whether there were significant differences between group medians. Upon detecting a significant difference between the medians of at least one pair of groups, we proceeded with the Dunn–Bonferroni post hoc test. Significance values have been adjusted by the Bonferroni correction for multiple tests. Spearman's rank correlation analysis was conducted between evaluated biochemical traits. We used Quade's method [56], a rank-based nonparametric ANCOVA,

to analyse the effect of two independent variables while accounting for covariates. The null hypothesis was assessed at a significance level of $\alpha = 0.05$ and rejected when the *p*-value fell below this threshold, indicating statistically significant results.

Biochemical parameters were incorporated as factors in a multivariate statistical analysis using hierarchical cluster analysis. This analysis, based on Euclidean distance and Ward's method, was performed to explore the similarities among metabolites and more precisely define distinct chemotypes.

To comprehensively assess the effects of bioclimatic parameters on leaf biochemistry, we performed principal component analysis (PCA) and generated a heat map using PAST version 4.03 software.

3. Results

3.1. The Chemotype Analysis of Leaves of Inventoried White and Black Mulberry Trees in Mesoregions of Slovenia

A total of 83 mainly historical white mulberry trees (*M. alba*) were inventoried, with the highest number of trees in Koper Hills region, followed by Karst Plateau region. In contrast, black mulberry (*M. nigra*) is an exceedingly rare species in Slovenia, only five specimens were identified, all within the Koper Hills mesoregion. Given the limited number and localised distribution of *M. nigra*, our comparative analysis of phenolic and protein composition in mulberry leaves was confined to this region. This focused approach helped minimise the influence of bioclimatic variability, as the Koper Hills exhibit relatively uniform climatic conditions.

The analysis of total protein and phenolic contents in the leaves of white and black mulberry trees demonstrated notable variability across the dataset. Protein content ranged from a minimum of 97.73 mg BSA/g DW to a maximum of 299.36 mg BSA/g DW, with an overall median of 157.82 mg BSA/g DW, reflecting significant diversity in nutritional potential. Similarly, phenolic content varied widely, with contents ranging from 7.42 mg GAE/g DW to 19.23 mg GAE/g DW and a median of 14.99 mg GAE/g DW.

The analysis of individual phenolics in white and black mulberry leaves revealed a total of eight distinct hydroxycinnamic acids and eleven flavonols (Table 1). Median values for total protein and phenolic content and individual phenolics are presented in the Supplementary Table S2. Among phenolic acids, caffeoylquinic acids—particularly chlorogenic acid—together with *p*-coumaroylquinic acid derivatives were the most abundant. In terms of flavonoids, quercetin and kaempferol glycosides predominated. Rutin was identified as the major quercetin glycoside, while kaempferol acetyl-hexoside represented the predominant kaempferol glycoside.

A comparison of the two species revealed that the median total protein and phenolic content in black mulberry leaves was significantly higher than in white mulberries (Figure 2). Among the white mulberries, the lowest protein content was observed in the unpruned mulberry tree SI23_00176 from Fijeroga (97.24 mg BSA/g DW), while the highest was found in the frequently pruned mulberry tree SI23_00164 from Šmarje (297.16 mg BSA/g DW), suggesting an influence of tree condition and pruning practices on protein content. Notably, the highest phenolic content was observed in black mulberry SI23_00217 (20.11 GAE/g DW), whereas the lowest was observed in white mulberry SI23_00162 (8.19 mg GAE/g DW).

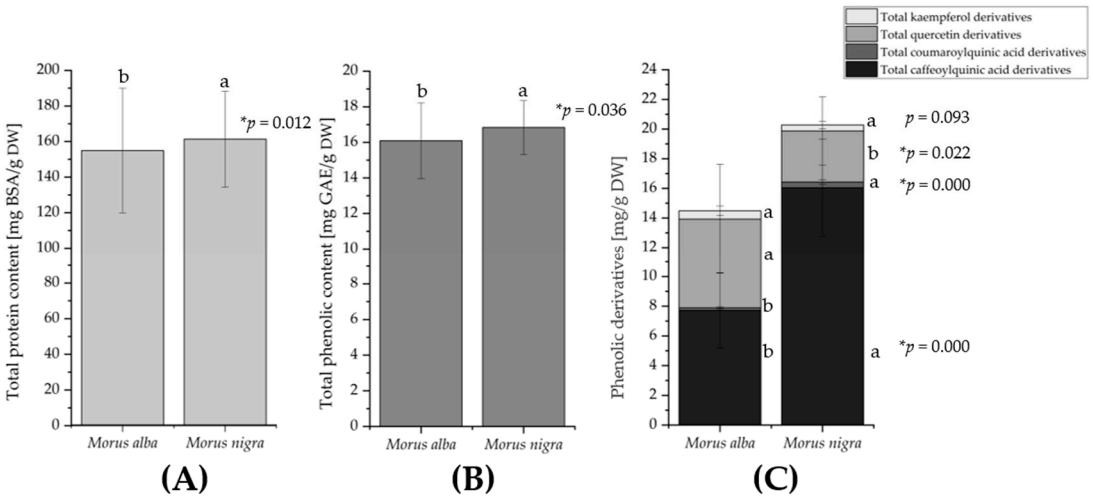

Figure 2. Content levels (median ± MAD) of (**A**) total protein content, (**B**) total phenolic content and (**C**) phenolic derivatives (mg/g DW) in white mulberry (*M. alba*) and black mulberry (*M. nigra*) leaves. Stacked graphs represent contents of different phenolic derivatives: caffeoylquinic acid derivatives, coumaroylquinic acid derivatives, quercetin derivatives, and kaempferol derivatives. Different letters (a,b) indicate significant differences ($p < 0.05$), which were determined using the post hoc Dunn–Bonferroni test. Asterisks above *p*-values in the figures indicate statistical significance at the 0.05 level ($p < 0.05$).

Analysis of individual phenolics revealed that black mulberry leaves contained significantly higher levels of total caffeoylquinic acid derivatives (16.05 mg/g DW) compared to white mulberries (7.72 mg/g DW). Similarly, the highest concentration was found in black mulberry tree SI23_00217 (22.11 mg/g DW), while the lowest was found in white mulberry tree SI23_00173 (3.49 mg/g DW). The content of coumaroylquinic acid derivatives differed significantly between the two mulberry species, with white mulberry exhibiting a lower median content (0.17 mg/g DW) compared to black mulberry leaves (0.39 mg/g DW). In contrast, white mulberry leaves exhibited statistically higher concentrations of total quercetin glycoside derivatives, with a median of 6.04 mg/g DW compared to a median of 3.44 mg/g DW in black mulberries. The range of total quercetin glycoside derivatives in white mulberry leaves spanned from 0.38 to 27.00 mg/g DW. There were no statistically significant differences in total kaempferol glycoside derivatives between mulberry species, with median values of 0.57 mg/g DW in white mulberry leaves and 0.41 mg/g DW in black mulberry leaves.

Spearman's rank correlation (ρ) was conducted to assess the relationships between total protein, total phenolic contents and individual phenolic compounds in mulberry leaves. Values in parentheses represent Spearman's rank correlation coefficients. Correlations marked with double asterisks (**) are statistically significant at the 0.01 level, while those marked with a single asterisk (*) are significant at the 0.05 level. Total protein content showed no statistically significant correlations with any of the evaluated phenolic constituents, suggesting an independent accumulation pattern in relation to phenolic metabolism.

Total phenolic content, however, exhibited multiple significant positive correlations with individual phenolic compounds. Moderate positive correlations were observed with total caffeoylquinic acid derivatives (0.46**), total coumaroylquinic acid derivatives (0.45**), total quercetin glycoside derivatives (0.39**) and total kaempferol glycoside derivatives (0.55**). Among individual compounds, total phenolic content was moderately correlated with chlorogenic acid (0.47**) and quercetin-3-glucoside (0.53**), as well as with quercetin malonyl hexoside (0.53**). Weaker, yet statistically significant, correlations were also found

between TPC and 5-caffeoylquinic acid (0.27*), the *cis*-5-*p*-coumaroylquinic acid (0.28*), and *p*-coumaric acid hexoside (0.24*). Among quercetin- glycoside derivatives, positive weak correlations were recorded with quercetin dirhamnosyl glycoside (0.24*), quercetin acetyl-rhamnosyl-hexoside (0.30**), and quercetin acetyl hexoside (0.36**). Regarding kaempferol derivatives, a moderate correlation was found with kaempferol acetyl-hexoside (0.55**), and a weaker one with kaempferol dirhamnosyl-hexoside (0.21*). All other correlations are presented in Supplementary Table S3.

To further explore these patterns, Ward's hierarchical clustering analysis using Euclidean distance was conducted to identify distinct chemotypes among the analysed white and black mulberry trees in regard to the analysed metabolites in leaves, including total protein content, total phenolic content, and individual phenolic compounds (caffeoylquinic acids, coumaroylquinic acids, quercetin derivatives, and kaempferol derivatives). The dendrogram with an integrated heatmap (Figure 3) presents a comprehensive overview of the biochemical characteristics of white and black mulberry leaves across different chemotype groups. The clustering analysis revealed three main clusters (A, B, and C), which were further divided into seven subclusters: A1, A2, A3, B1, B2, C1, and C2.

Cluster A encompassed mulberries with a mean total protein content of 163.20 mg BSA/g DW (ranging from 97.90 to 299.36 mg BSA/g DW) and a mean total phenolic content of 14.87 mg GAE/g DW (ranging from 8.19 to 18.04 mg GAE/g DW). Moderate levels of total caffeoylquinic acid derivatives (mean 8.55 mg/g DW, range: 3.49–16.88 mg/g DW), total coumaroylquinic acid derivatives (mean 0.23 mg/g DW, range: 0.09–0.75 mg/g DW), total quercetin glycoside derivatives (mean 6.12 mg/g DW, range: 0.40–18.96 mg/g DW), and total kaempferol glycoside derivatives (mean 0.63 mg/g DW, range: 0.04–1.38 mg/g DW) were observed. Mulberries from Koper Hills dominated this cluster, accounting for 97.3% of the samples, with mulberries from Drava Plain contributing the remaining 2.7%. This pattern highlights the bioclimatic influence, as mulberries from the Koper Hills exhibit similar biochemical profiles. Four of the five black mulberries were grouped into cluster A, underscoring its unique biochemical and species diversity.

Within cluster A, mulberries in subcluster A1 exhibited the highest protein content with a mean of 189.93 mg BSA/g DW (range: 179.35–200.51 mg BSA/g DW) and a mean phenolic content of 16.58 mg GAE/g DW (range: 16.33–16.84 mg GAE/g DW). Elevated levels of caffeoylquinic acid derivatives (mean: 13.56 mg/g DW, range: 12.29–14.83 mg/g DW) were accompanied by moderate levels of coumaroylquinic acid derivatives (mean: 0.51 mg/g DW, range: 0.28–0.75 mg/g DW), quercetin glycoside derivatives (mean: 6.22 mg/g DW, range: 4.32–8.11 mg/g DW), and kaempferol glycoside derivatives (mean: 0.57 mg/g DW, range: 0.48–0.67 mg/g DW). Both mulberry trees (one black mulberry), originated from Koper Hills and were classified in subcluster A1 due to their high protein and phenolic content accompanied by elevated caffeoylquinic acid derivatives.

White mulberry trees in subcluster A2 displayed slightly lower protein content (mean: 158.72 mg BSA/g DW, range: 97.90–299.36 mg BSA/g DW) and phenolic content (mean: 15.80 mg GAE/g DW, range: 11.58–18.04 mg GAE/g DW). Moderate levels of caffeoylquinic acid derivatives (mean: 8.31 mg/g DW, range: 4.71–11.47 mg/g DW) and quercetin glycoside derivatives (mean: 8.61 mg/g DW, range: 4.95–15.44 mg/g DW) were observed, while kaempferol glycoside derivatives (mean: 1.04 mg/g DW, range: 0.44–1.38 mg/g DW) reached the highest mean value within the cluster A. This subcluster primarily included trees from Koper Hills (90%) and Drava Plain (10%).

Subcluster A3 exhibited intermediate protein content (mean: 162.85 mg BSA/g DW, range: 103.87–247.16 mg BSA/g DW) and phenolic content (mean: 14.37 mg GAE/g DW, range: 8.19–17.87 mg GAE/g DW). Moderate levels of caffeoylquinic acid derivatives (mean: 8.25 mg/g DW, range: 3.49–16.88 mg/g DW) and quercetin glycoside derivatives

(mean: 5.11 mg/g DW, range: 0.40–18.96 mg/g DW) were observed, alongside kaempferol glycoside derivatives (mean: 0.46 mg/g DW, range: 0.04–1.16 mg/g DW). Exclusively represented by mulberries from Koper Hills, this subcluster included the majority of black mulberries, emphasising their unique composition.

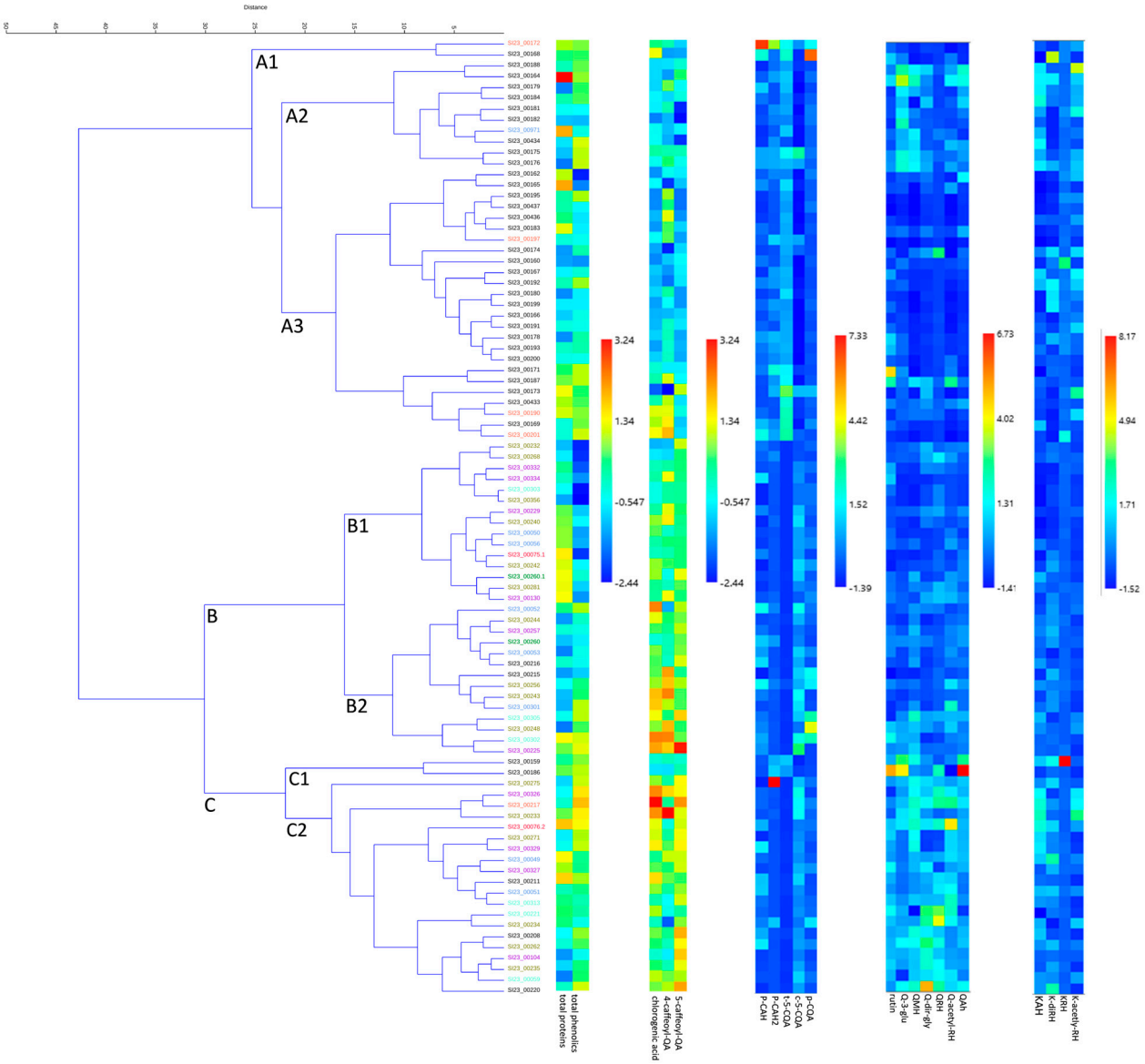

Figure 3. Ward's dendrogram using Euclidean distance with an integrated heatmap illustrating all analysed mulberry trees. The inventory number of each mulberry tree is assigned according to its mesoregion, indicated by a colour code: Drava Plain (blue), Slovenian Hills (turquoise), Gorica Hills (red), Vipava Hills (purple), Karst Plateau (olive), Brkini Hills and Reka Valley (green), and Koper Hills (black), with black mulberries shown in orange. The dendrogram highlights the clustering of mulberry trees based on their biochemical profiles, while the heatmap visualises the intensity of individual phenolic and protein compounds across the analysed mesoregions. All data are z-standardised. Abbreviations: 4-caffeoyl-QA: 4-caffeoylquinic acid; 5-caffeoyl-QA: 5-caffeoylquinic acid; c-5-CQA: *cis*-5-coumaroylquinic acid; t-5-CQA: *trans*-5-coumaroylquinic acid; *p*-CAH: *p*-coumaric acid hexoside; *p*-CAH2: *p*-coumaric acid hexoside 2; *p*-CQA: *p*-coumaroylquinic acid; Q-3-glu: quercetin-3-glucoside; QMH: quercetin malonyl-hexoside; Q-diR-gly: quercetin dirhamnosyl-glycoside; QRH: quercetin rhamnosyl-hexoside; Q-acetyl-RH: quercetin acetyl-rhamnosyl-hexoside; QAH: quercetin acetyl hexoside; KAH: kaempferol acetyl-hexoside; K-diRH: kaempferol dirhamnosyl-hexoside; KRH: kaempferol rhamnosyl-hexoside; K-acetyl-RH: kaempferol acetyl-rhamnosyl-hexoside.

White mulberry trees in cluster B exhibited a mean total protein content of 160.58 mg BSA/g DW (range: 97.73–224.41 mg BSA/g DW) and a mean total phenolic content of 12.86 mg GAE/g DW (range: 7.42–18.20 mg GAE/g DW). Elevated levels of caffeoylquinic acid derivatives (mean: 13.74 mg/g DW, range: 6.62–20.93 mg/g DW) were observed, along with moderate levels of coumaroylquinic acid derivatives (mean: 0.15 mg/g DW, range: 0.07–0.28 mg/g DW), quercetin glycoside derivatives (mean: 6.10 mg/g DW, range: 1.78–11.86 mg/g DW), and kaempferol glycoside derivatives (mean: 0.49 mg/g DW, range: 0.13–0.86 mg/g DW). This cluster included mulberries from Karst Plateau (34.5%), Vipava Hills (20.7%), Drava Plain (17.2%), Slovenian Hills (10.3%), Koper Hills (6.9%), Brkini Hills and Reka Valley (6.9%) and Gorica Hills (3.5%).

White mulberries in subcluster B1 showed high protein content (mean: 180.11 mg BSA/g DW, range: 112.47–224.41 mg BSA/g DW) but lower phenolic content (mean: 10.45 mg GAE/g DW, range: 7.42–13.00 mg GAE/g DW). Moderate levels of caffeoylquinic acid derivatives (mean: 11.57 mg/g DW, range: 6.62–14.91 mg/g DW) and quercetin glycoside derivatives (mean: 5.91 mg/g DW, range: 2.42–11.50 mg/g DW) were observed. This subcluster included trees from Karst Plateau (40.0%), Vipava Hills (26.7%), Drava Plain (13.2%), Brkini and Reka Valley (67%), Slovenian Hills (6.7%) and Gorica Hills (each 6.7%).

White mulberry trees in subcluster B2 exhibited lower protein content (mean: 139.65 mg BSA/g DW, range: 97.73–220.10 mg BSA/g DW) and higher phenolic content (mean: 15.43 mg GAE/g DW, range: 12.03–18.20 mg GAE/g DW). The highest levels of caffeoylquinic acid derivatives (mean: 16.06 mg/g DW, range: 10.10–20.93 mg/g DW) within cluster B were observed. This subcluster included mulberries from Karst Plateau (28.6%), Drava Plain (21.4%), Vipava Hills (14.3%), Brkini and Reka Valley (7.1%), Slovenian Hills (14.3%), and Koper Hills (14.3%).

Mulberries in cluster C exhibited the highest phenolic diversity among all groups, with a mean total phenolic content of 16.73 mg GAE/g DW (range: 13.36–20.11 mg GAE/g DW) and a mean total protein content of 165.17 mg BSA/g DW (range: 100.41–237.89 mg BSA/g DW). Elevated levels of total caffeoylquinic acid derivatives (mean: 14.44 mg/g DW, range: 8.03–22.86 mg/g DW) and quercetin glycoside derivatives (mean: 11.50 mg/g DW, range: 3.59–26.15 mg/g DW) were observed, along with moderate levels of coumaroylquinic acid derivatives (mean: 0.19 mg/g DW, range: 0.09–1.01 mg/g DW) and kaempferol glycoside derivatives (mean: 0.93 mg/g DW, range: 0.15–1.57 mg/g DW). Mulberries in this cluster were predominantly represented by Karst Plateau (27.3%), Koper Hills (27.3%), Vipava Hills (18.2%), Slovenian Hills (13.6%), Drava Plain (9.1%), and Gorica Hills (4.5%).

White mulberries in subcluster C1 showed high total protein content (mean: 180.94 mg BSA/g DW, range: 171.74–190.15 mg BSA/g DW) and phenolic content (mean: 17.25 mg GAE/g DW, range: 16.92–17.58 mg GAE/g DW). The chemotype is characterised by the highest levels of quercetin glycoside derivatives in the cluster (mean: 19.56 mg/g DW, range: 12.98–26.15 mg/g DW) alongside moderate levels of caffeoylquinic acid derivatives (mean: 9.07 mg/g DW, range: 8.03–10.11 mg/g DW) and kaempferol glycoside derivatives (mean: 0.83 mg/g DW, range: 0.39–1.27 mg/g DW). All samples in this subcluster were exclusively from Koper Hills.

Mulberry trees in subcluster C2 demonstrated intermediate protein content (mean: 163.59 mg BSA/g DW, range: 100.41–237.89 mg BSA/g DW) while exhibiting the highest phenolic content within cluster C (mean: 16.68 mg GAE/g DW, range: 13.36–20.11 mg GAE/g DW). This subcluster was characterised by elevated levels of caffeoylquinic acid derivatives (mean: 14.98 mg/g DW, range: 9.75–22.86 mg/g DW) and quercetin glycoside derivatives (mean: 10.70 mg/g DW, range: 3.59–13.71 mg/g DW), alongside moderate levels of kaempferol glycoside derivatives (mean: 0.94 mg/g DW,

range: 0.15–1.57 mg/g DW). The subcluster included mulberries from Karst Plateau (30%), Koper Hills (20%), Vipava Hills (20%), Slovenian Hills (15%), Drava Plain (10%), and Gorica Hills (5%). Notably, chemotype C2 encompassed the black mulberry tree SI23_00217, which displayed a distinct biochemical profile. This black mulberry tree exhibited a protein content of 181.50 mg BSA/g DW and the highest phenolic content within the subcluster (19.85 mg GAE/g DW). Elevated levels of caffeoylquinic acid derivatives (17.65 mg/g DW) and quercetin glycoside derivatives (12.43 mg/g DW) were also observed, along with moderate kaempferol glycoside derivatives (1.15 mg/g DW). These findings highlight the unique biochemical diversity within subcluster C2 and underscore the exceptional profile of black mulberry tree SI23_00217.

3.2. Comparison of Selected Bioclimatic Parameters in the Sub-Mediterranean and Sub-Pannonian Mesoregions of Sampled Mulberries

Selected bioclimatic data from seven distinct mesoregions, where leaf samples of white mulberry trees were collected, were analysed to assess variations in air temperature, total insolation, and precipitation (Figure 4) These analyses aimed to evaluate the influence of climatic factors on the protein and phenolic composition of mulberries. A detailed profile of bioclimatic parameters of sampled white mulberries across the mesoregions is provided in Table 2.

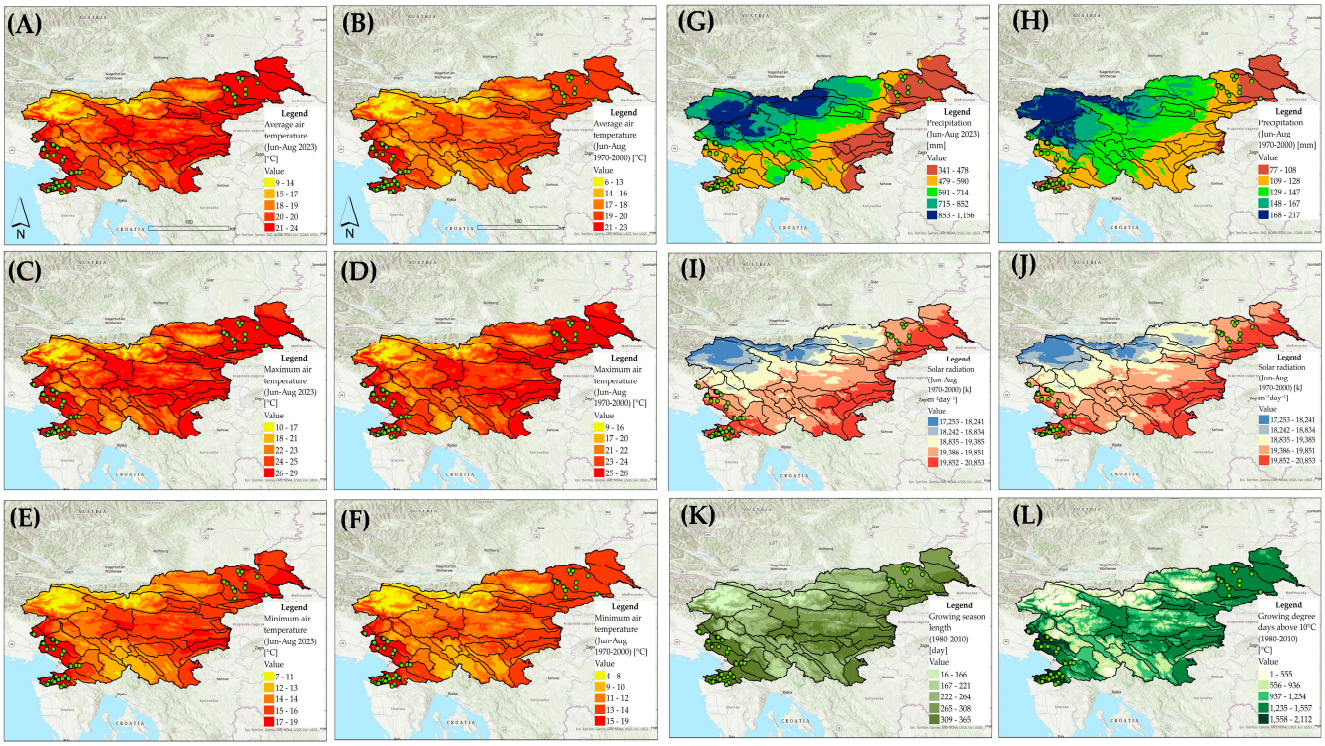

Figure 4. Geospatial bioclimatic maps with attributed location points representing inventoried white mulberry (*M. alba*) trees (green dots). (**A**) Average air temperature (June–August 2023) [°C], (**B**) Average air temperature (June–August 1970–2000) [°C], (**C**) Maximum air temperature (June–August 2023) [°C], (**D**) Maximum air temperature (June–August 1970–200) [°C], (**E**) Minimum air temperature (June–August 2023) [°C], (**F**) Minimum air temperature (June–August 1970–200) [°C], (**G**) Precipitation (June–August 2023) [mm], (**H**) Precipitation (June–August 1970–2000) [mm], (**I**) Total insolation (June–August 2023) [h], (**J**) Solar radiation (June–August 1970–2000) [kJ m^{-2} day^{-1}], (**K**) Growing degree days above 10 °C (1980–2010) [°C], (**L**) Growing season length (1980–2010) [number of days]. The maps were created using ArcGIS Pro with Esri basemaps [55].

Table 2. Median ± MAD of bioclimatic parameters for the mesoregions corresponding to the sampled mulberry trees. Different letters (a–e) indicate significant differences ($p < 0.05$), determined by the post hoc Dunn–Bonferroni test for mesoregional comparisons (lowercase letters) and by the Wilcoxon signed-rank test for comparisons between the 2023 sampling season and the long-term trend (uppercase letters). Comma is used as the thousands separator and dot as the decimal marker.

Mesoregion	Drava Plain	Slovenian Hills	Gorica Hills *	Vipava Hills	Karst Plateau	Koper Hills	Brkini Hills and Reka Valley *
Average air temperature (June–August 2023) [°C]	21.2 ± 0.1 Ac	21.0 ± 0.1 Ac	22.7 ± 0.0 Aab	22.9 ± 0.3 Aa	21.4 ± 0.7 Ac	22.2 ± 0.4 Ab	21.0 ± 0.0 Ac
Average air temperature (June–August 1970–2000) [°C]	18.9 ± 0.1 Bc	18.7 ± 0.2 Bd	21.4 ± 0.0 Bab	21.3 ± 0.3 Ba	19.7 ± 0.9 Bbc	21.7 ± 0.5 Ba	19.0 ± 0.0 Bbc
Maximum air temperature (June–August 2023) [°C]	26.1 ± 0.1 Ac	25.7 ± 0.1 Ac	27.9 ± 0.0 Aab	28.4 ± 0.2 Aa	26.2 ± 0.9 Ac	27.0 ± 0.6 Ab	26.0 ± 0.0 Ac
Maximum air temperature (June–August 1970–2000) [°C]	24.8 ± 0.1 Bc	24.5 ± 0.2 Bc	27.0 ± 0.0 Ba	26.5 ± 0.4 Ba	24.8 ± 0.9 Bc	26.2 ± 0.4 Bb	24.2 ± 0.0 Bc
Minimum air temperature (June–August 2023) [°C]	15.6 ± 0.1 Ac	15.7 ± 0.2 Ac	17.2 ± 0.0 Aab	17.3 ± 0.1 Aa	16.1 ± 0.8 Ac	16.9 ± 0.3 Ab	15.4 ± 0.0 Ac
Minimum air temperature (June–August 1970–2000) [°C]	13.0 ± 0.1 Bd	12.8 ± 0.2 Bd	15.8 ± 0.0 Bc	16.1 ± 0.2 Bb	14.7 ± 0.8 Bc	17.1 ± 0.5 Aa	13.9 ± 0.0 Bc
Precipitation (June–August 2023) [mm]	438.5 ± 21.2 Ad	440.9 ± 31.1 Ad	584.4 ± 0.0 Aa	612.4 ± 33.8 Aa	543.9 ± 20.7 Ab	493.5 ± 21.5 Ac	550.8 ± 0.0 Ab
Precipitation (June–August 1970–2000) [mm]	114.8 ± 2.8 Bb	110.7 ± 4.2 Bc	129.7 ± 0.0 Ba	120.3 ± 2.8 Bb	124.8 ± 5.0 Ba	91.7 ± 5.0 Bd	119.0 ± 0.0 Bb
Total insolation (June–August 2023) [h]	786.9 ± 3.5 d	772.6 ± 5.9 e	835.8 ± 0.0 c	839.3 ± 10.6 c	873.7 ± 4.3 b	924.7 ± 9.4 a	864.7 ± 0.0 c
Solar radiation (June–August 1970–2000) [kJ m^{-2} day^{-1}]	19,782.0 ± 112.7 bc	19,796.0 ± 147.8 bc	19,724.7 ± 0.0 bc	19,673.8 ± 47.0 c	19,801.0 ± 31.6 b	20,388.3 ± 111.6 a	19,860.3 ± 0.0 b
Growing degree days above 10 °C (1980–2010) [°C day]	1,417.5 ± 12.7 b	1358.6 ± 47.3 b	1,732.5 ± 0.0 ab	1756.4 ± 37.8 a	1438.9 ± 126.3 b	1788.6 ± 91.2 a	1387.3 ± 0.0 b
Growing season length (1980–2010) [day]	296.0 ± 4.6 b	290.5 ± 10.0 b	365.0 ± 0.0 a	365.0 ± 0.0 a	365.0 ± 0.0 a	365.0 ± 0.0 a	365.0 ± 0.0 a

* small sample size.

Average air temperatures during the sampling period (June to August 2023) across mesoregions were ranging from 21.0 °C in Slovenian Hills as well as Brkini Hills and Reka Valley to 22.9 °C in Vipava Hills that showed significantly highest median air temperature. Maximal air temperatures across the regions reached up to 28.4 °C in Vipava Hills, while the lowest minimal air temperatures of 15.4 °C were observed in Brkini Hills and Reka Valley.

In comparison, long-term summer air temperature averages (1970–2000) ranged from 18.7 °C in the Slovenian Hills to 21.7 °C in the Koper Hills, with both the Vipava and Koper Hills exhibiting the highest average air temperatures. Maximum air temperatures during this 30-year reference period reached 27.0 °C in the Gorica Hills, whereas the lowest minimum temperature of 12.8 °C was recorded in the Slovenian Hills. Overall, the comparison revealed a marked increase in summer air temperatures in 2023 relative to the 1970–2000 reference period, with both mean and minimum values showing a significant upward shift across mesoregions.

Precipitation varied significantly among the mesoregions, with statistically significant differences observed. Median values ranged from 438.5 mm in Drava Plain to 612.4 mm in Vipava Hills. Regions with higher precipitation, such as Vipava Hills and Gorica Hills, likely provided greater water availability for mulberry trees, consequently supporting enhanced nutrient uptake, assimilation and growth. In contrast, regions with lower precipitation levels, including Drava Plain and Slovenian Hills, experienced water stress, which stimulates the production of specific phenolic compounds as part of stress-induced metabolic responses In comparison, the precipitation amount during the long-term summer period (1970–2000) ranged from 91.7 mm in the Koper Hills to 129.7 mm in the Gorica Hills. Overall, a significant increase in precipitation was observed in 2023 compared to the long-term reference period.

During the summer of 2023 (June–August), total insolation showed marked regional differences. The highest values were recorded in the Koper Hills (924.7 h), followed by the Karst Plateau and the Brkini Hills with the Reka Valley. Intermediate levels were observed in the Vipava Hills and the Gorica Hills, while the lowest insolation was measured in the Drava Plain (786.9 h) and the Slovenian Hills (772.6 h). These results indicate that coastal and sub-Mediterranean mesoregions receive substantially more sunlight than continental areas.

Long-term solar radiation averages (1970–2000) expressed as daily energy input (kJ m^{-2} day^{-1}) revealed a similar spatial gradient. The Koper Hills exhibited the highest radiation (20,388 kJ m^{-2} day^{-1}), significantly exceeding values from other regions. The Karst Plateau and Brkini Hills with the Reka Valley also showed elevated radiation, while the lowest values were recorded in the Vipava Hills (19,674 kJ m^{-2} day^{-1}) and Gorica Hills (19,725 kJ m^{-2} day^{-1}).

Two additional light-related parameters—the Growing Degree Days above 10 °C (in °C) and Growing Season Length [in days]—were included for the long-term period (1981–2010) comparison between mesoregions since such spatial monthly data were not available for the experimental year (2023). Growing Degree Days above 10 °C ranged from 1358.6 °C in the Slovenian Hills to 1788.6 °C in the Koper Hills. Similarly, the Growing Season Length was shortest in the Slovenian Hills, with 290.5 days, and longest in all Sub-Mediterranean mesoregions, where favourable conditions persisted throughout the entire year (365 days).

This variability in bioclimatic parameters prompted us to further examine the impact of key bioclimatic factors on the protein content and phenolic composition of mulberries across diverse mesoregions.

3.3. Variations in Protein and Phenolic Content in White Mulberry Leaves Across Sub-Mediterranean and Sub-Pannonian Mesoregions in Slovenia

The differences in total protein, total phenolics and individual phenolic contents in white mulberry leaves with respect to their geographical distribution across ecogeographical regions were evaluated. This analysis aimed to assess the impact of bioclimatic factors, such as total insolation, precipitation, and air temperature, on the composition of the white mulberry metabolites.

The total protein content ranged from 112.32 mg BSA/g DW (determined in white mulberry tree SI23_00244 from Karst Plateau) to 297.16 mg BSA/g DW (SI23_00164 white mulberry tree from Koper Hills), with an overall median value of 156.02 mg BSA/g DW. We identified statistically significant differences in total protein content (Figure 5A) among the mesoregions. Mulberries from Karst Plateau had the significant lowest total protein content of 137.99 mg BSA/g DW, while trees from Gorica Hills exhibited significant highest total protein content in leaves with a median value of 229.47 mg BSA/g DW.

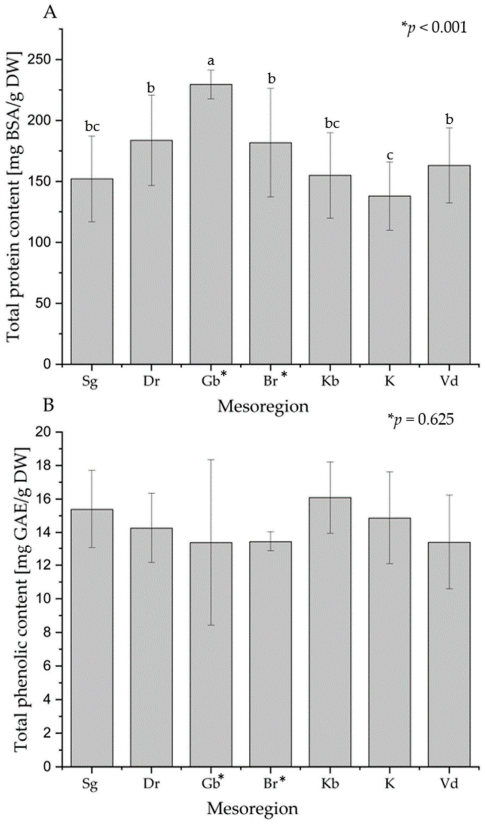

Figure 5. (**A**) Total protein and (**B**) total phenolic contents (median ± MAD, mg/g DW) in leaves of white mulberry trees across different mesoregions (Sg: Slovenian Hills; Dr: Drava Plain; Gb: Gorica Hills; Br: Brkini Hills and Reka Valley; Kb: Koper Hills; K: Karst Plateau; Vd: Vipava Hills). Different letters (a–c) indicate significant differences ($p < 0.05$), which were determined using the post hoc Dunn–Bonferroni test. with a small sample size are indicated with an asterisk (*).

The total phenolic content ranged from 7.09 mg GAE/g DW (determined in white mulberry tree SI23_00303 from Slovenian Hills) to 19.46 mg GAE/g DW (determined in white mulberry tree SI23_00326 from Vipava Hills), with an overall median of 14.93 mg GAE/g DW. There were no statistically significant differences in total phenolic content among the mesoregions, as shown in Figure 5B. However, a trend was observed, that mulberries from Koper Hills showed the highest median phenolic content (16.09 mg GAE/g DW), while those from Gorica Hills had the lowest median phenolic content (13.40 mg GAE/g DW).

The identified caffeoylquinic acid derivatives in mulberry leaves were *trans*-5-caffeoylquinic acid (chlorogenic acid), 4-caffeoylquinic acid, and *cis*-5-caffeoylquinic acid. Significant differences in median contents were observed among mulberries from different mesoregions as shown in Supplementary Table S3. Mulberries from Koper Hills had the statistically significantly lowest contents of caffeoylquinic acid derivatives with a median value of 7.72 mg/g DW. The highest median content (15.26 mg/g DW) was found in white mulberries from Slovenian Hills.

Similarly, chlorogenic acid levels were significantly lower in white mulberries of Koper Hills (6.04 mg/g DW), compared to those of Slovenian Hills, where the highest median content was 12.66 mg/g DW (Figure 6B). Among all the caffeoylquinic acid derivatives, *cis*-5-caffeoylquinic acid was present in the smallest quantities. Mulberries from Koper Hills showed the statistically lowest median content of 0.26 mg/g DW, while those from Vipava Hills exhibited the highest, with a median value of 0.52 mg/g DW.

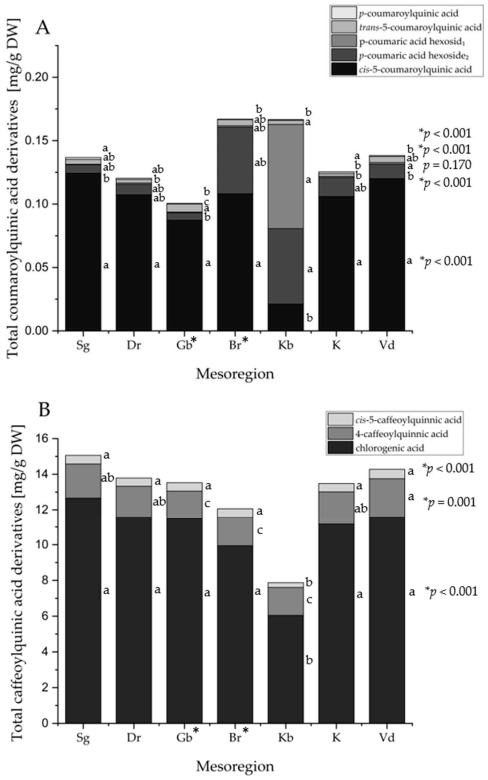

Figure 6. (**A**) Total coumaroylquinic acid derivatives and (**B**) total caffeoylquinic acid derivatives (median ± MAD, mg/g DW) in leaves of white mulberry trees from different mesoregions (Sg: Slovenian Hills; Dr: Drava Plain; Gb: Gorica Hills; Br: Brkini Hills and Reka Valley; Kb: Koper Hills; K: Karst Plateau; Vd: Vipava Hills). Each value represents within a mesoregion. Different letters (a–c) indicate significant differences ($p < 0.05$), which were determined using the post hoc Dunn–Bonferroni test. Stacked graphs represent contents of different phenolic acids. Mesoregions with a small sample size are indicated with an asterisk (*). Asterisks above *p*-values in the figures indicate statistical significance at the 0.05 level ($p < 0.05$).

In addition to caffeoylquinic acids, the study also identified several coumaroylquinic acid derivatives, including *cis*-5-coumaroylquinic acid, *trans*-5-coumaroylquinic acid, *p*-coumaric acid hexoside$_1$, *p*-coumaric acid hexoside$_2$, and *p*-coumaroylquinic acid. The total content of these compounds was significantly higher in white mulberries from Brkini Hills and Reka Valley, with a median value of 0.17 mg/g DW, compared to the lowest median value of 0.10 mg/g DW in mulberries from Gorica Hills. Among the coumaroylquinic acid derivatives, *cis*-5-coumaroylquinic acid was the most predominant (Figure 6A). We con-

firmed statistically significant differences between mesoregions, with the highest content in white mulberries from Slovenian Hills (0.12 mg/g DW) and the lowest in white mulberries from Koper Hills (0.02 mg/g DW). For *trans*-5-coumaroylquinic acid, the highest contents were recorded in white mulberries from Koper Hills (0.08 mg/g DW), whereas those from Slovenian Hills had the lowest content (0.0004 mg/g DW).

The study further identified seven quercetin glycoside derivatives: rutin, quercetin-3-glucoside, quercetin malonyl hexoside, quercetin rhamnosyl hexoside, quercetin dirhamnosyl glycoside, quercetin acetyl-hexoside, and quercetin acetyl rhamnosyl hexoside. No statistically significant differences were observed in the total quercetin glycoside content of mulberries across the regions (Supplementary Table S4). However, a trend was observed, that white mulberries from Slovenian Hills showing the highest median content of 10.12 mg/g DW, while those from Koper Hills exhibited the lowest median content of 6.04 mg GAE/g DW. Rutin and quercetin malonyl hexoside were the predominant quercetin glycoside, with similar trend being highest in white mulberries from Slovenian Hills (6.14 mg/g DW; 2.30 mg/g DW) and the lowest in white mulberries from Koper Hills (3.58 mg/g DW; 0.67 mg/g DW), shown in Figure 7A.

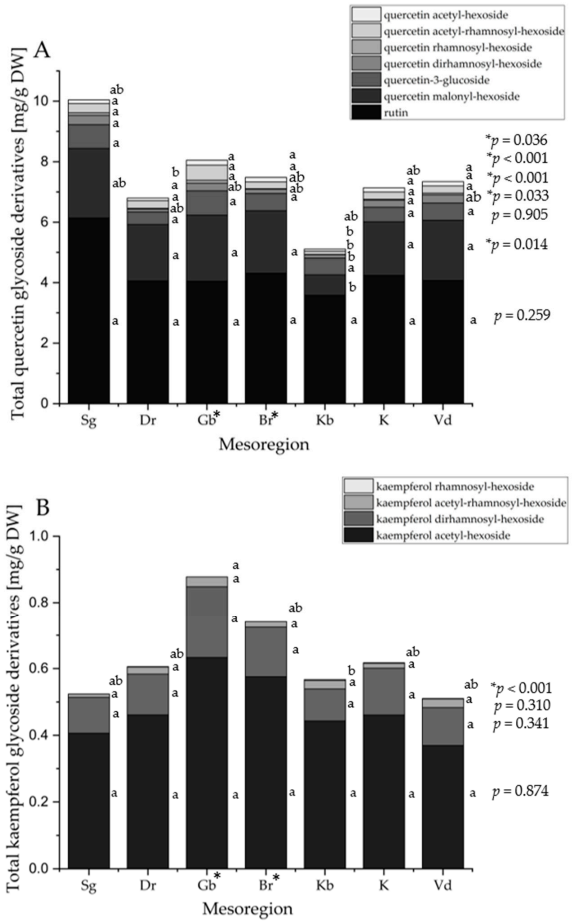

Figure 7. (**A**) Total quercetin glycosides derivatives and (**B**) total kaempferol glycosides derivatives (median ± MAD, mg/g DW) in leaves of white mulberry trees from different mesoregions (Sg: Slovenian Hills; Dr: Drava Plain; Gb: Gorica Hills; Br: Brkini Hills and Reka Valley; Kb: Koper Hills; K: Karst Plateau; Vd: Vipava Hills). Different letters (a–b) indicate significant differences ($p < 0.05$), which were determined using the post hoc Dunn–Bonferroni test. Stacked graphs represent contents of different flavonoid contents Mesoregions with a small sample size are indicated with an asterisk (*). Asterisks above *p*-values in the figures indicate statistical significance at the 0.05 level ($p < 0.05$).

The total kaempferol glycoside derivatives measured comprised kaempferol dirhamnosyl-hexoside, kaempferol rhamnosyl-hexoside, kaempferol acetyl-rhamnosyl-hexoside, and kaempferol acetyl-hexoside. There were no statistically significant differences in the content of these derivatives across the various mesoregions, with an overall median content of 0.60 mg/g DW. The lowest median level of kaempferol glycosides was observed in white mulberries from Koper Hills and Slovenian Hills (0.57 mg/g DW), while the highest was found in white mulberries from Gorica Hills (0.88 mg/g DW). Kaempferol acetyl-hexoside was the predominant kaempferol derivative, with an overall median content of 0.44 mg/g DW (Figure 7B). The lowest value was determined in white mulberries from Vipava Hills (0.37 mg/g DW), while the highest concentration of 0.63 mg/g DW was observed in white mulberries from Gorica Hills.

3.4. Effect of Pruning and Mesoregion on Protein and Phenolics Content in White Mulberry Leaves

A two-way ANOVA on ranked values (ANCOVA) was conducted to evaluate the effects of mesoregion, pruning frequency, and their interaction on total protein, total phenolic and total phenolic derivatives (coumaroylquinic acids, caffeoylquinic acids, quercetin, kaempferol) contents in white mulberry leaves.

Both mesoregion and the interaction between mesoregion and pruning frequency significantly influenced the total protein contents (Figure 8A). Unpruned mulberries had statistically significantly lower total protein content compared to both yearly and frequently pruned mulberries across all mesoregions. The lowest median protein content in unpruned trees was observed in Karst Plateau (126.14 mg BSA/g DW), while the highest was recorded in Drava Plain (179.25 mg BSA/g DW). Yearly pruned mulberries exhibited the highest total protein content, with the maximum recorded in Drava Plain (244.17 mg BSA/g DW). Mulberries from Gorica Hills consistently showed high protein content (229.47 mg BSA/g DW). The lowest median protein content for yearly pruned trees was found in Karst Plateau (162.35 mg BSA/g DW). Across all mesoregions, the median protein content of yearly pruned trees was 187.24 mg BSA/g DW. Frequently pruned mulberries demonstrated intermediate total protein levels across all mesoregions (178.45 mg BSA/g DW). The lowest median protein content for frequently pruned trees was recorded in Karst Plateau (122.08 mg BSA/g DW), while the highest was observed in Vipava Hills (190.89 mg BSA/g DW).

There were no statistically significant differences in total phenolic content between mesoregions or pruning frequencies. However, a trend towards decreased total phenolic content was noticed when trees were pruned (Figure 8B). Among the unpruned mulberries, the highest total phenolic content was observed in Slovenian Hills (16.11 mg GAE/g DW). For yearly pruned mulberries, the highest phenolic content was recorded in Koper Hills (16.42 mg GAE/g DW). Frequently pruned mulberries showed the highest phenolic content in Karst Plateau (15.64 mg GAE/g DW).

Both mesoregion and the interaction between mesoregion and pruning frequency significantly influenced the content of total caffeoylquinic acid derivatives (Figure 8C). Among unpruned trees, the lowest median total caffeoylquinic acid derivative content was observed in Koper Hills (7.64 mg/g DW), while the highest was recorded in Vipava Hills (14.59 mg/g DW). Across all mesoregions, unpruned mulberries showed the highest total caffeoylquinic acid derivative content (13.36 mg/g DW), whereas yearly pruned mulberries exhibited the lowest content (11.55 mg/g DW). Specifically, the lowest value for yearly pruned mulberries was measured in Koper Hills (7.51 mg/g DW), and the highest was found in Slovenian Hills, with a median of 14.15 mg/g DW.

No significant interactions were observed for total coumaroylquinic acid derivatives, total kaempferol glycoside derivatives, or total quercetin derivatives with respect to the combined effect of mesoregion and pruning frequency.

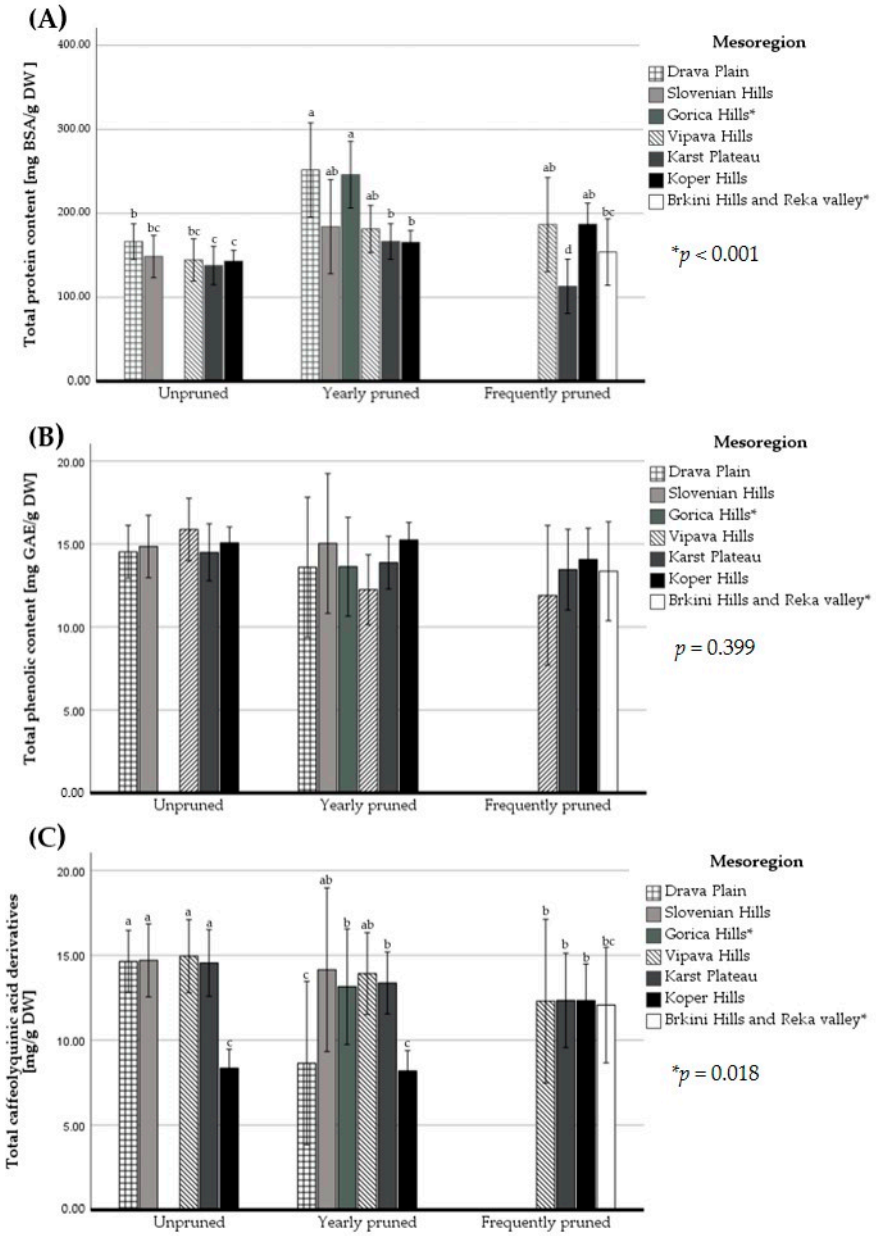

Figure 8. (**A**) Total protein; (**B**) total phenolic contents; and (**C**) total caffeoylquinic acid derivatives (median ± MAD, mg/g DW) in leaves of white mulberry trees from different mesoregions in regard to pruning. Different letters (a–c) indicate significant differences ($p < 0.05$), which were determined using the post hoc Dunn–Bonferroni test. Mesoregions with a small sample size are indicated with an asterisk (*). Asterisks above *p*-values in the figures indicate statistical significance at the 0.05 level ($p < 0.05$).

3.5. Principal Component Analysis of White Mulberry Basic Descriptors, Biochemical and Selected Bioclimatic Parameters

The PCA based on geographic distribution of inventoried white mulberry trees, selected climatic parameters, and evaluated basic tree descriptors (circumference range, tree vigour, pruning practice, leaf size ratio) along with biochemical composition (total protein, total phenolic and individual phenolic derivatives) revealed distinct clustering patterns (Figure 9). The two principal components (PC1 and PC2) account for 67.55% of the total variance, with PC1 explaining 43.76% and PC2 representing 23.79% of the variance.

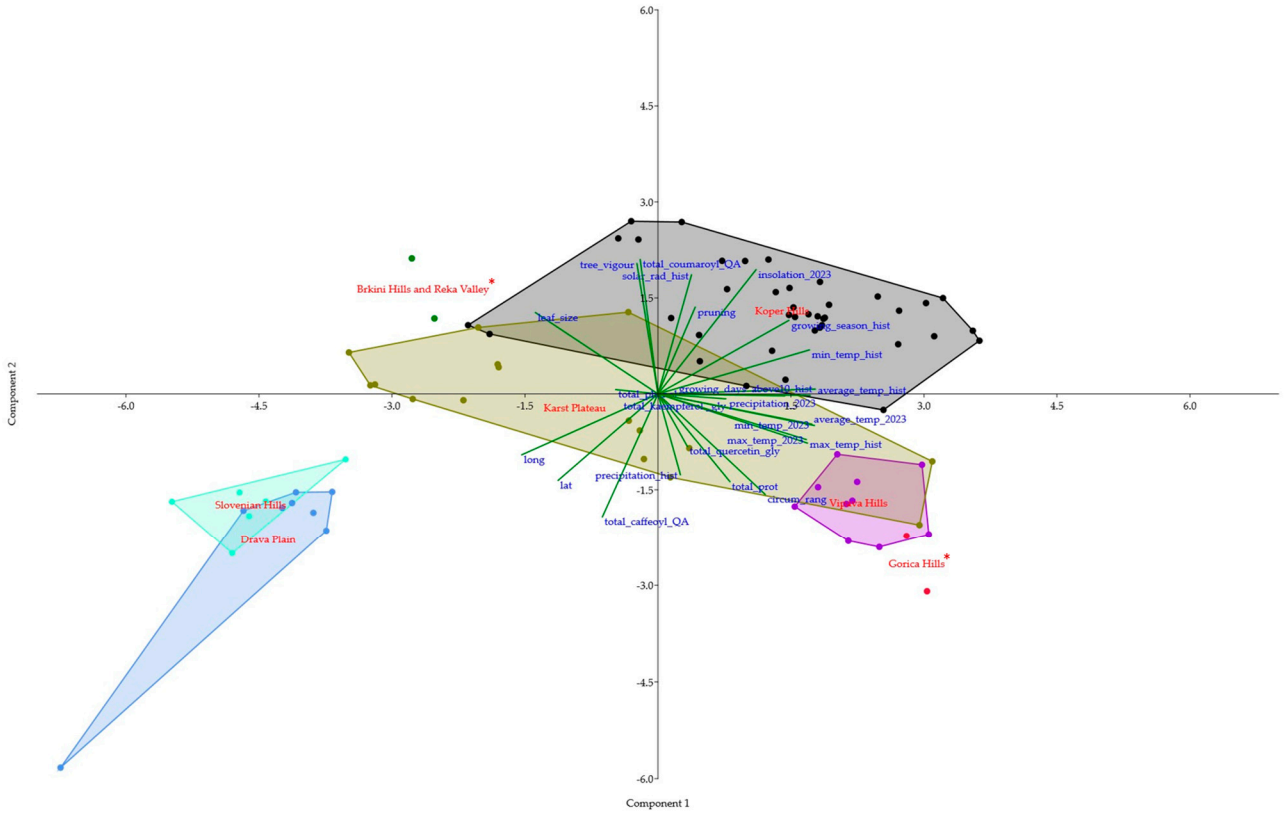

Figure 9. Principal component analysis (PCA) plot comprising the geographic scale, selected climatic parameters (average, minimum, and maximum air temperatures; total insolation; and precipitation during the 2023 sampling season [June–August]), long-term climatic trends (average, minimum, and maximum air temperatures; solar radiation; and precipitation from 1970 to 2000 [June–August]), as well as growing degree days above 10 °C and growing season length (1981–2010), basic tree descriptors (circumference range, tree vigour, pruning, leaf size ratio) and biochemical parameters (total protein, total phenolic and individual phenolic derivatives) of leaves of inventoried white mulberry trees across mesoregions: Drava Plain (blue), Slovenian Hills (turquoise), Gorica Hills (red, Karst Plateau (olive), Brkini Hills and Reka Valley (green) and Koper Hills (black) and Vipava Hills (purple). Mesoregions with a small sample size are indicated with an asterisk (*).

Principal component 1 (PC1) reflected a climatic gradient, that was weighted most strongly with air temperature (June–August 2023 and 1970–2000), precipitation and insolation during the 2023 sampling season, growing degree days above 10 °C and growing season length (1980–2010). PC1 showed a positive association with total protein content, as well as with the quercetin and kaempferol derivatives. It also correlated positively with tree circumference range and pruning practices, while showing a negative correlation with total caffeolyquinic acid derivatives, longitude, latitude, leaf size (see Supplementary Table S5).

The second component (PC2) was negatively associated with tree vigour, pruning practice, leaf size ratio, which coincide with total coumaroylquinic acid derivatives and total kaempferol glycoside content. Among selected bioclimatic parameters a negative correlation was determined for total insolation (June–August 2023) and precipitation (June–August 2023). Positively correlations were found with total caffeoylquinic acid derivatives, circumference range, and latitude, suggesting that the clinal variation is mostly driven by composition of caffeoylquinic acids.

The PCA diagram revealed a clear separation between the Sub-Pannonian and Sub-Mediterranean macroregions, highlighting that local climatic conditions and latitude, affect phenolic profiles. The analysed trees from the Drava Plain and Slovenian Hills mesoregions, both part of the Sub-Pannonian macroregion, showed 70% separation and were clustered

in the lower-left quadrant. These trees are characterised by the effect of the geographic scale, high levels of total caffeoylquinic acid derivatives and total phenolics, suggesting that the bioclimatic conditions of this region—characterised by lower total insolation and precipitation—specifically enhance phenolic acid profiles in mulberry leaves.

In contrast, trees from Sub-Mediterranean macroregion (e.g., Koper Hills, Gorica Hills, Vipava Hills) clustered in the lower right quadrant, showing a positive association with higher total insolation (June–August 2023) and solar radiation (June–August 1970–2000) and precipitation, as well as elevated levels of total kaempferol derivatives. Mulberries from Koper Hills exhibited a strong alignment with total insolation playing a significant role in enhancing total phenolic and coumaroylquinic acid contents. It further shows a strong correlation with pruning frequency. Trees from Gorica Hills are attributed to both higher precipitation (June–August 2023 and from 1970 to 2000) and air temperature (June–August 2023 and from 1970 to 2000) contributing to negative correlation between high protein contents and low total phenolic contents characterised by distinctive individual phenolic ratio.

Overall, this analysis demonstrates that air temperature, precipitation, and total insolation are key factors of mulberry protein and phenolic content across Slovenian mesoregions. The Sub-Pannonian regions favour higher caffeoylquinic acid derivatives content under cooler conditions, while the Sub-Mediterranean regions exhibit flavonoid profiles shaped by warmer, more insulated, and more humid climates. These findings underscore the influence of local climate conditions on mulberry characteristics, providing valuable insights into the biogeographical impact on phenolic composition.

4. Discussion

4.1. Comparative Analysis of Leaves of Black and White Mulberries

The comparative analysis of leaves of black and white mulberries from the mesoregion of Koper Hills uncovered significant differences in protein and phenolic composition. Leaves of black mulberries exhibited higher protein content compared to white mulberries. This observation contrasts with broader trends in the literature, which generally report higher protein levels in white mulberries [25,57]. However, Sánchez-Salcedo et al. [57] reported black mulberry genotype having a higher protein content (18.7%) than white mulberry clones, which had an average protein content of 16.5% across four different genotypes. Iqbal et al. [58] also determined that black mulberry contains more protein than white mulberry. Urbanek Krajnc et al. [3] attributed the typically elevated protein content in white mulberries to chemotype-specific metabolic pathways which are closely correlated with main amino acids, threonine, arginine, asparagine, serine, and glutamine as the most prominent free amino acids. The divergence observed in our study may result from local environmental conditions, genotype-specific traits and cultivation practices.

Similarly to proteins, the total phenolic content was significantly higher in black mulberries compared to white mulberries from Koper Hills. This finding aligns with existing literature, which consistently reports higher phenolic concentrations in black mulberries, contributing to their superior antioxidant properties [57–60]. For instance, Iqbal et al. [58] found that black mulberry leaves contained significantly more total phenolics (24.37 mg GAE/g DW) compared to white mulberry leaves (16.21 mg GAE/g DW), highlighting the greater potential of black mulberries for preventative health applications. Similarly, Sánchez-Salcedo et al. [61] observed that among the genotypes they studied, white mulberry clones had the lowest phenolic content (12.81–15.50 mg GAE/g DW), while black mulberries displayed slightly higher values (13.48–16.13 mg GAE/g DW). Additionally, Radojković et al. [62] reported lower levels of total phenolics in white mulberry leaves compared to black mulberry leaves, further supporting these findings.

The analysis of specific phenolic compounds revealed distinct patterns between the two species. Black mulberries exhibited significantly higher levels of caffeoylquinic acid derivatives, predominantly chlorogenic acid, compared to white mulberries. These findings align with previous studies [61,63], which consistently identified chlorogenic acid as the predominant individual phenolic compound, with black mulberries containing higher concentrations than white mulberries.

In contrast, white mulberries exhibited significantly higher concentrations of total quercetin glycoside derivatives, with rutin being the predominant flavonoid. This finding aligns with the results of Połumackanycz et al. [59], who compared various extraction methods and confirmed, using HPLC analysis, that methanolic extracts of white mulberries contained higher levels of rutin compared to black mulberries.

4.2. Correlations Between Protein and Phenolic Contents and Chemotype Variation in Black and White Mulberry Leaves

In the present study, no significant correlations were observed between total protein and total phenolic contents. This finding is consistent with previous research on local mulberry genotypes and other plants walnut [64]. In contrast, multiple significant positive correlations were identified between total phenolic content and individual phenolic compounds largely consistent with our previous findings on local mulberry genotypes Šelih et al., [4]. Values in parentheses represent Spearman's rank correlation coefficients. Correlations marked with double asterisks (**) are statistically significant at the 0.01 level, while those marked with a single asterisk (*) are significant at the 0.05 level. Among these, total phenolic content showed a moderate correlation with chlorogenic acid (0.47**), suggesting that this compound contributes substantially to the overall phenolic profile of mulberry leaves. Similar findings have been reported in other plant species. For instance, previous studies in apples and ciders found an exceptionally strong correlation between total phenolic content and chlorogenic acid content (0.99*), both across different cultivars and within individual varieties [65]. Likewise, significant positive associations were observed in yellow- and white-fleshed potatoes [66,67], further supporting the key role of chlorogenic acid as a major contributor to phenolic accumulation in various plant systems. We also determined a significant positive correlation between total phenolic content and quercetin glycoside derivatives (ρ0.39**) as well as total kaempferol glycoside derivatives (0.55**). This is consistent with findings from a previous study on cruciferous vegetables, which reported a significant positive correlation between total phenolic content and total flavonoids—including quercetin and kaempferol glycosides—with Spearman's ρ = 0.741 [68]. The strong correlations observed between quercetin and kaempferol glycoside contents are consistent with previous findings on local mulberry genotypes Šelih et al., [4]. In that study, the strongest correlations were reported between rutin and isoquercetin, as well as between quercetin malonylhexoside and kaempferol acetylhexoside. In our analysis, we similarly identified moderate positive correlations between quercetin-3-O-rutinoside and both quercetin malonylhexoside and kaempferol acetylhexoside. Furthermore, isoquercetin showed strong correlations with quercetin malonylhexoside and kaempferol acetylhexoside, which is in line with the correlations previously reported Ward's hierarchical clustering revealed three major chemotypes (Clusters A, B, and C) based on the biochemical composition of mulberry leaves, including total protein, total phenolic content, and key phenolic subclasses such as caffeoylquinic acids, coumaroylquinic acids, and quercetin and kampferol glycosides. These groupings reflect distinct metabolic patterns among mulberry trees and are here referred to as chemotypes—a term denoting chemically differentiated forms within the same species, shaped by environmental conditions, genetic variation, or both [11].

Chemotypes—distinct chemical phenotypes within a species—are increasingly recognised as important markers of ecological adaptation and genetic divergence in both herbaceous and woody plant species [69]. In mulberries, such chemical profiles may reflect not only current bioclimatic conditions but also the legacy of historical cultivation and breeding [3,4].

Notably, Cluster A comprised mulberries with the lowest average content of total caffeoylquinic acids, while also containing individuals with the highest levels of total protein. This cluster included nearly all black mulberries and the majority of trees from the Sub-Mediterranean Koper Hills region, suggesting a distinct chemotype potentially shaped by high solar radiation and reduced precipitation typical of this environment. Given the historical context, it is possible that these mulberries were propagated from grafted, high-yielding varieties originating from Italy, where grafting was more common [1].

Cluster B was composed of a mix of Sub-Pannonian and Sub-Mediterranean white mulberries, distinguished by high levels of caffeoylquinic acid derivatives. The distribution across several eco-regions suggests this group could represent either locally adapted spontaneous varieties or older cultivated lines selected for resilience across different climates.

Cluster C represented the most chemically diverse group, with elevated levels of total phenolic content and flavonol glycosides and broad regional distribution. The wide geographical spread of mulberries—from Karst Plateau to Vipava Hills and Slovenian Hills—suggests multiple origins descend from historically diverse germplasm pools. These distinct chemical types may also mirror underlying genetic structure and historical germplasm distribution. In Slovenia, centuries-old white mulberry trees have been documented across several eco-geographical regions, with the highest densities in the Sub-Mediterranean zone [4]. These trees likely represent remnants of sericultural selection lines, preserved through generations for their leaf quality and adaptability [3]. Similar patterns have been reported globally, where chemotypic and genotypic clustering often align with geographic origin and historical breeding [70,71].

In this context, our chemotype classification complements the spatial and climatic analyses by highlighting how biochemical traits can serve as integrative markers of both environmental imprint and genetic legacy.

4.3. Biochemical Profile of White Mulberry Leaves Across Sub-Pannonian and Sub-Mediterranean Mesoregions

Beyond comparative analysis, the nutritional profile of white mulberry leaves has attracted considerable interest due to their high total protein and phenolic content, which underpin their health-promoting properties and diverse applications [72]. Notably, white mulberry leaves are characterised by a substantial protein content ranging from 13% to 31%, enriched with amino acids such as threonine, arginine, asparagine, serine, and glutamine and leucine [3,73,74]. Šelih et al. [4] reported that pruning had a significant impact on the levels of asparagine, alanine, and serine with higher concentrations observed in annually pruned trees, regardless of morphotype. Using the Lowry method, previous studies have reported protein concentrations in mulberry leaves of various varieties to be between 90 and 300 mg/g DW [24,34,75]. In our study, the total protein content of white mulberry leaves was found to range from 97.72 to 299.36 mg/g DW (9.8–29.9%), with a mean value of 162.51 mg/g DW (16.2%). These findings align closely with the values reported in earlier research, further validating the nutritional richness of white mulberry leaves.

The mean total phenolic content in leaves of all studied white mulberries was 14.59 mg GAE/g DW, aligning with findings from Alidee et al. [60] reporting contents of 16.5 mg/g DW in white mulberry leaves. In addition, the observed range (7.42 to 19.23 mg GAE/g DW) is consistent with Urbanek Krajnc et al. [3], who documented values between 7 and 20 mg GAE/g DW in local mulberry varieties, and with Sánchez-

Salcedo et al. [61], who found a narrower range of 12.81 to 15.50 mg GAE/g DW. Polumackanycz et al. [59], who characterised mulberry leaves as a promising food source of phenolic compounds with antioxidant activity, reported values in range from 2.14 mg GAE/g DW (in infusions) to 5.36 mg GAE/g (DW) in decoctions. These results highlight the phenolic richness of mulberry leaves, confirming their potential as a significant source of bioactive compounds.

Among the predominant phenolic compounds found in white mulberry leaves, chlorogenic acid stands out as the most abundant, with contents reported between 2.45 and 10.24 mg/g DW [76]. This compound is recognised for its strong antioxidant activity, which plays a crucial role in protecting cells from oxidative stress [77,78]. Memon et al. [79] confirmed that regardless of the mulberry species (*M. alba*, *M. nigra* and *M. laevigata*), mulberry leaves contain the most chlorogenic acid, between 60.5% and 67.2% out of analysed phenolics. In our study, chlorogenic acid was similarly identified as the predominant phenolic compound, with concentrations ranging from 2.77 mg/g to 17.41 mg/g DW and an average of 9.48 mg/g DW. In our previous study evaluating phenolics of local mulberry trees the content of chlorogenic acid ranged between 1.80 and 6.89 mg/g DW, whereas the maximum content was of 18.98 mg/g DW [4].

Seasonal and harvest-specific variations in chlorogenic acid content have also been previously documented. Sugiyama et al. [80] analysed once- and twice-harvested mulberry trees of different varieties and reported concentrations ranging from 6.16 mg/g to 10.14 mg/g DW, indicating that environmental factors and harvest frequency significantly influence phenolic accumulation. Similarly, Pothinuch and Tongchitpakdee [63] analysed total caffeoylquinic acid derivatives in leaves of three cultivars of different leaf ages, reporting values between 5.70 and 16.30 mg/g DW, highlighting the importance of leaf maturity. Notably higher chlorogenic acid levels have been observed in some studies. Jarienė et al. [81] reported concentrations ranging from 15.29 mg/g to 17.41 mg/g DW in leaves of two *M. alba* cultivars, while Flaczyk et al. [75,80] documented a maximum concentration of 23.30 mg/g DW in a Polish variety.

Out of coumaroylquinic acid derivatives *cis*-5-coumaroylquinic acid has been identified as predominant in white mulberry leaves, with concentrations reaching levels of 0.26 mg/g DW. These findings are consistent with those reported by Jelen & Urbanek Krajnc [25], who previously highlighted *cis*-5-coumaroylquinic acid as the predominant derivative within the coumaroylquinic acids in leaves of different analysed mulberry species and varieties.

Flavonoids such as quercetin and kaempferol glycosides are significant bioactive constituents of mulberry leaves, contributing to their health-promoting properties [32,82]. Among these, rutin—a flavonoid glycoside abundant in white mulberry leaves—is particularly notable for its potent antioxidant activity and diverse health benefits, including anti-inflammatory and anti-diabetic effects [83]. While white mulberry is renowned for its health benefits in humans, it also serves as the sole food source for silkworms. Recent studies have further elucidated the metabolic processing of flavonoids in silkworms, highlighting a glycoside hydrolase that facilitates the uptake and utilisation of quercetin glycosides, enhancing their biological roles in silkworm physiology [84].

Silkworm larvae exhibit the ability to differentiate between quercetin glycosides in mulberry leaves. Isoquercetin, a specific quercetin glycoside, has been identified as a feeding stimulant for silkworms, whereas its rhamnose conjugate may deter feeding behaviour [85]. Notably, isoquercetin has been shown to exert a positive effect on the growth and development of silkworm larvae, highlighting its role in larval nutrition and performance [85]. In contrast, studies have not confirmed any significant influence of

rutin on the growth and development of silkworm larvae, although rutin acts as a feeding stimulant for many insect species [85].

Different studies have reported rutin content in mulberry leaves ranging from 0.53 mg/g to 4.34 mg/g DW, depending on the extraction method and leaf variety [86,87]. In our study, rutin was identified as the second most predominant phenolic compound in white mulberry leaves, with concentrations ranging from 0.25 to 19.29 mg/g DW and an average of 4.52 mg/g DW. While our results align with previous findings, particularly in the lower and mid-range values, we also observed significantly higher rutin levels in certain mulberry tree leaves. Notably, three mulberry trees (SI23_00171, SI23_00186, and SI23_00187) from the Koper Hills region exhibited rutin concentrations exceeding 10 mg/g DW. This elevated content may be attributed to both genotype-specific traits and environmental factors, as variations in rutin levels are often influenced by altitude, air temperature, and light exposure, alongside genetic diversity [83,87].

Overall, our study highlights the substantial variation in rutin content among white mulberry leaves. By confirming known trends and revealing high rutin concentrations in specific mulberry trees, our findings enhance the understanding of environmental and genetic influences on phenolic composition. These insights underscore the potential of mulberry leaves as a valuable natural antioxidant source, with practical applications in agriculture, nutrition, and the pharmaceutical industry [88].

Quercetin glycosides are further known to play an important role in plant–microbe interactions, in particular the synthesis is accelerated under fungal infection [89]. From the point of view of sericulture the effect of flavonols on the growth and development of silkworm larvae and cocoon formation has been intensively studied [63,84,85,90]. In low concentrations, they have a beneficial effect on growth and development, whereas high doses might have an antinutrient effect [91].

In our analysis of white mulberry leaves, kaempferol acetyl-hexoside was identified as the predominant kaempferol glycoside derivative, with concentrations ranging from 0.007 to 1.28 mg/g DW and an average of 0.48 mg/g DW. These results align with findings from previous studies. For example, Pothinuch and Tongchitpakdee [63] identified kaempferol acetyl-hexoside as a major kaempferol derivative in mulberry leaves, although specific concentrations were not provided. Similarly, Šelih et al. [4] highlighted kaempferol acetyl hexoside as one of the principal phenolic compounds in mulberry leaves, which reached the highest amount of 0.98 mg/g DW, emphasising its biochemical and physiological significance.

4.4. Bioclimatic Comparison Between Sub-Pannonian and Sub-Mediterranean Mesoregions

The Sub-Mediterranean region (southwest Slovenia) is characterised by milder winters, hotter summers, and higher annual precipitation, along with pronounced seasonal rainfall variability and frequent summer droughts. In contrast, the Sub-Pannonian region (northeast Slovenia) has a more continental climate, with hot summers, colder winters, and significantly lower annual precipitation [10,92,93]. These climatic differences are consistent with our results for mesoregions of the sampling season (June–August 2023) as well as with long-term trends from 1970 to 2000, although direct comparisons are limited to the summer period.

Both the Sub-Mediterranean and Sub-Pannonian regions have experienced statistically significant increases in average summer temperatures since 2000, with warming rates of approximately 0.7–0.9 °C per decade. In the Sub-Mediterranean region, average July daytime heat index values now regularly exceed 30 °C, with some locations reaching over 32 °C and daily maximums as high as 47 °C. This has led to an increased frequency and severity of heat stress, with one-third to more than half of summer days now classified

under strong or very strong heat stress categories [94,95]. This trend was also evident when comparing air temperatures from June–August 2023 to historical averages, as temperatures during our sampling season were statistically significantly higher. Nevertheless, the pattern across mesoregions remained relatively stable.

Precipitation patterns have also shifted. The Sub-Mediterranean region has seen declines in summer precipitation and river discharge, contributing to more frequent droughts and water scarcity. Conversely, autumn precipitation has increased, raising flood risks outside the primary growing season. The Sub-Pannonian region has likewise experienced more extreme weather events, including heatwaves and intense rainfall, but also benefits from longer periods favourable for agricultural and outdoor activities [93,95,96]. Notably, the summer season of 2023 recorded unusually high precipitation amounts compared to long-term climatic averages.

The Sub-Mediterranean region also receives more annual solar radiation and higher average temperatures than the Sub-Pannonian region [95,97]. This was confirmed in our dataset, which showed significant differences in total insolation among mesoregions for both the 2023 season and the historical 1970–2000 period.

Additionally, significant differences were found between the Sub-Pannonian and Sub-Mediterranean mesoregions in Growing Degree Days above 10 °C and Growing Season Length.

Overall, the data highlight a consistent distinction between coastal/sub-Mediterranean and continental mesoregions, with higher insolation and solar radiation, reflecting their climatic advantage for energy input and plant growth

Furthermore, the combination of high air temperatures and solar exposure in Sub-Mediterranean regions Koper Hills and Vipava Hills suggests conditions that may favour unique phenolic profiles in mulberries compared to regions with lower sunlight exposure, such as Slovenian Hills. Additionally, the range of precipitation levels could further differentiate metabolic responses, with regions experiencing higher water availability likely promoting growth, nitrogen assimilation and protein synthesis, while drier regions may induce stress-related decrease in proteins and accumulation of phenolics.

4.5. Effect of Pruning on Protein and Phenolic Content in White Mulberry Leaves

We also evaluated the impact of pruning on protein and phenolic content, given its recognised role in enhancing leaf nutritional quality. Consistent with previous studies [3,4], our findings confirmed that pruned mulberry trees—especially those pruned annually—exhibited significantly higher protein levels compared to unpruned trees. This supports the notion that regular pruning stimulates physiological processes associated with protein synthesis, including improved nitrogen assimilation and amino acid accumulation. In contrast, unpruned trees consistently had the lowest protein content, highlighting the relevance of pruning as a practical cultivation strategy to improve mulberry leaf quality for sericultural and nutritional applications.

The interaction between pruning and nitrogen metabolism is increasingly recognised as a key driver of protein enhancement in plants. Pruning alters source–sink dynamics, promotes compensatory growth, and modulates hormonal signalling pathways—all of which influence nitrogen uptake, assimilation, and amino acid biosynthesis. Recent studies across diverse crop species such as rice, wheat, maize, and legumes have shown that manipulating nitrogen metabolism—through genetic, biochemical, or agronomic approaches—can significantly enhance protein accumulation. These effects are mediated by the upregulation of key nitrogen-assimilating enzymes such as glutamine synthetase and glutamate synthase, expansion of free amino acid pools, and activation of transcriptional networks responsible for protein synthesis [98–102].

Moreover, integrated multi-omics analyses reveal that pruning and nitrogen supply can jointly regulate carbon–nitrogen metabolic pathways, hormonal responses, and post-translational mechanisms, leading to improved nitrogen use efficiency and protein yield [98,103–105]. These insights carry important implications for sustainable agricultural practices, crop quality optimisation, and food system resilience under variable climatic conditions.

Pruning acts as a stressor, triggering complex hormonal and metabolic responses. In *Ginkgo biloba* and *Populus tremula*, pruning upregulates genes involved in flavonoid and phenolic biosynthesis, leading to increased accumulation of these compounds [106,107]. In tea and peach, similarly to our study, pruning downregulates secondary metabolite pathways, reducing phenolic content [46,108,109]. The response is mediated by changes in auxin signalling, tryptophan metabolism, and the phenylpropanoid pathway, with key enzymes such as phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), and hydroxycinnamoyl transferase (HQT) playing central roles [46,106]. Interestingly, the total phenolic content showed no statistically significant differences between the mesoregions or pruning frequencies. However, a trend was observed where unpruned trees generally had higher phenolic levels, which was also reported in previous study by Šelih et al. [4]. The less obvious effect of pruning is most likely amplified by the combined effect with bioclimatic factors. Slovenian Hills recorded the highest phenolic content among unpruned trees, likely due to cooler bioclimatic conditions that promote secondary metabolite accumulation. In plants, cold stress enhances the activity of PAL, a key enzyme in the phenylpropanoid metabolic pathway, which plays a crucial role in phenolic compound synthesis [110].

Pruned trees, regardless of pruning frequency, exhibited slightly reduced phenolic levels, suggesting that pruning may prioritise primary metabolic processes over secondary metabolite production.

Previous studies determined that the effect of pruning on chlorogenic acid is variable and species-dependent. In peach and tea, pruning often leads to a decrease in chlorogenic acid and related phenolics, associated with downregulation of key biosynthetic genes such as HQT and chalcone isomerase (CHI) [44,46]. In our study, the content of caffeoylquinic acid derivatives with predominating chlorogenic acid demonstrated significant variation influenced by both mesoregion and pruning frequency. Unpruned mulberries, particularly those from Vipava Hills, exhibited the highest levels of caffeoylquinic acid derivatives. Conversely, yearly pruned trees, particularly in Koper Hills, showed the lowest levels. These results suggest that the production of caffeoylquinic acid derivatives is highly responsive to the interaction between environmental conditions and cultivation practices.

In contrast, no statistical interactions with the mesoregions or pruning frequency were found for the derivatives of coumaroylquinic acid, quercetin glycosides or kaempferol glycosides.

4.6. Influence of Selected Bioclimatic Factors and Pruning Practices on the Biochemical Composition of White Mulberry Leaves Across Different Mesoregions in Slovenia

Our study highlights the significant influence of selected bioclimatic factors and pruning practices on the biochemical composition of white mulberry leaves across different mesoregions in Slovenia. We observed notable variability in total protein and phenolic content, with distinct regional patterns influenced by air temperature (June–August 2023), total insolation (June–August 2023), and precipitation (June–August 2023), long-term climatic averages (average, minimum, and maximum air temperatures; solar radiation; and precipitation from 1970 to 2000 [June–August]), as well as growing degree days above 10 °C and growing season length (1981–2010), demonstrating that environmental stressors play a crucial role in shaping the metabolic profile of plants [105–107].

To capture and interpret the complexity of these interactions, we employed Principal Component Analysis (PCA), that revealed distinct clustering patterns, with the first two components explaining 67.55% of total variance (PC1: 43.76%, PC2: 23.79%), confirming that biochemical traits of mulberry leaves are tightly linked to both environmental and management factors (Figure 9).

Among the key drivers of PC1 were average air temperatures (both for the sampling period, June–August 2023, and the long-term trend from 1970 to 2000) precipitation (sampling period, June–August 2023, and the long-term trend from 1970 to 2000), growing degree days above 10 °C and growing season length (1981–2010), all of which showed strong positive correlations with total protein content and kaempferol glycoside levels. This highlights a clear link between thermal accumulation and both primary (protein synthesis) and secondary (phenolic biosynthesis) metabolism in white mulberries. In contrast, PC2 was primarily driven by total insolation (June–August 2023) and long-term solar radiation (1970–2000), which were closely associated with coumaroylquinic acid accumulation, pruning response, and leaf canopy traits—possibly reflecting adaptive strategies for photoprotection and growth regulation under high light stress. Notably, historical climate variables (1970–2000) exhibited strong positive loadings that closely aligned with those from the 2023 sampling season—particularly for air temperature, precipitation, and solar radiation—indicating stable long-term climatic patterns. As these historical and current variables were similarly expressed, we discuss them in PCA, since their influence on biochemical traits followed comparable trends.

In defining the effects of mesoregions on the nutrient profile of mulberry leaves, several environmental factors such as climate, soil quality and hydrology in different regions should be considered [111]. Clinal variation has been identified as a tool for understanding climatic effects [34,112]. Clinal variations are observable gradients at the geographic scale (latitude and altitude) that reflect a continuous change in the biological characteristics of species across geographic ranges. Since clinial variations represent active genomic responses at the population level, clines provide the opportunity to answer questions related to climate effects and the responses at both phenotypic and genotypic levels of mulberries in different regions [113].

Environmental conditions, particularly photo-thermal fluctuations, play crucial roles in the biosynthesis of phenolic compounds in plants. Air temperature and light duration modulates the variation in phenolic compounds profile in relation to photosynthesis and consequently protein synthesis [114]. Połumackanycz et al. [59] reported that the phenolic profile of mulberry leaves is influenced by various environmental stressors, including air temperature changes, drought, and UV light exposure. Similarly, Jin et al. [115] highlighted the potent antioxidant properties of phenolics in mulberry leaves, which are significantly shaped by external conditions like air temperature. While this enhances the synthesis of total phenolic content. Our results further showed that phenolic acids, such as caffeoylquinic acids, may accumulate more in regions with less light exposure. The interaction between light and water availability further emphasises the critical role of environmental factors. Specific light spectra could enhance the production of bioactive compounds in mulberry leaves, underscoring the synergistic importance of insolation and water availability for optimal phenolic biosynthesis [116].

Particularly UV-B spectrum, has been shown to play a dual role in plant physiology. Excessive exposure to UV-B radiation can cause damage to cellular structures, including membranes and DNA, leading to oxidative stress and impaired function. Moderate UV-B exposure stimulates the biosynthesis of secondary metabolites, including flavonoids, which act as photoprotective agents [117]. When exposed to UV-B stress, plants respond with enhanced expression of genes for flavonoid production, especially the key-enzyme chalcone

synthase [118]. The main role of quercetin and kaempferol glycosides is the antioxidant activity, which enable them to scavenge reactive oxygen species (ROS) in particular under high intensity of sunlight. Stored in the vacuoles of the epidermal cells they act as UV filters by absorbing the short-wave light. This protects the below chlorenchyma cells and their photosynthetic system from deteriorating effect of UV light [89].

On the basis of the above-mentioned studies, a multivariate analysis integrating metabolite composition and environmental parameters led us to conclude that the increased total phenolic content observed in the Koper Hills region is primarily driven by photo-thermal stress. This region experiences the highest duration of total insolation among all mesoregions, coupled with relatively high air temperatures and low precipitation. Similarly, the elevated levels of total kaempferol glycoside derivatives in Karst Plateau can be attributed to prolonged insolation exposure.

Distinct cluster of mulberries from the Slovenian Hills region, which is characterised by low sun exposure and air temperatures, had the highest levels of total quercetin derivatives and the highest levels of caffeoylquinic acid derivatives. It has been shown that low insolation promotes the production of caffeoylquinic acid derivatives. Kim et al. [119] reported increased concentrations of 3,4-di-O-caffeoylquinic acid under limited light conditions.

Our analysis revealed that mulberries from the Gorica Hills region characterised by higher precipitation during the summer sampling period and moderate total insolation, exhibited the highest protein content. Conversely, mulberries from Koper Hills, which experienced lower precipitation and the highest total insolation, had the lowest protein levels. These findings highlight that while precipitation enhances protein synthesis, excessive insolation may negatively impact protein accumulation [111]. Drought stress inhibits nitrate reductase activity, which is critical for nitrogen assimilation, as reduced transcription of nitrate reductase genes has been observed [104,120]. Additionally, oxidative stress caused by drought leads to the accumulation of reactive oxygen species, which damages nitrate reductase and impair their function [121]. Stomatal closure during drought reduces carbon dioxide availability, leading to decreased photosynthesis and limited energy supply for nitrate reductase activity [122]. Collectively, these factors diminish nitrogen assimilation and protein synthesis in plants under drought stress.

Principal Component Analysis (PCA) identified total insolation and precipitation as significant factors affecting protein levels in mulberry leaves. The results suggest that while pruning enhances protein content, environmental factors such as precipitation and total insolation are critical in determining the extent of this enhancement. The interaction between these factors indicates that optimal agricultural practices must consider local climatic conditions to maximise the nutritional benefits of mulberry leaves [111].

Similarly, moderate water stress caused by reduced precipitation, as observed in Slovenian Hills, has been linked to increased phenolic compound synthesis. Sharma et al. [123] reported that drought stress in many plant species influences the activity of key enzymes in the phenylpropanoid metabolic pathway, including Phenylalanine Ammonia-Lyase (PAL), chalcone synthase (CHS), and flavanone 3-hydroxylase (F3H), resulting in an overall increase in phenolic compound content. However, the signalling and regulatory mechanisms governing individual flavonoids in mitigating drought stress in combination with photo-thermal stress remain unclear [118,123–126].

5. Conclusions

This study highlights the complex interplay between bioclimatic factors, geography, and cultivation practices in shaping the biochemical composition of leaves of mulberry trees across Sub-Mediterranean and Sub-Pannonian macroregions of Slovenia. The observed

clinal variation serves as a valuable tool for understanding trait changes across geographical and environmental gradients.

Furthermore, variability in protein and phenolic content across mesoregions underscores the pivotal influence of air temperature, total insolation, and precipitation in shaping the nutritional profile of white mulberry leaves, s.

Principal component and cluster analyses showed distinct regional patterns of mulberry metabolite profile. Mulberries from Sub-Pannonian areas, like the Drava Plain and Slovenian Hills, had higher caffeoylquinic acid derivatives, while mulberries from Sub-Mediterranean regions showed more kaempferol derivatives. Mulberries from Koper Hills, characterised by high radiation and low rainfall, exhibited elevated total phenolics, while those from the Slovenian Hills were distinguished by particularly high levels of caffeoylquinic acids. Pruning was shown to significantly increase protein content and reduce phenolic content, confirming its importance as a cultivation practice for the sericultural use of mulberry trees. Together, these findings provide a basis for developing optimised agricultural strategies that enhance both nutritional and ecological benefits, while supporting biodiversity conservation and innovative applications of this versatile species.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae11091096/s1, Table S1: Comprehensive metadata for all sampled mulberry individuals, including *Morus*APP identifiers (ID), detailed macroregional and mesoregional classifications, global navigation satellite system (GNSS) location coordinates (latitude, longitude), tree circumference range, pruning practices, number of individuals per sampling site, and estimated leaf size classes. The dataset includes both white mulberry (*Morus alba*) and black mulberry (*Morus nigra*) individuals, with black mulberries listed separately at the bottom of the table. Table S2: Detailed biochemical composition of each sampled mulberry tree, including quantified values of individual phenolic compounds. Each value represents the mean of at least two independent measurements. Below each column, the minimum, maximum, and mean values across all sampled trees are provided. Abbreviations: 4-caffeoyl-QA—4-caffeoylquinic acid; 5-caffeoyl-QA—5-caffeoylquinic acid; 5-total CQA—total caffeoylquinic acid derivatives; c-5-CQA—cis-5-coumaroylquinic acid; t-5-CQA—trans-5-coumaroylquinic acid; *p*-CAH—p-coumaric acid hexoside; *p*-CAH2—*p*-coumaric acid hexoside 2; *p*-CQA—*p*-coumaroylquinic acid; total CQA—total *p*-coumaroylquinic acid derivatives; Q-3-glu—quercetin-3-glucoside; QMH—quercetin malonyl hexoside; Q-diR-gly—quercetin dirhamnosyl glycoside; QRH—quercetin-rhamnosyl hexoside; Q-acetyl-RH—quercetin-acetyl rhamnosyl hexoside; QAH—quercetin acetyl hexoside; total quercetin-gly—total quercetin glycoside derivatives; KAH—kaempferol acetyl hexoside; K-diRH—kaempferol dirhamnosyl hexoside; KRH—kaempferol rhamnosyl hexoside; K-acetyl-RH—kaempferol-acetyl rhamnosyl hexoside. Table S3: Total phenolic derivatives contents in leaves of local white mulberry trees across different mesoregions. Each value represents median ± MAD within a mesoregion in mg/g DW. Different letters (a–b) indicate significant differences ($p < 0.05$), which were determined using the post hoc Dunn–Bonferroni test Table S4: Spearman's correlation matrix of total protein content, total phenolic content, and in-dividual phenolic compounds in white and black mulberry (*Morus* sp.) leaves. Table S5: Principal Component Analysis (PCA) loadings, eigenvalues, and explained variance for the first two principal components (PC1 and PC2). The table shows the contributions (loadings) of each original variable to PC1 and PC2. Variables include morphological traits (e.g., tree circumference range, vigour, pruning, leaf size ratio), geographic coordinates (longitude, latitude), biochemical parameters (e.g., total protein, total phenolic content, caffeoylquinic and coumaroylquinic acid derivatives, quercetin and kaempferol glycosides), and climatic factors (e.g., average, maximum, and minimum temperatures, total solar insolation, precipitation). The first two components together explain 68.76% of the total variance in the dataset, with PC1 accounting for 38.06% and PC2 for 30.69%. Positive and negative loadings indicate the direction and strength of correlation between the variable and the principal components.

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