Acta Societatis Botanicorum Poloniae

DOI: 10.5586/asbp.3614

Publication history
Received: 2018-02-27
Accepted: 2018-11-05
Published: 2019-04-02

Handling editor
Łukasz Łuczaj, Faculty of Biotechnology, University of Rzeszów, Poland

Authors' contributions
RLB, TB, and AUK designed the experiments; AUK, MF, TU, JR, AP, RLB, and TB performed the sampling excursions; TU and AUK carried out the analyses of single amino acids; MF, AUK, and VB carried out the analyses of total proteins, total phenolics, and single phenolics; MMP identified the phenolic profile via HPLC-MS analysis; FJ, AP, and MF performed the statistical evaluation of the results; AUK, MF, TU, and JR prepared the manuscript

Funding
This research was financed by Slovenian Research Agency (ARRS, N1-0041, P-0164).

Competing interests
No competing interests have been declared.

Citation
Urbanek Krajnc A, Ugulin T, Paušič A, Rabensteiner J, Bukovac V, Mikulič Petkovšek M, et al. Morphometric and biochemical screening of old mulberry trees (*Morus alba* L.) in the former sericulture region of Slovenia. Acta Soc Bot Pol. 2019;88(1):3614. https://doi.org/10.5586/asbp.3614

ORIGINAL RESEARCH PAPER

Morphometric and biochemical screening of old mulberry trees (*Morus alba* L.) in the former sericulture region of Slovenia

Andreja Urbanek Krajnc[1]*, Tina Ugulin[2], Andrej Paušič[3], Johannes Rabensteiner[2], Vesna Bukovac[4], Maja Mikulič Petkovšek[5], Franc Janžekovič[6], Tamás Bakonyi[7,8], Rebeka Lucijana Berčič[7,8], Mateja Felicijan[1]

[1] Department of Botany and Plant Physiology, Faculty of Agriculture and Life Sciences, University of Maribor, Pivola 10, 2311 Hoče, Slovenia
[2] Division of Plant Sciences, Institute of Biology, University of Graz, Schubertstrasse 51, 8010 Graz, Austria
[3] University Botanical Garden, Faculty of Agriculture and Life Sciences, University of Maribor, Pivola 90A, 2311 Hoče, Slovenia
[4] Department of Agrochemistry, Faculty of Agriculture and Life Sciences, University of Maribor, Pivola 10, 2311 Hoče, Slovenia
[5] Department of Agronomy, Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, 1000 Ljubljana, Slovenia
[6] Department of Biology, Faculty of Natural Sciences and Mathematics, University of Maribor, Koroška 160, 2000 Maribor, Slovenia
[7] Department of Microbiology and Infectious Diseases, University of Veterinary Medicine, Istvan utca 2, 1078 Budapest, Hungary
[8] University of Veterinary Medicine, Veterinärplatz 1, 1210 Vienna, Austria

* Corresponding author. Email: andreja.urbanek@um.si

Abstract

Over centuries, in many European countries, the white mulberry trees (*Morus alba* L.) became an integral part of the cultural landscape, bearing witness to past sericulture activities. The distribution records of white mulberry trees in the cultural landscape are incomplete and in general poorly documented. The aim of the presented research was to collect data regarding geographical locations of mulberry trees and to define their morphological and biochemical variability in Goriška region, one of the historical sericulture regions in Slovenia. Principal component analysis of all morphometrical leaf traits allowed us to characterize two separate groups of morphotypes. Recordings of the tree pruning management revealed that annual base cutting is traditionally used in Goriška region. Significant correlations between pruning management and leaf morphology traits showed that frequently pruned trees form larger leaves. Biochemically, mulberry leaves are shown to be rich in proteins containing threonine, arginine, asparagine, serine, and glutamine as the most prominent free amino acids. The main phenolic compounds were identified as caffeoylquinic acid derivatives, quercetin malonyl-hexoside, rutin, kaempferol acetyl-hexoside, quercetin-3-glucoside, and *p*-coumaric acid derivatives. The difference in concentrations of the investigated metabolites is correlated either with the pruning management or the morphotype. Pruning significantly affected the levels of asparagine, alanine, and serine, which were higher in the annually pruned trees regardless of the morphotype. Furthermore, we were able to confirm a significant effect of pruning on total phenolics as well as on the levels of rutin, quercetin malonyl-hexoside, and quercetin-3-glucoside contents. Multivariate analysis allowed us to determine seven chemotypes with distinctive biochemical traits. Our results are the basis for defining superior high-yielding genotypes with optimum metabolic composition for both silkworm feeding as well as for innovative usage in food processing and pharmaceutical industries.

Keywords
free amino acids; crop intraspecific diversity; leaf traits; morphological variability; *Morus alba* L.; phenolics; proteins; sericulture

Introduction

The natural range of white mulberry (*Morus alba* L.) is probably located in the central and western provinces of China and adjacent regions of Central Asia (western Tien Shan range, the region between the Amu Darya and Syr Darya rivers), where it has been widely cultivated for more than 4,500 years. The white mulberry was brought to Europe in the twelfth century in order to establish a functioning European sericulture since the leaves are the preferred food of the silk-producing caterpillars of *Bombyx mori* L. [1].

First attempts to promote the cultivation of mulberry trees in Slovenia were already undertaken during the sixteenth century, when sericulture was first introduced to the Goriška region. This influence has spread throughout the former Austro-Hungarian Monarchy, when Empress Maria Theresia of Austria started a state support program in 1749 in order to gain more independence of expensive silk imports from France and Italy. Regional sericulture attempts had reached their peak in the middle of the nineteenth century, known as "the golden century of Gorizian sericulture". Since then, it went into a huge decline because of a bacterial infection of the silkworms, which spread through Europe from France, as well as late frost diebacks and fungal and bacterial epidemics of mulberry trees. Despite all taken measures, no lasting results could be achieved, and the silk-producing industry ended with the beginning of the twentieth century, also effected by the introduction of synthetic fibers [2–5]. However, former regions of sericulture retained a number of nowadays centuries-old mulberry trees, which represent both a valuable natural heritage of intraspecific genetic diversity and outstanding monuments of the very early attempts of the industrial society in Europe. There is an urgent need for a broader public awareness towards a conservation of historical remains of sericulture, particularly regarding the historical genotypes of mulberries in context of general tendency to an immense loss of crop plant diversity.

Although the mulberry leaves play an indispensable role in silkworm rearing, they are currently also more frequently used as a supplementary food for livestock [6,7]. Furthermore, *M. alba* is important in traditional herbal medicine. In Chinese medicine, it has been widely used since 659 AD [8]. Previously, many authors [8–16] studied mulberry leaves of different varieties regarding their human pharmacological potential.

The mulberry leaves were shown to have high protein content (18–25 g/100 g DW). Beside the proteins and their unique composition of amino acids, dominated by aspartic acid, glutamic acid, phenylalanine, lysine, and arginine, the main noteworthy components of the leaves are carbohydrates, calcium, iron, ascorbic acid, carotenoids, thiamine, folic acid, and phenols [17].

A certain diversity of phenolic compounds was found in mulberry leaves, and they have been reported to have multiple biological effects, including antioxidant activity [8–16]. The main phenolic acids were identified as chlorogenic acid followed by other caffeolyquinic acid (CQA) derivatives and *p*-coumaric acid (*p*-CA) derivatives [10,17–21]. The flavonols fraction contained rutin, quercetin, as well as kaempferol glycosides [10,12,22–27].

The aim of the presented research was to collect data regarding geographical locations of historical mulberry trees of the Goriška region, to define their morphological variability and to screen their biochemical patterns regarding important primary (proteins and amino acids) metabolites and phenolics. The statistical evaluation allowed us to demonstrate the most suitable differentiating traits, to closer define morphotypes, and to present correlations between measured parameters with respects to potential leaf-harvest related pruning management. All genotypes included in these morphometric and biochemical analyses are part of a broader study aimed at defining high-yielding

and nutritive richer mulberry cultivars from the local gene pool, which will be selected and propagated as highly recommendable feed for silkworms.

Material and methods

Study area

We chose a study area of about 140 km^2 in the western part of Slovenia. The area of research includes the Goriška Brda hilly area, Vipava River valley, and the western edge of Vipava valley, the Podsabotin calcareous transition zone. The study area is naturally limited to the north by the Trnovo forest karst plateau, to the east by the Dinaric Nanos range (plateau). To the south, the study area is limited by the high elevated plateau of Kras (Karst). Western limit of our study coincides with the Brda municipality (Fig. 1A). The average altitude of the area of investigation is 200 m a.s.l. The sub-Mediterranean climate regime of the study area is characterized by annual average precipitation of about 1,000 mm and an average temperature of 12°C [28] (Fig. 1B,C). The geological base of the region is represented by a karst solution plain, formed mainly by calcareous rocks, limestone, and rarely dolomites. On the surface, these rocks are weathering into chromic cambisols and luvisols, which sporadically even completely cover them. The soil quality is all over the region of our investigations practically identical, the chromic (calco) cambisols and deep rendzinas in the dolines and alluvial ravines [29]. The potential natural vegetation of the area is *Quercus-Carpinus* forest [30].

During field excursions, we recorded the exact GPS locations of 85 mainly historical trees, which were identified as *Morus alba* L. Nine genotypes were taken in the region of Goriška Brda (Gorizia hills) (B), 13 genotypes in the Miren-Kostanjevica (MK) municipality, six in Šempeter-Vrtojba (SV) municipality, 52 in Ajdovščina (A), and five in the Nova Gorica (NG) municipality (Tab. 1, Fig. 1). The average stem diameter at breast height (DBH) was more than 180 cm. Large-diameter trees (DBH > 300 cm) represented 11% of trees, 25% of trees had an average DBH between 200 cm and 300 cm. As the shape and the size of leaves may vary according to eventual pruning management [31], the trees were split up to three categories with respect to pruning frequency: 1 – unpruned trees, 2 – trees pruned within last 3 years, and 3 – trees pruned yearly. Pruning recordings revealed that base cutting is traditionally used in the Goriška region, since 51.8% of trees underwent yearly pruning procedure, 28.2% of trees were pruned once within 3 years, whereas 20% of studied individuals represented unpruned trees.

Morphometrical analyses

Twenty to thirty fully developed sun-exposed current year leaves on the fifth position from the top of branches from this years's growth were sampled from each tree. Eight morphometrical parameters of each leaf (leaf area, peduncle length, leaf length, leaf width, left and right leaf width, length of left and right basal vein) were measured and evaluated with the Cell^A 2.4 software (Olympus BioSystems, Houston, USA, 2006). As the shape and the size of leaves may vary according to eventual pruning management [31], the trees were split up to three categories with respect to pruning frequency: 1 – unpruned trees, 2 – trees pruned within last 3 years, and 3 – trees pruned yearly (Fig. 2B).

Biochemical analyses

For biochemical analyses, five to seven fully developed sun-exposed current year leaves (seventh leaf counted starting from apex of branches from this year's growth) were collected randomly from each tree and used as one sample. Immediately after collection, samples were stored on dry ice, transferred to freezer at –80°C, subsequently lyophilized and ground prior to the biochemical analysis. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

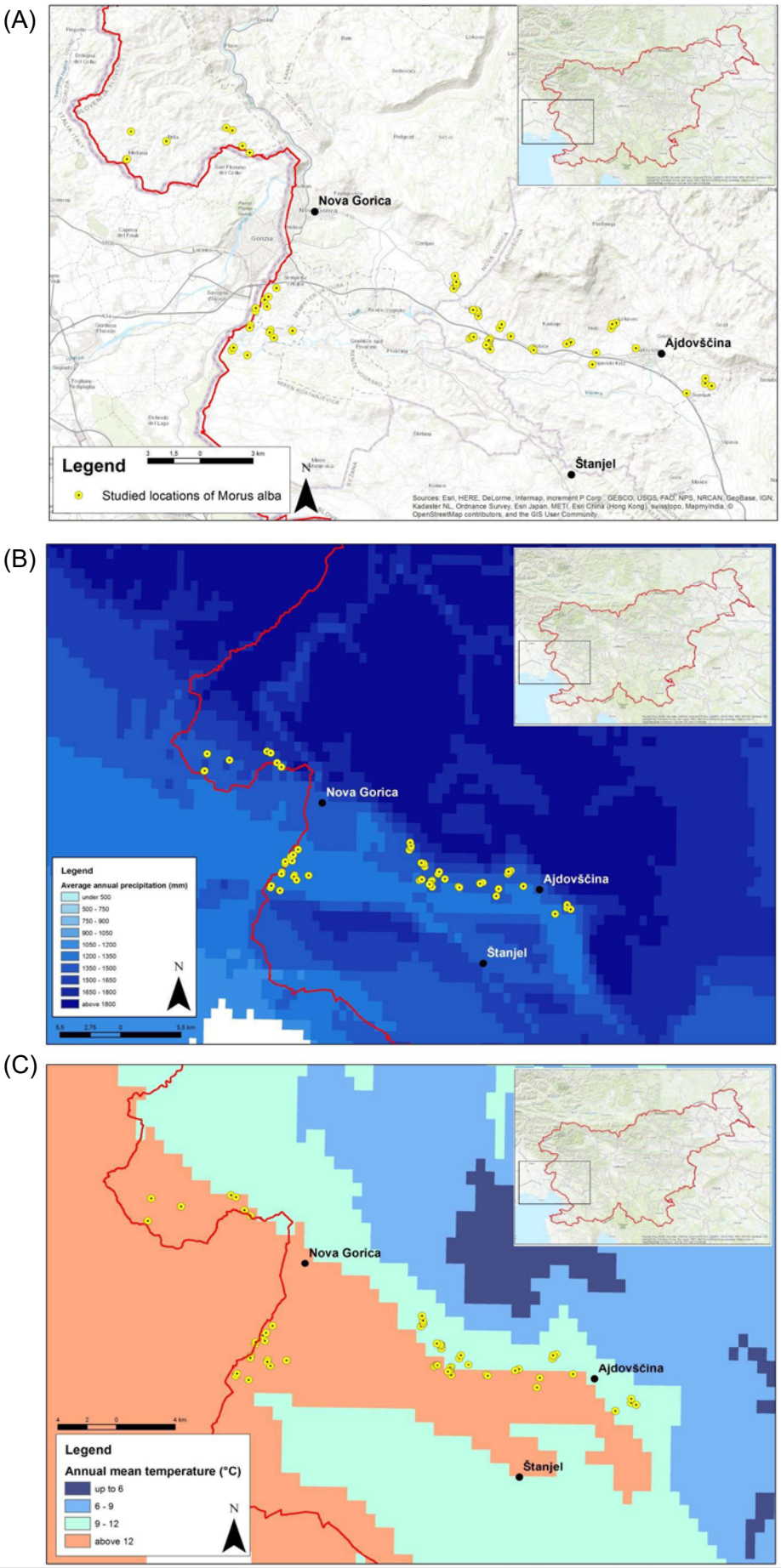

Fig. 1 Map showing (**A**) sampling localities of historical mulberry (*Morus alba* L.) trees, (**B**) average annual precipitation (mm) of the study area (W Slovenia), and (**C**) annual mean temperatures (°C) of the study area. For sample identities see Tab. 1.

Tab. 1 Location and geographic coordinates of historical mulberry trees from five different municipalities in the Gorizia region, sampled in July 2016.

Municipality	Sample ID	Latitude (° N)	Longitude (° E)	Municipality	Sample ID	Latitude (° N)	Longitude (° E)
Goriška Brda	B1	45.99652	13.52310	Ajdovščina	A16	45.88728	13.86758
Goriška Brda	B2	45.98202	13.51987	Ajdovščina	A17	45.89239	13.84870
Goriška Brda	B3	45.98236	13.52049	Ajdovščina	A18	45.89245	13.84904
Goriška Brda	B4	45.99175	13.54941	Ajdovščina	A19	45.89154	13.84582
Goriška Brda	B5	45.99177	13.54941	Ajdovščina	A20	45.89154	13.84582
Goriška Brda	B6	45.99946	13.59320	Ajdovščina	A21	45.88871	13.82042
Goriška Brda	B7	45.99820	13.59771	Ajdovščina	A22	45.88871	13.82042
Goriška Brda	B8	45.99010	13.60534	Ajdovščina	A23	45.89500	13.80458
Goriška Brda	B9	45.98667	13.61073	Ajdovščina	A24	45.89500	13.80458
Miren–Kostanjevica	MK1	45.89847	13.61328	Ajdovščina	A25	45.89913	13.79646
Miren–Kostanjevica	MK2	45.89847	13.61328	Ajdovščina	A26	45.90099	13.79777
Miren–Kostanjevica	MK3	45.89847	13.61328	Ajdovščina	A27	45.88809	13.82181
Miren–Kostanjevica	MK4	45.89719	13.61287	Ajdovščina	A28	45.89344	13.78936
Miren–Kostanjevica	MK5	45.89719	13.61287	Ajdovščina	A29	45.89344	13.78936
Miren–Kostanjevica	MK6	45.88315	13.61158	Ajdovščina	A30	45.89344	13.78936
Miren–Kostanjevica	MK7	45.88568	13.60026	Ajdovščina	A31	45.89344	13.78936
Miren–Kostanjevica	MK8	45.88543	13.60023	Ajdovščina	A32	45.89360	13.78960
Miren–Kostanjevica	MK9	45.89604	13.64466	Ajdovščina	A33	45.89290	13.78860
Miren–Kostanjevica	MK10	45.89560	13.62773	Ajdovščina	A34	45.89057	13.78876
Miren–Kostanjevica	MK11	45.89623	13.62813	Ajdovščina	A35	45.88944	13.78883
Miren–Kostanjevica	MK12	45.89496	13.62754	Ajdovščina	A36	45.88949	13.78898
Miren–Kostanjevica	MK13	45.89222	13.63069	Ajdovščina	A37	45.88813	13.78964
Šempeter–Vrtojba	SV1	45.90577	13.61685	Ajdovščina	A38	45.89030	13.78775
Šempeter–Vrtojba	SV2	45.90728	13.61710	Ajdovščina	A39	45.89026	13.78667
Šempeter–Vrtojba	SV3	45.90808	13.62519	Ajdovščina	A40	45.89037	13.78666
Šempeter–Vrtojba	SV4	45.91148	13.62343	Ajdovščina	A41	45.89342	13.77469
Šempeter–Vrtojba	SV5	45.91317	13.62623	Ajdovščina	A42	45.89440	13.77618
Šempeter–Vrtojba	SV6	45.91761	13.63194	Ajdovščina	A43	45.89463	13.77709
Ajdovščina	A1	45.87228	13.94796	Ajdovščina	A44	45.89437	13.77744
Ajdovščina	A2	45.87492	13.94811	Ajdovščina	A45	45.90508	13.78070
Ajdovščina	A3	45.87112	13.95257	Ajdovščina	A46	45.90508	13.78070
Ajdovščina	A4	45.87112	13.95257	Ajdovščina	A47	45.90729	13.78030
Ajdovščina	A5	45.86707	13.93436	Ajdovščina	A48	45.90720	13.78024
Ajdovščina	A6	45.88985	13.89660	Ajdovščina	A49	45.90715	13.78019
Ajdovščina	A7	45.90258	13.88187	Ajdovščina	A50	45.90776	13.77919
Ajdovščina	A8	45.89642	13.87300	Ajdovščina	A51	45.90810	13.77659
Ajdovščina	A9	45.89974	13.87837	Ajdovščina	A52	45.90813	13.77664
Ajdovščina	A10	45.89976	13.87822	Nova Gorica	NG1	45.91874	13.76226
Ajdovščina	A11	45.89977	13.87829	Nova Gorica	NG2	45.92052	13.76432
Ajdovščina	A12	45.90124	13.87981	Nova Gorica	NG3	45.92115	13.76424
Ajdovščina	A13	45.90124	13.87981	Nova Gorica	NG4	45.92230	13.76417
Ajdovščina	A14	45.90185	13.87891	Nova Gorica	NG5	45.92529	13.76311
Ajdovščina	A15	45.88121	13.86495				

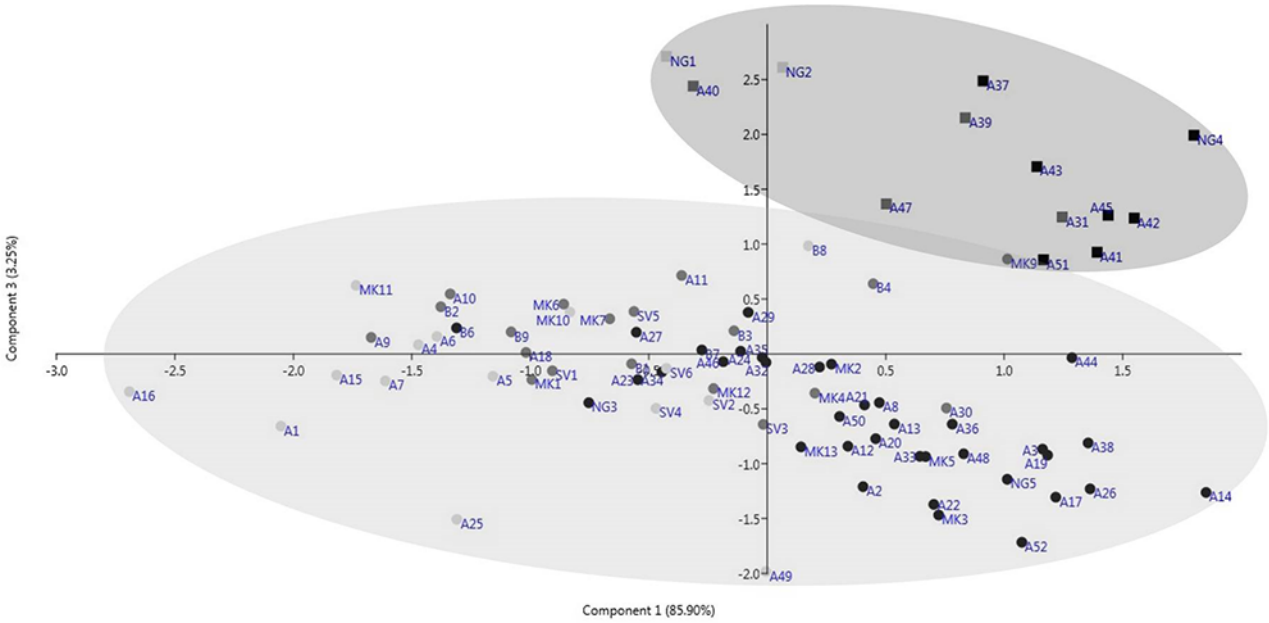

Fig. 2 Projection of the trees on the first and third principal components according to the principal component analysis of leaf morphological traits in *Morus alba* L. Trees of Morphotype 1 (M1) are marked with dots, trees of Morphotype 2 (M2) are marked with square. The ellipses delimit spaces that included samples of M1 and M2 with $p < 0.05$. Unpruned trees are colored with light gray, trees pruned within last 3 years with dark grey marks, and those yearly pruned trees with black marks.

Extraction and determination of total protein content

Total proteins were quantitatively determined by using the Lowry's method [32]. Previously, the proteins were precipitated with trichloro acetic acid (TCA) according to Kumar et al. [33]. Twenty-five mg of dry leaf powder was homogenized in 5 mL of 80% ethanol. Supernatant was discarded and pellet was suspended in 5 mL of 10% TCA for 30 minutes. After centrifugation, the supernatant was discarded. Pellet was then washed with 5% TCA to remove interfering amino acids and phenols. Protein precipitate was then dissolved in 1 M sodium hydroxide in hot water bath for 30 minutes. Extracted proteins in 1 M NaOH were diluted 10 times with distilled water. Five hundred µL of diluted protein sample was taken in a test tube and 700 µL of Lowry reagent was added. Following the 20-min incubation at room temperature, 100 µL of Folin phenol reagent was added to the samples. After 30-min incubation at room temperature, absorbance was measured spectrophotometrically at 750 nm.

The total protein content in each sample was calculated based on a standard curve, which was prepared using bovine serum albumine (BSA, 25–100 µg mL^{-1}) and expressed as grams of BSA equivalent per 100 g of dried leaf sample (g BSA/100 g DW).

Determination of amino acids

A HPLC method with OPA (*o*-phthalaldehyde) reagent precolumn derivatization was modified according to Noctor et al. [34] and Perucho et al. [35]. The OPA was prepared freshly (24 h before first use) by dissolving OPA at 5.4 mg mL^{-1} in methanol and adding 200 µL to 1.8 mL 0.5 M sodium borate (pH 9.5) and 40 µL 2-mercaptoethanol. The reagent was filtered into an autosampler vial and used for up to 3 days [34]. For material extraction, HCl (2 mL of 0.1 M, 32%) was used by adding 60 mg PVP. Eighty mg of lyophilized sample was added to the extraction solvent and homogenized. The separation and determination of amino acids was carried out using a gradient HPLC method on the HPLC system Waters 2695, Waters 2475 Multi Fluorescence detector (excitation 340 nm, emission 450 nm wavelength), colon ProntoSIL-AA-FMOC 5.0 µm, 250 × 4.0 mm. Solvent A was sodium acetate (50 mM, pH 4.2) dissolved in water with the addition of tetrahydrofuran (5%, v/v). Solvent B was acetonitrile (100%) and the gradient was 5% of solvent B to 15% of solvent B in 13 minutes, 100% of solvent

B for 10 minutes, and 5% of solvent B for another 10 minutes. The flow rate was 1.58 mL min^{-1}.

Determination of total phenolics

The total amount of phenolic compounds (TPC) was determined using the Folin–Ciocalteu method following the procedure of Ainsworth and Gillespie [36]. The absorbance was measured at 765 nm against a reagent blank (95% methanol) using the Varian Cary UV/VIS spectrophotometer. Gallic acid was used as the reference standard (GAE, 0.025–0.25 mg mL^{-1}). Measurements were done at least four times for each separate sample and in duplicate. The TPC was expressed as mg of gallic acid equivalents per gram of dried mulberry leaves (g GAE/100 g DW).

Determination of phenolics

The methanolic (95%) leaves extracts were analyzed by a gradient HPLC method modified according to Bukovac et al. [37] using Waters Alliance 2695 HPLC System, coupled with a 2996 photodiode array detector (PDA). For the separation of phenolic compounds Phenomenex (Torrance, CA, USA) HPLC column C18 (Synergi Hydro RP 150 × 4.6 mm, 4 μm) with attached Phenomenex security guard column was used. The column temperature was 35°C. The elution solvents were 0.1% H_3PO_4 (solvent A) and 100% methanol (solvent B). Gradient of mobile phases was: 90% to 78% of solvent A for 11 min, 78% to 50% of solvent A for 10 min, 50% to 34% of solvent A for next 9 min, 34% of solvent A for 4 min, and 90% solvent A for another 6 minutes. The flow rate was 1.0 mL min^{-1} and the injection volume was 10 μL. Detection of phenolic compounds was done at 320 nm (CQA derivatives), 309 nm (*p*-CA derivatives), 255 nm (quercetin and kaempferol glycosides). Concentrations of compounds were calculated from chromatogram peak areas on the basis of calibration curves and the results were expressed in g/100 g DW leaves. Each analysis was performed in triplicate.

All phenolic compounds presented in our results were identified by a mass spectrometer (Thermo Finigan, San Jose, CA, USA) with electrospray interface (ESI) operating in negative ion mode. The analyses were carried out using full scan data dependent MS^2 scanning from *m*/*z* 110 to 1,500. Column and chromatographic conditions were identical to those used for the HPLC-DAD analyses. The capillary temperature was 250°C, the sheath gas and auxiliary gas were 60 and 15 units, respectively, the source voltage was 3 kV, and normalized collision energy was between 20% and 35%. Spectral data were elaborated using the Excalibur software (Thermo Scientific). The identification of compounds was confirmed by comparing retention times and their spectra as well as by adding the standard solution to the sample and by fragmentation. Quantification was achieved according to the concentrations of corresponding external standard. For the compounds for which the standards were not available, related compounds were used as standards. Therefore, quercetin-3-glucoside (Q-3-glu) and quercetin malonyl-hexoside (QMH) were quantified in equivalent of quercetin, kaempferol acetyl-hexoside (KAH) in equivalent of kaempferol, CQA derivatives in equivalent of chlorogenic acid and *p*-CA derivatives in equivalent of *p*-coumaric acid.

Statistics

From the leaf measurements, the mean value (N = 20–30) of each morphological parameter was calculated for each tree, which was used as input data for analyses. Assumptions of normal distribution of measured trait data were checked with the Kolmogorov–Smirnov test. Normality for trait leaf area was obtained after logarithmic transformation of measured values. Additionally, linear regression was applied to explain the percentage of variance between the leaf parameters. The analysis confirmed two morphologically distinct groups (Fig. S1) which were further separately analyzed.

In order to enable a comprehensive assessment of the effects of leaf morphology and to closer define the morphotypes, we conducted a principal component analysis using

the PAST 3.17 [38] software (Fig. 2, Tab. S1). To prevent dominance in the PCA by large values at the expense of small ones, data were z-standardized using the formula: $z = (x - \mu)\sigma^{-1}$.

Furthermore, the analysis of variance was used to verify the differences between the effect of the pruning management (categories from 1 to 3) and morphotype (Fig. 3). Significant differences ($p < 0.05$) between mean values of each parameter were determined using the post hoc Duncan test. The effect of pruning management on the morphotype was further evaluated by two-way analysis of variance (ANOVA) (Tab. S2).

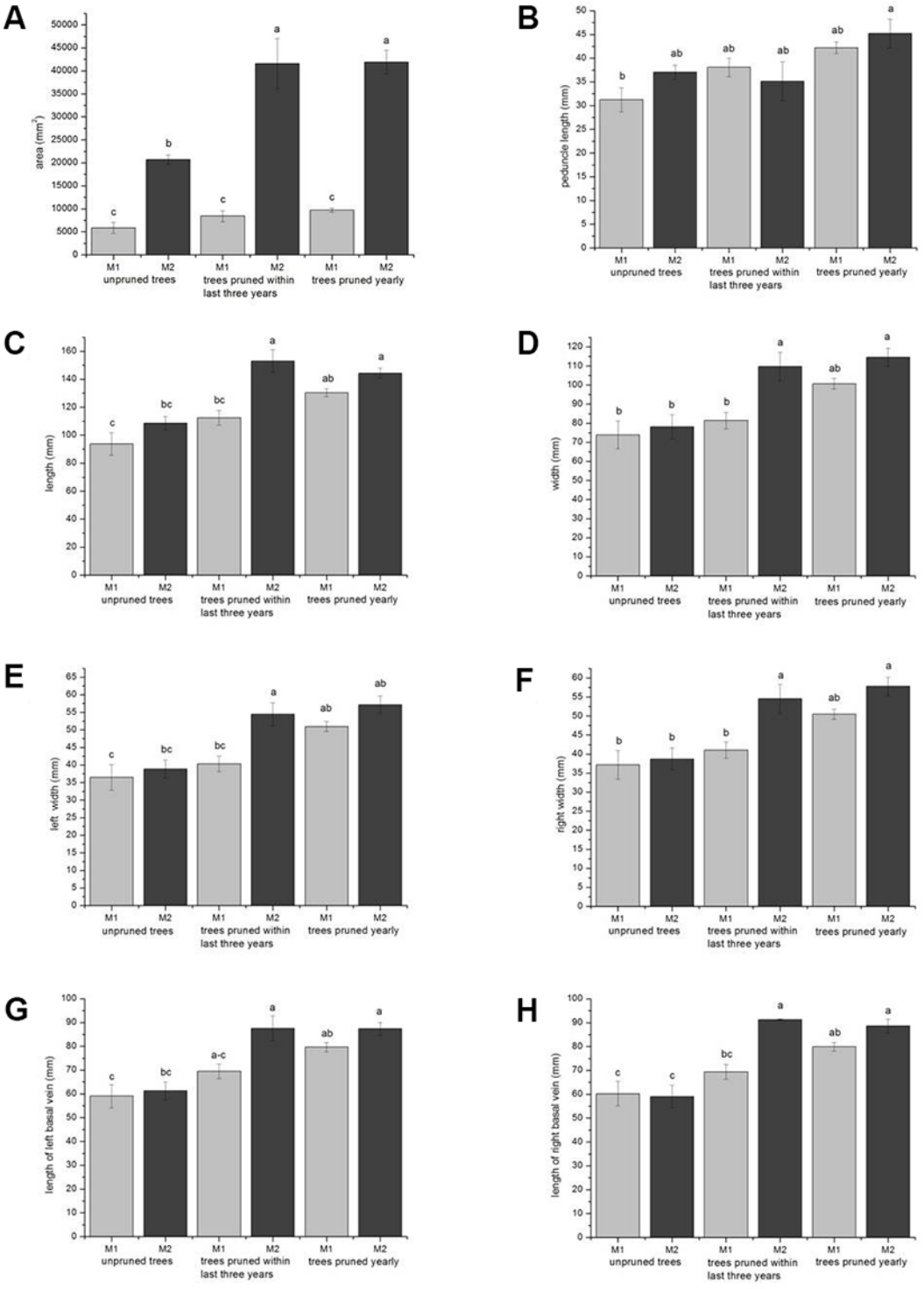

Fig. 3 The effect of pruning management on each studied morphometric trait within both morphotypes (M1, M2). Each value represents mean ±*SE* of each leaf parameter within each pruning management/morphotype category. Different letters (a–c) indicate significant differences ($p < 0.05$), which were determined using the post hoc Duncan test.

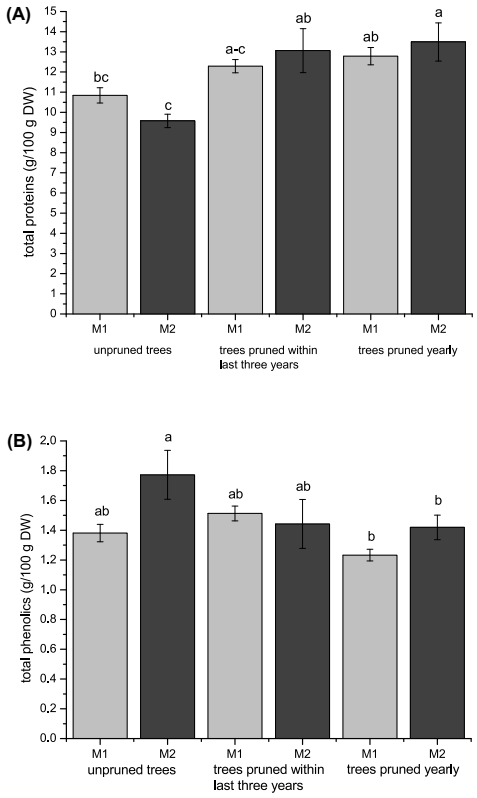

Fig. 4 The effect of pruning management on the concentration of the (**A**) total proteins (g/100 g DW) and (**B**) total phenolics (g/100 g DW) within both morphotypes (M1, M2). Each value represents mean ±*SE* of each leaf parameter within each pruning management/morphotype category. Different letters (a–c) indicate significant differences ($p < 0.05$), which were determined using the post hoc Duncan test.

Assumptions of normality for biochemical traits (total proteins, single free amino acids, TPC, single phenolics) were checked with Kolmogorov–Smirnov test. The normality for the free amino acids was obtained after logarithmic transformation of the measured values.

The biochemical traits of mulberry leaves within both morphotypes with respect to pruning management are represented by means and standard errors, and were statistically evaluated by one-way analysis of variance (ANOVA). Significant differences between mean values were determined using the post hoc Duncan test. Significant differences ($p < 0.05$) are indicated by different letters (Fig. 4).

In order to analyze correlations between the measured biochemical parameters and the morphometric traits, Pearson correlation coefficient was applied (Tab. S3). The morphometric index (MI) was generated, which per se is a transformation of values of PCA residuals from Axis 1. We think the MI index is a strong conglomerate of all morphometric traits and is therefore crucial for further statistical analysis of all the other biochemical traits in this paper. The index itself has an explanatory variance rate of 86% for the measured morphological traits. In the Tab. S3, asterisks denote that the correlation is significant at the 0.05 level (*) and at the 0.01 level (**; 2-tailed), respectively [39].

In the final step, the morphometric traits as well as biochemical parameters were included as factors in multivariate statistical analysis using hierarchical cluster analysis based on Euclidian distance and Ward's methods, in order to interpret the similarities between the metabolites with respect to morphotype and pruning and to closer define distinct chemotypes.

IBM SPSS Statistics 21 (New York, USA, 2012), StatSoft, Inc. STATISTICA 8.0 (Victoria, Australia, 2007), and PAST 3.17 software were used for statistical analysis.

Results

Morphometrical analyses of mulberry leaves

The results of the morphometrical analysis show that all studied parameters have a significant impact on the variability of *M. alba* leaves. Linear regression of morphometrical traits allowed us to characterize two separate morphotype groups, characterized by different regression coefficient, whereas the slope between both regression lines is equal (0.008). Both morphotype groups are characterized by high *R*-squared values (Fig. S1).

In order to enable a comprehensive assessment of leaf traits and to closer define the morphotypes, we conducted a principal component analysis. Principal Component 1 (PC1) accounts for 85.90% variance explained, where all the parameters contribute more or less equally to the overall grouping (distribution). The third PC accounts for 3.25% variance and is associated mostly with leaf area, peduncle length, and leaf length. Thus, PC3 merges all genotypes with small leaves and separates them from those which have larger leaf dimensions (Fig. 2, Tab. S1). The PCA analysis clearly separates both morphotype groups. Morphotype 1 (M1) comprises a group of mulberry trees that are located in Goriška Brda, Šempeter–Vrtojba, Miren–Kostanjevica, Nova Gorica, and also include certain mulberry trees from the Ajdovščina municipality. Morphotype 2 (M2) was characterized by 11 samples, three originating from Nova Gorica (NG1, NG2, NG4; Tab. 1) and the others from Ajdovščina municipality (A31, A37, A39, A40–43, A45, A47, A51; Tab. 1, Fig. 2). One is able to see a separation of the genotypes within both morphologically distinct groups according to the pruning frequency. Most frequently pruned trees formed leaves with larger dimensions. But this separation is not as clear as the separation between both morphotypes. Analysis of variance was used to identify whether pruning has any impact on the studied parameters within both morphotypes. Morphotype 2 formed significantly larger leaves with respect to all measured traits

when trees were pruned yearly. For M1, the significant effect of pruning management was confirmed for leaf length, left width, and left and right basal vein. However, there was no statistically significant difference in any of the measured parameters between yearly pruned trees and those which were pruned within 3 years (Fig. 3).

Total proteins and single amino acids

Significantly higher levels of total proteins were determined in pruned trees belonging to M2 when compared to unpruned trees. The highest mean protein value (20.2 g/100 g DW) was determined in leaves of genotype A40 (Tab. 2). For M1, the effect of pruning on the content of total proteins was not significant (Fig. 4A).

We measured the concentration of 15 different free amino acids in the mulberry leaves. Their composition varied between the observed genotypes. Threonine, asparagine, and arginine were proven to be the most predominant, followed by glutamine, serine, glycine, lysine, methionine, and alanine. We could further detect valine, leucine, isoleucine, phenylalanine, tyrosine, and histidine in lower concentrations (Fig. 5A).

The concentration of threonine in mulberry leaves showed a rather high genotype-dependent variability reaching levels up to 865.0 mg/100 g DW in genotype A23 (Tab. 2) and representing up to 80% of free amino acid pool. Unpruned trees of M1 had significantly lower threonine concentrations than pruned individuals (Fig. 5A).

Asparagine reached concentrations up to 481.4 mg/100 g DW in genotype A37 (Tab. 2), which in this genotype represented 67% of the total free amino acid pool. Yearly pruned trees of M2 showed significantly higher concentrations of asparagine when compared to other categories. Within the yearly pruned and unpruned category, M2 was characterized by significantly higher asparagine content in comparison to M1 (Fig. 5A).

The leaf-content of arginine reached levels up to 633.8 mg/100 g DW in genotype A44 (Tab. 2), which resulted in up to 32% of the total free amino acid concentration. Trees that were pruned yearly showed a significant increase in arginine independent of the morphotype. Unpruned trees of M1 revealed the lowest concentrations of arginine (Fig. 5A).

Furthermore, trees that were pruned yearly had significantly higher glutamine, serine, and alanine contents when compared to unpruned trees. Glycine and lysine were found to be significantly lower in unpruned trees of M1 when compared to other pruning categories of M1. The concentrations of methionine did not significantly differ between pruning management and morphotypes. For other amino acids, which were found in lower concentrations, a similar trend towards increased concentrations in pruned trees was detected (Fig. 5A). The effect of pruning and morphotypes was also tested with a two-way analysis of variance. Pruning significantly affected the amino acids asparagine ($p = 0.00$), serine ($p = 0.03$), and alanine ($p = 0.03$), whilst no morphotype-specific influence on the composition of amino acids was found (Tab. S2).

Phenolics

The TPC in leaves within the sampled genotypes ranged from 0.7 to 2.0 g/100 g DW (Fig. 4B). The highest TPC concentration (2 g/100 g DW) was measured in B9 genotype, sampled in the Goriška Brda municipality (Tab. 2). Unpruned trees of M2 had significantly higher amount of TPC when compared to yearly pruned trees of the same morphotype. Similar trend, although insignificant, was also observed for M1 (Fig. 4B).

Twenty-six phenolic compounds were detected in mulberry leaves. These compounds were identified using HPLC-MS, by comparison of their retention times and PDA spectra with standards and based on mass fragmentation pattern from the literature (Tab. 3). Within the sampled mulberry genotypes, the main phenolics from the hydroxycinnamic group were CQA derivatives (with chlorogenic acid predominating) and *p*-CA derivatives. The main flavonoids were quercetin glycosides and kaempferol glycosides. The predominant quercetin glycosides were QMH, followed by rutin and Q-3-glu, whereas the predominant kaempferol glycoside was KAH. The quantitative proportion of single phenolics varied between morphotypes and pruning categories (Fig. 5B).

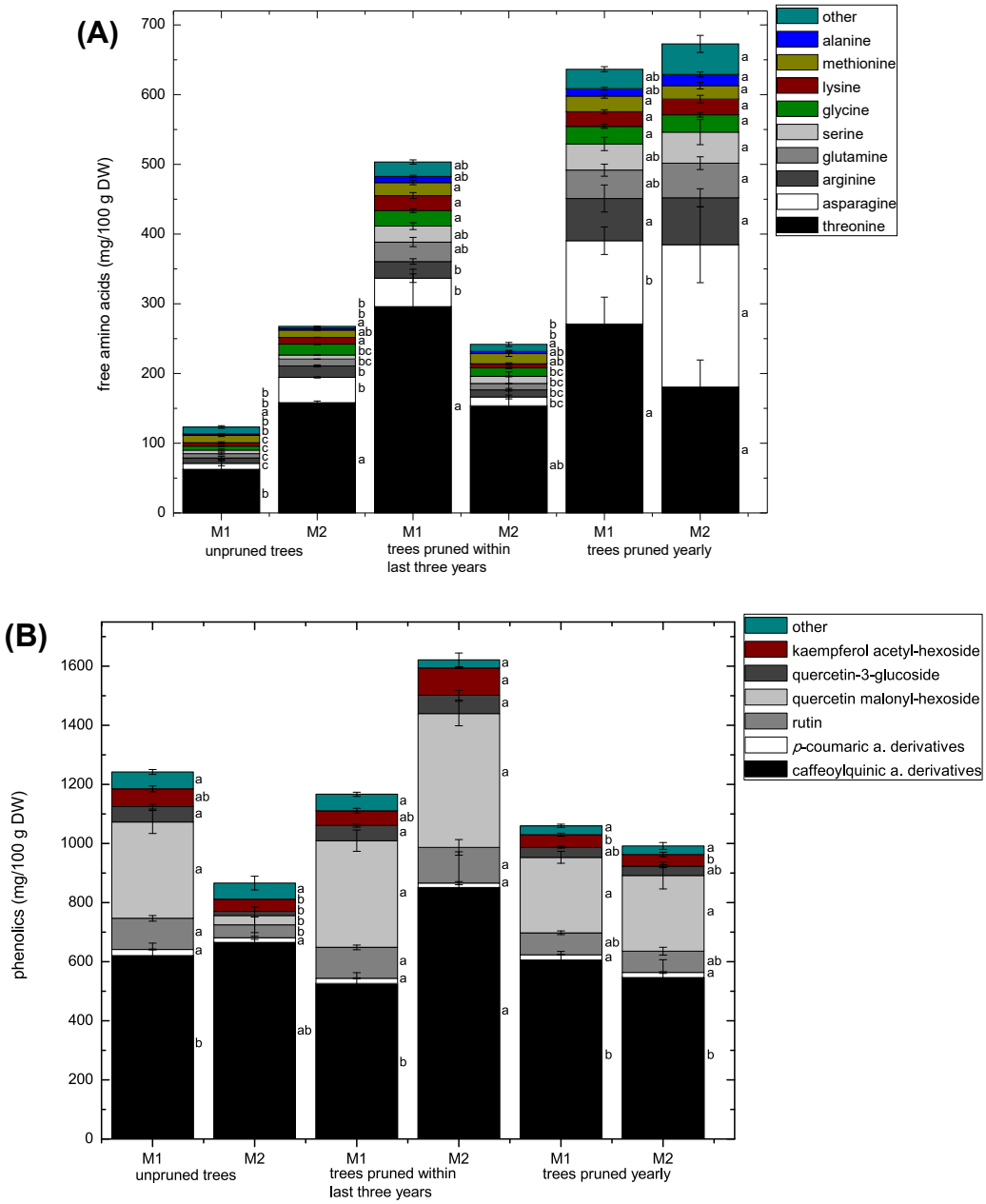

Fig. 5 Content levels of predominant (**A**) amino acids and (**B**) phenolics within both morphotypes (M1, M2) with respect to pruning management. Each value represents mean ±*SE* of each component within each pruning management/morphotype category. Different letters (a–c) indicate significant differences ($p < 0.05$), which were determined using the post hoc Duncan test.

The average content of CQA derivatives ranged between 356.8 and 1,175.2 mg/100 g DW. Genotype with the significantly highest average concentration (>83% of total) was A25 (Tab. 2). Yearly pruned trees of M2 were characterized by significantly lower contents of CQA derivatives (Fig. 6B). The content of *p*-CA derivatives reached levels up to 44.9 mg/100 g DW in A25 genotype (Tab. 2). Morphotype and pruning management did not have significant effect on the *p*-CA derivatives contents (Fig. 5B).

Rutin reached the highest mean concentration (186.1 mg/100 g DW) in genotype A16 (Tab. 2). Unpruned trees of M1 were characterized by significantly higher concentration of rutin in comparison to unpruned trees of M2 (Fig. 5B). Other quercetin derivatives and KAH were found to be the highest in those trees of M2 which were pruned within 3 years and lowest in unpruned trees (Fig. 5B). The highest concentration of QMH was determined in genotype A1, Q-3-glu reached maximum values in A40, whereas KAH was the highest in B9 (Tab. 2).

Tab. 2 List of predominant metabolites with their mean values (mg/100 g) ±*SD*, minimum and maximum values of superior genotypes, and variability coefficient (*SD*/mean × 100).

Trait	Mean	*SD*	Min	Max	Max sample ID	*VC* (%)
Total proteins	12,366.74	2,286.90	7,581.11	20,232.49	A40	18.49
Threonine	188.14	123.60	42.98	865.02	A38	65.70
Asparagine	80.13	46.74	28.97	481.43	A23	58.33
Arginine	34.23	11.68	14.44	633.80	A44	34.11
Glutamine	38.90	16.76	15.09	223.76	A37	43.08
Serine	29.36	13.68	10.73	294.09	A44	46.59
Glycine	17.65	8.78	3.09	63.04	B2	49.72
Lysine	15.48	9.10	3.45	63.34	B6	58.77
Methionine	16.75	6.33	6.66	89.21	A44	37.76
Alanine	9.34	4.31	2.43	63.50	A24	46.19
Total phenolics	13,489.45	2,629.94	7,445.63	20,077.91	B9	19.50
Caffeoylquinic acid derivatives	590.42	174.44	237.96	1175.21	A25	29.55
p-Coumaric acid derivatives	17.68	7.11	2.92	44.91	A25	40.23
Rutin	89.80	39.95	13.83	186.05	A16	44.49
Quercetin malonyl-hexoside	299.54	150.19	11.67	755.00	B9	50.14
Quercetin-3-glucoside	42.53	23.99	5.40	143.64	A1	56.40
Kaempferol acetyl-hexoside	52.08	32.41	1.87	145.91	A40	62.22

The effect of pruning and morphotype on the biochemical traits was additionally tested with a two-way analysis of variance. Pruning significantly affected TPC (p = 0.01), rutin (p = 0.04), QMH (p = 0.02), and Q-3-glu (p = 0.04) contents. The combined effect of pruning and morphotype is reflected in significantly different levels of CQA derivatives (Tab. S2).

The chemotype analysis

In order to analyze correlative relationship between the measured biochemical parameters and the morphometric traits, the MI was generated by summarizing the PCA residuals from axis PC1. A significant positive correlation was found between MI 1 and the content of total proteins and all amino acids accept for methionine. Morphotype 2 showed a medium positive correlation between MI 2 and the content of arginine (0.40), glutamine (0.40), and alanine (0.44), whereas a medium negative correlation was found with threonine (−0.48), although the correlations were not significant (Tab. S3).

For phenolics, a significant negative correlation was determined between MI 1 and TPC (* −0.30), rutin (** −0.44), and Q-3-glu (** −0.35). For M2 a positive correlation was found between MI 2 and TPC (0.38) and *p*-CA (0.30), whereas a negative correlation was found between MI 2 and the content of CQA (−0.31) and KAH (−0.39) (Tab. S3).

For M1, the correlations among all amino acids were found to be significant. Morphotype 2 was characterized by a significant negative correlation between total proteins and serine (* −0.58), whereas significant positive correlations were found among certain main amino acids. To point out, for M1 we were able to establish a strong negative correlation between rutin and total proteins (** −0.40), threonine (* −0.24), asparagine (** −0.50), arginine (** −0.44), glutamine (** −0.33), serine (** −0.37), and glycine (* −0.31), whereas for M2, a significant negative correlation was found only between rutin and asparagine (* −0.62). Morphotype 1 was further characterized by a negative correlation between total proteins and TPC (* −0.25), CQA (* −0.25), Q-3-glu (** −0.33). Morphotype 2 showed a significant positive correlation between threonine and CQA (** 0.69), whereas a strong negative correlation was determined between threonine and TPC (** −0.87) and *p*-CA (** −0.71).

Tab. 3 Identification of phenolic compounds in mulberry leaves in negative ion mode by HPLC-MS.

Phenolic compound	Peak No.	RT	[M–H] – (*m*/*z*)	MS^2 (*m*/*z*)
p-Coumaric acid hexoside 1	1	12.00	325	163, 119
3-Caffeoylquinic acid	2	12.00	353	191,179,135
p-Coumaric acid hexoside 2	3	14.03	325	163, 119
3-*p*-Coumaroylquinic acid	4	15.12	337	163
trans-5-Caffeoylquinic acid (chlorogenic acid)	5	15.51	353	191, 179
4-Caffeoylquinic acid	6	16.14	353	173, 179, 191
cis-5-Caffeoylquinic acid	7	17.44	353	191, 179, 191
p-Coumaric acid hexoside 3	8	17.44	325	163
Epicatechin	9	17.44	289	245
trans-5-*p*-Coumaroylquinic acid	10	19.46	337	191, 163, 173
Quercetin dirhamnosyl-hexoside	11	20.11	755	609, 301
Kaempferol dirhamnosyl-hexoside	12	21.75	739	593, 285
Quercetin rhamnosyl-hexoside	13	21.86	609	301
Quercetin-3-rutinoside	14	22.87	609	301
Kaempferol rhamnosyl-hexoside 1	15	23.42	593	447, 285
Quercetin-3-galactoside	16	23.42	463	301
Quercetin-3-glucoside	17	23.85	463	301
Quercetin acetyl-rhamnosyl-hexoside	18	24.12	651	463, 301
Kaempferol rhamnosyl-hexoside 2	19	24.52	593	447, 285
Quercetin-3-xyloside	20	24.52	433	301
Kaempferol acetyl-rhamnosyl-hexoside	21	25.46	635	447, 285
Kaempferol hexoside	22	25.46	447	285
Quercetin malonyl-hexoside	23	25.86	549	463, 301
Quercetin acetyl-hexoside	24	27.17	505	301
Kaempferol acetyl-hexoside 1	25	27.74	489	285
Kaempferol acetyl-hexoside 2	26	29.47	489	285

Hierarchical cluster analysis was applied, in order to examine certain chemically distinct groups. The dendrogram (Fig. 6) shows two distinct branches. In general, the chemotype group cA is composed by yearly pruned genotypes characterized by larger leaves, which are superior in total proteins and most of amino acids. All genotypes of M2 were pooled in the chemotype group cA, except the unpruned genotype NG1. The chemotype cB is characterized by small-leaved genotypes of M1 superior in TPC and flavonoids. Both clusters are further subclustered in chemically distinct groups with a certain gradient in single amino acids and phenolics. Cluster cA1 is characterized by genotypes with longest leaves, which are highest in the concentration of total proteins and superior in the amount of CQA derivatives and KAH. Subcluster cA2 is characterized by superior content of amino acids asparagine, glutamine, serine, and glycine, whereas the concentration of phenolics is the lowest among all subclusters. Subcluster cA3 represents samples with widest leaves, which are superior in arginine and alanine, as well as high in other amino acids. The contents of TPC, quercetin, and kaempferol glycosides are rather low.

The cB cluster clearly separates group cB1 which comprises trees with medium leaf size, lowest amount of amino acids, and also rather low amounts of TPC, quercetin, and kaempferol glycosides. The cB2 chemotype is represented by unpruned genotypes with smallest leaves, which are the lowest in total protein content, but superior in rutin and Q-3-glu concentration. Chemotype cB3 was found to be superior in threonine, lysine, and methionine contents as well as the highest in the content of TPC and QMH. Chemotype group cB4 is represented by samples being most superior in *p*-CA

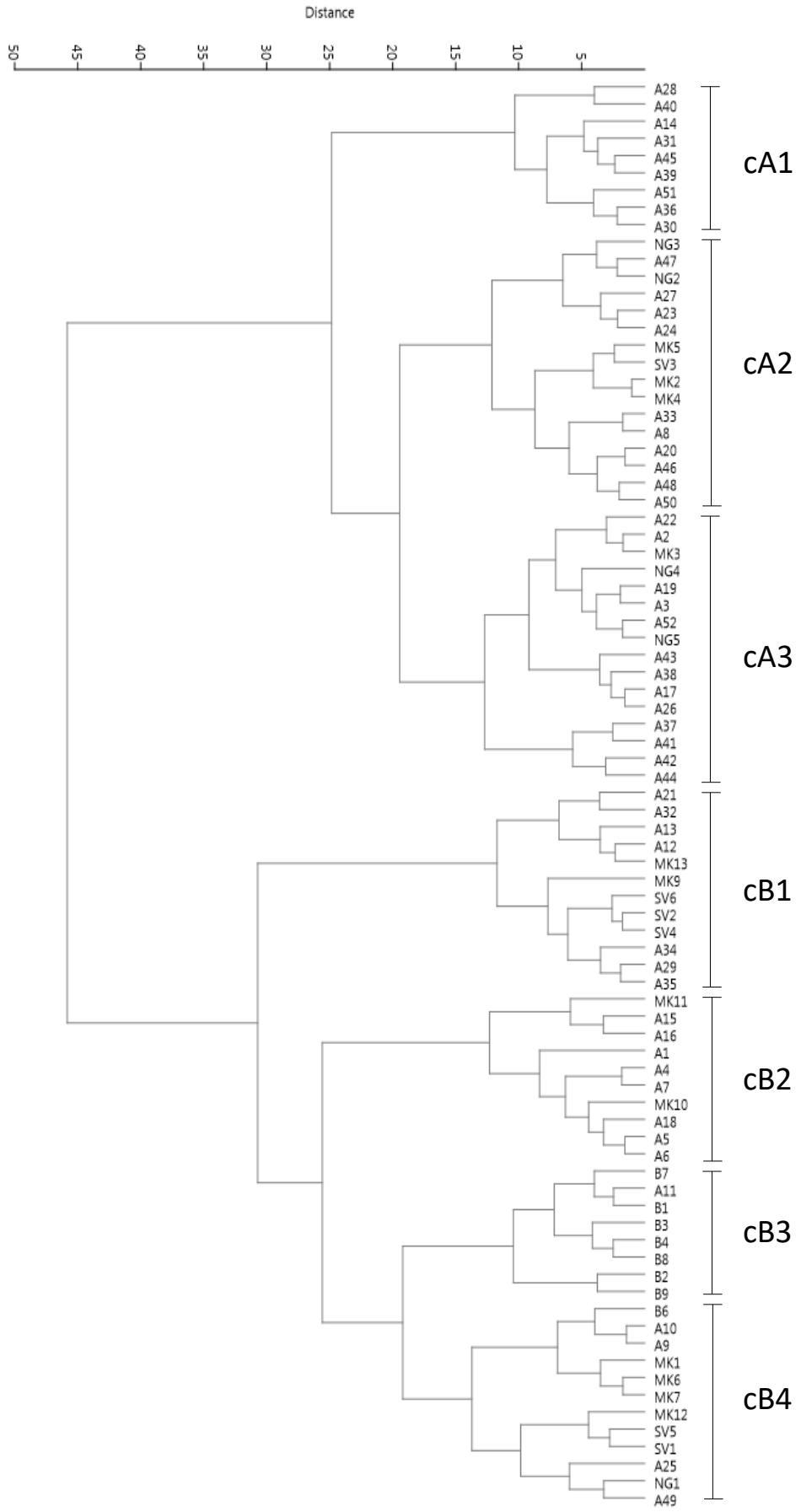

Fig. 6 Dendrogram combining the analyzed morphometrical and biochemical traits of studied local mulberry genotypes using hierarchical clustering method based on Euclidian distance.

derivatives. To point out, the total protein content of cB4 group was found to be the highest among the chemotype cB subclusters.

Discussion

Morphometrical analyses of mulberry leaves

The results of the morphometrical analysis show that it is possible to clearly identify two morphometrically distinct groups of white mulberry (*Morus alba* L.) trees within Goriška region. The statistical analysis shows that leaf area is the strongest differentiating trait between both morphotypes. The M2 has longer, bigger, and more elliptical leaves, whereas M1 has more broader leaves. A similar morphometrical study was conducted by Peris et al. [40] allowing the authors to distinguish between five mulberry accessions using 12 phenotypic traits. We suggest that the distinct groups may be caused by their different origin. Each morphotype may represent more or less closely related seedlings originating in different mulberry tree nurseries, which had been established during the Austro-Hungarian sericulture promotion attempts. There is little knowledge about the historical sources of seeds for the big-scale cultivation of white mulberry trees. Selective use and vegetative propagation by grafting of specified clones with better feeding characteristics is described for the region in the literature of the mid-nineteenth century [2,3]. In general, it is obvious that the origin and genetic relationship of mulberry trees is mainly affected by the time of planting and the historical use of different resources of planting material during the period of Gorizian sericulture.

Pruning is a cultural practice which is known to significantly alter growth and leaf parameters of mulberry trees [41,42]. Pruning recordings revealed that pruning practice of mulberries is still traditionally used in Goriška region [4,5]. Based on statistical evaluation, the influence of pruning on the morphotypically distinct groups is confirmed. However, the effect of pruning is more obvious for morphometric traits of M2. Frequent pruning is known to result in larger leaf dimensions and high productivity per unit area. On the other hand, it is known that trees which are left unpruned year after year does not make enough growth and bear leaves of small size and inferior quality [41,42]. A major factor determining productivity and hence the profitability in sericulture is the yield of mulberry leaf crop. Maximizing of mulberry leaf yield per unit area as a result of pruning allows to achieve the two most important economic aims of sericulture, namely increased cocoon production per hectare and reduced cost of production [43].

Total proteins and single amino acids

Mulberry leaves are characterized by a high protein and amino acid content when compared to main vegetables and leaf protein content is a major determinant of nutrient quality for silk worm larvae [8,44]. By using Lowry's method, other studies reported mean protein concentrations in leaves of different mulberry varieties varying from 9 to 30 g/100 g DW [33,45,46], which coincides with mean protein values of mulberry leaves analyzed in our experiment, where maximum total protein values reached levels of 20.2 g/100 g DW. The protein values are in accordance with Kumar et al. [33], who screened the total protein contents using Lowry's method after protein precipitation in tender, medium and coarse leaves of different white mulberry varieties and found the protein values ranged from 17.6 to 20.9 g/100 g DW in medium leaves.

It was previously confirmed that higher protein content in certain mulberry varieties had a direct bearing on larval growth, particularly on silk gland development and cocoon characters of silkworm [33]. Silkworms obtain 72–86% of their amino acids from mulberry leaves and more than 60% of the absorbed amino acids are used for silk production. It has been experimentally determined that silkworms require 18 amino acids for their adequate nutrition. The amino acids essential for silkworm larvae development are arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine [47]. Most of them are transformed to two main components of raw silk: fibroin and sericin [48,49]. Al-kirschi et al. [17] found that

the most represented amino acids in mulberry leaves are glutamic and aspartic acids followed by leucine, with concentrations of 3.3, 3.1, and 2.6 g/100 g DW, respectively. Our research was based on the estimation of free amino acid pool. We found threonine, asparagine, and arginine as the most prevalent. Threonine was recently found to be important for the expression of antibacterial activity in silkworms [50]. In the presented study, arginine and asparagine achieved the highest mean concentration in samples from Ajdovščina. Nutritional tests on silkworms showed that arginine has important role in growth and development of silk worms and the deficiency of asparagine leads to retardation of larval weight [44,51].

Pruning and morphotype had different effects on studied total proteins and amino acids. Total protein levels were significantly lower in M2 when trees were left unpruned. This was also confirmed by hierarchical analysis which clearly separates two chemically distinct clusters. Unpruned trees of M2 were classified in cluster cB, which was characterized by low total protein contents, the minimum amount of amino acids was determined in subcluster cB1. Cluster cA was characterized by higher total protein and amino acid concentrations. All genotypes of M2, except of genotype NG1, were pooled in chemotype cA. The subcluster cA2 was found to be superior in amino acids asparagine, glutamine, serine, and glycine and lowest in phenolics.

Some early investigations were focused on the effect of pruning management on the content of proteins and nutritional value of mulberry leaves. Krishnaswami et al. [52] found that crude protein, moisture, and minerals were higher in leaves of plants which received pruning. Chaluvachari and Bongale [53] recorded maximum protein content in leaves at 45 days after pruning; later on, the protein content was gradually decreasing.

The results of our study show that trees pruned every year have significantly higher asparagine, glutamine, serine, and arginine content when compared to unpruned trees, regardless of morphotype. It was previously confirmed by different authors that pruning significantly affects the levels of above-mentioned amino acids [54,55]. Yamashita [55] stated that the limitation of sugar supply after pruning and during the regeneration of buds enhances accumulation of arginine and asparagine.

Phenolics

Phenolic compounds have been pointed out as the main phytochemical compounds of mulberry leaves with confirmed biological properties [8–16,56,57]. In our study, the amount of TPC in leaves of studied mulberry genotypes ranged from 0.7 to 2.0 g/100 g DW. The maximum TPC values were documented in genotypes, originating from the Goriška Brda municipality, which were pooled in the cB3 chemotype cluster. The results are in accordance with those of Sánchez-Salcedo et al. [58], who reported TPC in range between 1.3 and 1.6 g GAE/100 g DW.

Plant phenolics are important antioxidants and are mainly involved in the defence mechanisms of plants against pathogens and insects [59,60]. Many flavonoids and isoflavonoids can serve as antinutrients to phytophagous insects. They reduce the nutrient value of plant tissues by binding amino acids and proteins and they can even reduce digestion of insects by binding to digestive gut enzymes [25]. It was confirmed by different authors that the phenolic content in leaves of different mulberry cultivars affects the growth of silkworm larvae. Certain phenolic compounds have been found to be transferred from the larval diet into the hemolymph and cocoons [60–62].

Among phenolics, the highest antioxidative activity is exhibited by phenolic acids and flavonoids [8–16]. In our study, the main phenolic acids were CQA and *p*-CA derivatives. Among CQA derivatives, which in average ranged between 526 and 851 mg/100 g, the predominant compound was chlorogenic acid, which represents up to 85% of phenolic acids. The results are in accordance with Sanchez-Salcedo et al. [59] who found chlorogenic acid in concentrations ranging between 530 and 720 mg/100 g DW. Similarly, Lee and Choi [26] reported chlorogenic acid values ranging from 328 to 965 mg/100 g DW, depending on the variety and harvesting time. Flaczyk et al. [12] reported much higher concentration in a Polish variety *Morus alba* L. var. *wielkolista zolwinska* (2,330 mg/100 g DW). In our analyzed genotypes, the highest CQA derivatives levels reached the concentration of 1,175 mg/100 g DW in genotype A25.

We were able to define 16 flavonoids, all of which were kaempferol and quercetin glycosides. The flavonoid profile is in accordance with that of Ju et al. [21], who performed the qualitative and quantitative analysis of flavonoids from 12 Korean mulberry varieties via UPLC-DAD-QTOF/MS. Among flavonoids, QMH was detected in the highest content in mulberry leaves. Previously, other authors [19–22] identified QMH as quercetin 3-(6-*O*-malonyl) glucoside (QMG). In the analyzed morphotypes, the mean concentration range of QMH was between 250 to 450 mg/100 g depending on pruning frequency. Our results are in accordance with other authors. Ju et al. [21] analyzed QMG in leaves of different varieties in range between 255 to 360 mg/100 g. Lee and Choi [26] analyzed six different varieties at three different harvest periods between May and September and found QMG at highest concentration in May ranging between 237 and 530 mg/100 g, while the lowest were identified in September, ranging between 110 and 450 mg/100 g. Furthermore, Choi et al. [23] analyzed 'Cheongil', as widely used mulberry cultivar for mulberry leaf tea, according to three different heat pretreatments and found QMG at lower values of 143.25 mg/100 g. The QMG was known as the main bioactive substance with antidiabetic and antiarteriosclerotic activity. Genotype B9, which was found as superior in QMH amount (755 mg/100 g) can be further propagated for pharmaceutical proposes.

The second most predominant flavonoid was rutin, reported also by other authors [19,21,24,25]. In our samples, the average rutin concentrations ranged between 44 and 121 mg/100 g DW depending on the morphotype and pruning frequency. The maximum rutin concentration of 189 mg/g DW was found in genotype A16. The contents are in accordance with several authors [11,12,25], who found rutin concentrations of approximately 190 mg/100 g DW. Lee and Choi [26] found rutin in range between 20 and 250 mg/100 g, depending on variety and sampling period. In contrast, Ju et al. [21] found rutin in range between 90 and 425 mg/100 g. Katsube et al. [19] determined rutin in concentration of 573 mg/100 g. Rutin has been reported to have cytoprotective effects against reactive oxygen species, anti-inflammatory and antioxidant properties within human cells. Furthermore, it is known for decreasing plasma glucose, increasing insulin levels, restoring the glycogen content and hexokinase activity [15,16]. Based on the results of Hunyadi et al. [63], chlorogenic acid and rutin might account for as much as half the observed antidiabetic activity of mulberry leaves, hence they can be considered as excellent markers for the quality control of mulberry products. These compounds, found at high concentration in certain individuals of chemotype cA1 and cB2, might significantly contribute to the overall antidiabetic action and can be further propagated for pharmaceutical purposes.

The third predominant quercetin glycoside was Q-3-glu (isoquercetin), which in our morphotypes ranged in average between 14 and 62 mg/100 g DW, with maximum value of 144 mg/100 g in genotype A1. Lee and Choi [26] found Q-3-glu in range between 27 and 110 mg/100 g, which is in accordance with our results. Similarly, Ju et al. [21] found Q-3-glu ranging between 75 and 297 mg/100 g DW.

In our genotypes, the seven identified kaempferol glycosides (kaempferol dirhamnosyl-hexoside, kaempferol rhamnosyl-hexoside 1 and 2, kaempferol acetyl-rhamnosyl-hexoside, kaempferol hexoside, kaempferol acetyl-hexoside 1 and 2) were previously described by Ju et al. [21]. Among them, we found KMH as predominant kaempferol glycoside. In earlier studies, authors were able to identify astragalin [19,24,26], kaempferol 7-*O*-glucoside [25], kaempferol-3,7-glucopyranoside [24], kaempferol-3-*O*-α-L-rhamnopyranosyl(1–6)-β-D-glucopyranoside [24], kaempferol-3-*O*-glucopyranosyl-(1,6)β-D-glucopyranoside [25], and kaempferol-3*O*-(6-*O*-malonyl)-β-D-glucoside [25,26].

We confirmed that pruning has a significant effect on TPC. Unpruned trees of M2 were characterized by significantly higher amount of TPC when compared to yearly-pruned trees. A significant effect of pruning on leaf phenolic contents was previously confirmed by Thangamalar [31]. It is also well known for cultivated bush tea (*Anthrixia phylicoides*) that the phenolics remain higher in unpruned tea plants [64]. Furthermore, unpruned trees of M2 and those pruned within 3 years were found to be significantly higher in the concentrations of CQA derivatives. Previously, Sugiyama et al. [65] investigated the effect of pruning on the amounts of phenolic components; namely, chlorogenic acid content was found to be higher in the twice-harvested mulberries in comparison

to the once-harvested group. In contrast, the authors found no pruning-specific effect on the concentration of TPC.

In our study, we were able to statistically define chemotype cB characterized by superior amounts of main flavonoids. Rutin content reached the highest mean concentration in genotype A16, which refers to the chemotype subcluster cB2. Within the same chemotype group, genotype A1 was found to be superior in QMH. Within chemotype group cB3, we found the genotype B9 superior in the concentrations of TPC and KAH. To point out the chemically distinct group cA1, which is characterized by the longest leaves, the genotype with significantly highest concentration of total proteins and KAH was found to be A40. The above discussed genotypes appear to be promising and will be further explored in terms of their active constituents.

It is known that genetics, environment, and agricultural practices greatly affect the phytochemical profiles of plants. However, there is limited evidence of the impact of these factors on the biochemical compounds of mulberry leaves. Such information would help growers to select cultivars to produce mulberry leaves and related products with the greatest levels of bioactive compounds. The multivariate analysis helped us to define those high-yielding genotypes with a superior metabolite composition, with high concentration of main amino acids and low of phenolics which can be further propagated as highly recommendable feed for silkworm. Those varieties with high concentration of certain phenolics can be further propagated as useful for pharmaceutical purposes.

Conclusion

We can conclude that the studied morphometrical traits distinguish the white mulberry into different morphological groups, which will be of great use in further selection of valuable genotypes within the local historical gene pool. A broader genetic analysis will provide additional insights into the variability and diversity of the studied genotypes, and it is expected to confirm the results of our morphometric study.

Pruning, which is traditionally used in Goriška region, has significant effect on the morphometrical and certain biochemical parameters. From the perspective of sericulture, the results of our study clearly confirm that pruning measures not only maximize leaf yield, but also contribute to nutritional quality of leaves. Morphotype 2 was found as promising in moricultural use. Multivariate analysis allowed us to further define distinct chemotypes which were evaluated from the perspective of superior composition of certain single amino acids and phenolics. The obtained results would help us to select and propagate those superior local varieties with high concentration of the main amino acids and specific composition of phenolics and recommend them for silkworm rearing or even for pharmaceutical purposes.

Acknowledgments

The authors thank Silvia Cappellozza from the Sericulture Section of Padova (Livestock Experimental Institute of the Ministry for Agriculture and Forestry Policies, Padova, Italy) for effective collaboration during the project's research as well as valuable suggestions and support during the experimental work. The authors also thank Anja Ivanus, Veronika Cresnar, and Julija Korez for assistance in morphometric measurements, as well as Katja Urbanek and Bernard Brecko for maintaining the gene bank collection of vegetatively propagated field samples.

Supplementary material

The following supplementary material for this article is available at http://pbsociety.org.pl/journals/index.php/asbp/rt/suppFiles/asbp.3614/0:

Fig. S1 The linear regression between the logarithm values of leaf area and leaf length.

Tab. S1 Standardized scores for the first three PCA axis.

Tab. S2 Two-way ANOVA test results for the effect of pruning and two morphological distinct groups on different biochemical traits.

Tab. S3 Pearson correlation coefficient between morphometric index (MI) and measured biochemical parameters of M1 and M2.

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