

DE GRUYTER OPEN

Variations in leaf total protein, phenolic and thiol contents amongst old varieties of mulberry from the Gorizia region

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ABSTRACT

Preserving the plant genetic resources of genus *Morus* is insufficient but undoubtedly vital for conservation of the world's germplasm for our successors. This research was focused on old mulberry varieties from the Gorizia region in Slovenia which were assessed for their contents on crucial metabolites (proteins, phenolics and thiols) in leaves regarding their antioxidant and nutraceutical potentials. Total proteins were measured spectrophotometrically by following the procedure of Bradford, the total phenolic contents were determined using the Folin-Ciocalteu method and thiols were established with monobromobimane fluorescent dye. The presented metabolite screening showed that some of the evaluated genotypes had higher concentrations of glutathione and were superior in contents of proteins and phenolics when compared to the results of other authors and could be propagated as highly recommendable feed for silkworms, and other animals.

Key words: feeds, glutathione, *Morus*, phenols, proteins

INTRODUCTION

Preserving the plant genetic resources of *Morus* genus is still insufficient but undoubtedly vital for the conservation of the world's germplast for our successors. Plant material can be preserved in herbariums and museums, while the main strategies of conservation can be divided into *in situ* conservation, *ex situ* conservation, *in vitro* conservation, and DNA banking (Vijayan et al. 2011a). Old varieties are a chance for sustainable leaves production for the growing silk industry. The main method of mulberry propagation is through stem cuttings or grafting of dormant buds (Vijayan et al. 2011b).

Mulberry trees originate from Asia and are currently cultivated in subtropical, tropical, and moderate environments. *Morus* L. (Moraceae) is a genus that is comprised of 10 to 15 species (Nepal, 2008). After expansion from Venice, sericulture and mulberry cultivation within the Gorizia region of Slovenia started to flourish in the middle of the 16th century (Ipavec 2008). Today sericulture in Slovenia has perished, and with plantations gone, only individual trees can be found, mostly near houses. The number of these types of trees in Slovenia is at the present time unknown. Still, sericulture is today existent in 70 other countries amongst which the leaders are China, India, Vietnam, Uzbekistan, Brazil, and Thailand.

Old genotypes are interesting for research because of their adaptations to environmental conditions. As a result of naturalisation the classification of cultivars based on morpho-phenological attributes is problematic. Leaf shapes are often diverse, even on the same tree, and are influenced by several factors suchas plant maturity, growth, leaf position on the branch, and period of growth. Mulberry trees can be monoecious or dioecious which is a challenge for phylogeny.

Throughout history when in the cases of drought or when typical fodder wasn't available, mulberry leaves were given to livestock. Fresh leaves were provided to goats, cattle, horses and pigs, although in small quantities. Mulberry leaves are a well-known fodder in sericulture, where it is the sole source because of its suitable nutritional composition which includes proteins, carbohydrates, chlorophyll and carotenoids. These nutritional parameters of mulberry leaves and silk production are directly proportional to each other (Kumar et al. 2010). Leaves have a high protein content (18 to 25 % DM) and high digestibility (75 to 85 %) (Ba et al.2005).

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The type of leaves preferred by silkworms are characterized by lower levels of minerals and crude fibers (Krishnaswami et al. 1970). They can also be used in human diet as non-toxic effects on internal organs were found (Srivastava et al. 2003). Regarding the high contents of proteins in mulberry leaves, they have potential as a forage supplement for animals such as sheep (Liu et al. 2001), goats (Bakshi and Wadhava 2007), and rabbits (Martinez et al. 2005).

Different parts of mulberry preparations have been used in traditional phytomedicine mainly due to high concentrationsof antioxidants such as phenolics (Nakamura et al. 2009). Among them phenolic acids and flavonoids possess the highest antioxidant potential. Data also shows that mulberry leaves are abundant in caffeoylquinic acids (6.8 – 8.5 mg/g DM) and flavonols (3.7 – 9.8 mg/g DM) as recorded by Sánchez-Salcedo et al. (2015). Because of the high antioxidant properties of *Morus* species, these leaves have the potential of being used as dietary supplements and food products for human consumption also. On the grounds of the high phenolic content of mulberry extracts there is a potential exploitation of a phytochemical nature.

Morus alba is widely valued in traditional medicine, where it is known by the name *Mori folium*, for diabetes, protecting the liver, and lower blood pressure (Kim et al. 2014). Confections containing *Morus alba* extracts contribute to the prevention and qualities of life for pre-diabetic and diabetic patients (Nakamura et al. 2009; Ma et al. 2014). Mulberry leaf polyphenols possess antiatherogenesis effects that could be studied further (Yang et al. 2011). Yang et al. (2011) came to the conclusion that a single administration of a water extract from leaves of *M. alba* lowers systolic blood pressure in a dose-dependent way. Another study showed that a long-term administration of *M. alba*leaves resulted in decrease in body weight and hepatic lipid accumulation (Oh et al. 2009).

Part of the main objective of the study was to screen the biochemical contents of important primary (proteins) and secondary metabolites (phenolics, cysteine, glutathione) in leaves of local mulberry varieties obtained from the Gorizia region, in order to test their relevance as animal feed.

MATERIALS AND METHODS

Plant material

The samples of mulberry leaves were collected from 22 selected old mulberry genotypes from the Gorizia region in Slovenia. Five fully developed sun-exposed leaves (7th leaf from apex) were gathered randomly from each tree on August 20th 2015 and used as one sample. The mulberry trees were of old genotype origin with perimeters ranging from 80 to 370 cm. The meteorological information is given in the Table 1.

Meteorological information was obtained from the nearest weather station BILJE on August 20th 2015 from 07:00 to 21:00 hours: Temperature: 16.8 – 26.9 °C; Relative humidity: 48 – 99 %; Wind direction: NE, W, WNW; Wind speed: 0.6 – 2.1 m/s.

Table 1: Coordinates of mulberry habitat

Sample number	Coordinates	Additional information
1 - 6	45.89846038818359, 13.61314487457275	Miren 91
7	45.88313293457031, 13.61183166503906	Vrtoče
8 - 9	45.88530731201172, 13.60030746459961	Miren 219
10	45.88692092895508, 13.60146427154541	Miren 192
11	45.90574645996094, 13.61686134338378	Njiva pri Vrtojbi
12	45.90725708007812, 13.61715316772461	Njiva pri Vrtojbi B
13	45.90815353393555, 13.62518024444580	Njiva pri Vrtojbi C
14	45.91115126082022, 13.62616417914690	Njiva pri Vrtojbi D
15	45.91320419311523, 13.62628936767578	Njiva – Krožna cesta
16	45.91765213012695, 13.63195037841796	Vrtojba
17	45.89608383178711, 13.64458942413330	Bilje 149
18 - 20	45.89556884765625, 13.62782192230224	Pot Bilje - Miren
21 - 22	45.89221954345703, 13.63083553314209	Bilje – pri vodnjaku

Sample preparation for biochemical analysis

Immediately aftercollection, samples were stored in liquid nitrogen. Afterwards they were transferred into the freezer at -80 °C. The tissue samples were subsequently lyophilized and ground. The prepared samples were then stored in air tight vials at -20 °C prior to biochemical analysis.

Extraction and determination of total protein content (tPr)

Total proteins were determined by using the Bradford reagent following the procedure of Bradford (Bradford 1976). The standards were prepared with Bovine Serum Albumine (BSA). TRIS/HCl (1 ml, 0,1 M) (Douchefa) was added to 25 mg of each sample. After being vortexed for 1 min, the samples were centrifuged for 10 min at 14000 RPM. 80 µl of water was added to the 20 µl of mixture. Bradford reagent (900 µl) (Sigma-Aldrich) was added to the samples, standards, and the reference blank. The mixture was then vortexed for 1 min and proteins were spectrophotometrically determined at 595 nm.

Extraction and determination of total phenolic content (tPh)

The total amount of phenolic compounds was determined using the Folin-Ciocalteu method following the procedure of Ainsworth and Gillespie (2007). Briefly, 1.5 ml of 95 % methanol was added to the 25 mg of each lyophilized sample. After 5 min of homogenisation at 30 Hz in an ultrasonic bath, the samples were centrifuged at 4 °C and 14000 RPM. Subsequently 100 µl of supernatant was pipetted and 200 µl of F-C reagent (10 %) (Merck) was added and vortexed. After exactly 3 minutes 800 µl of 700mM Na_2CO_3 (Sigma-Aldrich) was applied and the mixture was maintained at room temperature in the dark for 120 min. The absorbance was measured at 765 nm against a reagent blank using the Varian Cary UV/VIS spectrophotometer. Gallic acid (Sigma-Aldrich) was used as the reference standard. The total phenolic content was expressed as mg of gallic acid equivalents per gram of each extract on dry basis (mg GAE/g DM).

Extraction and determination of thiols (CYS, GSH)

For the glutathione and cysteine analysis a method with the thiol-specific fluorescence dye monobromobimane was used as described by Tausz et al. (2003). HCl (2 ml of 0.1 M, 32 %) (Sigma-Aldrich) was added to 60 mg PVP (Sigma-Aldrich) and after being allowed to rest overnight the mixture expanded. PVP was used here as a method for excluding any disruptive phenols. 40 mg of lyophilizedsample was added to the mixture and homogenized with Ultra-Turrax for 20 sec and afterwards centrifuged for 15 min at 3000 RPM at 4 °C. For the extraction of reduced thiol forms (GSH and CYS) 280 µl of each extract was incubated along with 420 µl of CHESS puffer [5,2 g CHES (Sigma-Aldrich) + 100 ml bi-distilled water + 40 mg EDTA (Sigma-Aldrich) with the pH 9.3) and 70 µl of 5mM DDT [19,2 mg DTT (Fluka) + 25 ml bi-distilled water] solution. After one hour of incubation thereaction started with the application of 50 µl of 8mM monobromobimane[25 mgmonobromobimane (Invitrogen) + 12 ml acetonitrile (Sigma-Aldrich)], followed by 15 min incubation in the dark at room temperature. The derivatisation was stopped with the addition of 600 µl of methansulphonic acid (0.25 %, v/v) (Merck). The suspension was centrifuged for 45 min at 14000 RPM at 4 °C and ultimately 1 ml of each extract was pipetted into vials.

The separation and determination of thiols was carried out using the gradient method, namely, liquid chromatography on the HPLC system Waters 2695, Waters 2475 Multi Fluorescence detector (excitation: 380 nm; emission: 480 nm), column Spherisorb S5 ODS2 25 x 4.6 mm, column temperature 23 ± 1 °C. Solvent A: acetic acid (0.25 %, v/v) in water with the addition of methanol (5 %, pH 3.9) (Sigma-Aldrich). Solvent B: methanol (90 %, v/v) in water with the addition of acetic acid (0.2 %, v/v) gradient: 5 % solvent B to 15 % solvent B in 20 minutes, 100 % solvent B for 6 minutes, and 5 % solvent B for another 8 minutes. Flow rate was 1 mL min^{-1}.

Statistical analysis

The results of biochemical analyses represented the means and standard deviations (S.D.) of three replicate measurements of each sample. The results were evaluated by one-way ANOVA using the program IBM SPSS Statistics 22. Additionally, correlation between secondary metabolites was analyzed. The differences between the samples were compared with the Duncan test at $p \leq 0.05$. Significant differences were indicated by different lowercase letters (a-k).

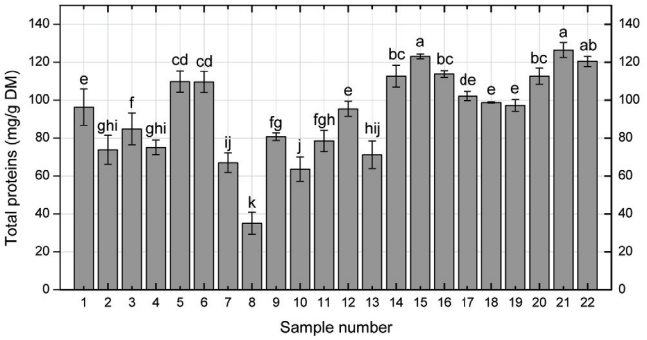

Fig. 1: Total proteins' concentrations

RESULTS AND DISCUSSION

Total protein content

Amino acids represent a primary class of nutrients. The highest levels of total protein content were analyzed in samples 21, 15 and 22, and the lowest in sample 8, as shown in Figure 1. The protein contents were determined within a range from 35 to 126 mg/g DM.

Kandylis et al. (2008) performed a study of the crude protein content in mulberry leaves with concentrations reaching 200 g/kg DM. The major protein fractions are represented by prolamins (44.1 %) and albumins (11.1 %) followed by globulins (9.7 %) and glutelins (8.5 %). It is known that the total protein contents change with mulberry leaf aging (Matei et al. 2006). As the leaves' content of protein decreases with maturity, it is possible this is the source of differences in concentrations between our study and the one conducted by Kandylis. In another study, it was found that amino acids' content increase during leaf maturity thought the differences were insignificant. The most represented amino acids were reported to be glutamine, asparanin and leucine (Yao et al. 2000).

Total phenolic content

Phenolics are a group among three main categories of secondary plant compounds. Mulberries possess antioxidant properties, mainly because of the content of phenolic compounds (Thirumalaisamy 2009, Memon et al. 2010, Radojković et al. 2012, Flaczyk et al. 2013).The DPPH radical-scavenging potential was determined to have a positive correlation (R = 0,803) with the amount of total phenolics (Memon et al. 2010). In leaves of *M. alba* the main phenolic

components were identified as chlorogenic acid, followed by caffeic, vanillic, sinapic, ferulic and gallic acid. The flavonols fraction contains rutin, quercetine, and kaempferol. Meanwhile, Radojković et al. (2012) determined ferulic acid as the predominant phenolic compound in *Morus alba* leaf extract, followed by rutin, sinapic, chlorogenic and gallic acid. Quercetine was not detected. However, in another study conducted by Katsube et al (2005) the predominant flavonol glycoside was indeed quercetine. Mulberry leaves are free of tannins, as reported by Singh and Makkar (2002).

In the presented study, the maximum amount of total phenolic content was documented among samples 17, 11, 19, 18 and 7, where the concentration reached 15.7 g GAE/100 g extract. The lowest levels were analyzedin samples 14, 13, 5, 4, 10, 2, 6, 3, 22 and 21, as shown in Figure 2.

Our results are similar to those reported by Flaczyk et al. (2013), who analyzed total concentration of phenolic compounds equal to 14.4 g GAE/100 g in *M. alba* leaf extracts. In another study a much lower total phenolic concentration was determined in *M. alba* leaves reaching a maximum value of 4.56 % (Thirumalaisamy 2009). Some of our genotypes expressing higher tPh contents could be valuable sources of genes in future breedings. Additional research is required focused on single phenolic compounds in order to determine the predominant phenols and their concentration within each genotype.

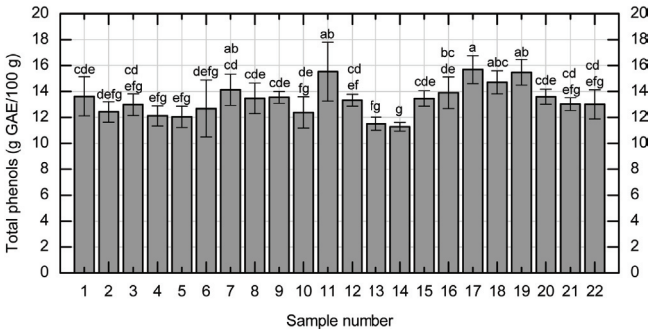

Fig. 2: Total phenols content

Reduced glutathione and cysteine content

Glutathione is the primary low-molecular-weight thiol in most cells. Ordinarily, glutathione is mostly present in its reduced form (GSH), which was measured in this study, with only a small part present in its oxidized state (GSSH) (Dixon et al. 1998). In the absence of stress, plant tissues, including leaves, usually maintain a ratio between GSH:GSSG of a minimum of 20:1 (Noctor et al., 2011). GSH is crucial for plant development by influencing critical functions in embryo and meristem development (Frottin et al. 2009).

Statistically significant differences in the reduced glutathione and cysteine content were determined ($p \leq 0.05$). The data was then grouped into homogeneous subsets, 10 groups for cysteine and 11 for glutathione, as shown in Figure 3. The highest rate of cysteine was measured in sample 15 reaching almost 73 nmol/g DM and the lowest consisted in the samples 16, 17, 14, 7 and 8, with concentrations of 24nmol/g. The highest reduced glutathione concentrations were found in samples 5 and 3, with levels of 2000nmol/g, while the lowest levels were found among samples 8, 16, 17, 14, 10 and 19, with concentrations above 700 nmol/g.

In comparison with beech trees (*Fagus sylvatica* L.) mulberries have manifold higher concentrations of glutathione (Herbinger et al. 2005).

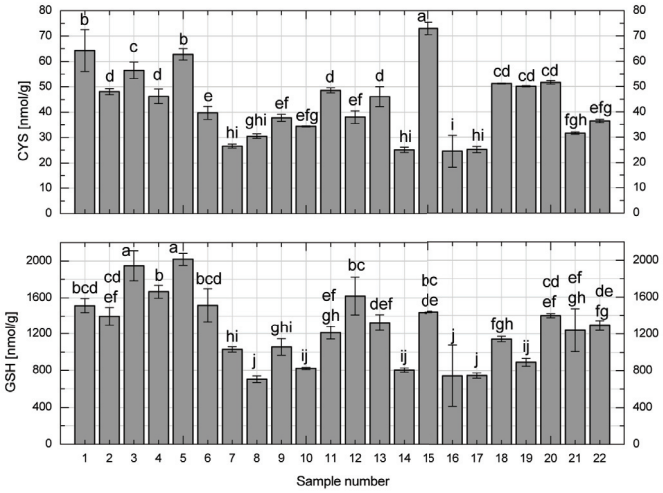

Fig. 3: Cysteine and reduced glutathione concentrations

Correlation between studied biochemical components

The correlation was calculated with the Pearson correlation coefficient, as shown in Table 2. Glutathione is made up of the amino acids glutamine, cysteine and glycine so a high correlation factor between GSH and cysteine was expected. Statistically significant correlation was documented between glutathione and cysteine, with the coefficient reaching 0.707. A lower but statistically significant link was noted between glutathione and proteins, with the coefficient of 0.293. The correlation between cysteine and proteins was 0.241, which was not statistically significant.

Phenolics are known as important antioxidants. As a result of scavenging activities phenolics become oxidized to phenoxyl radicals. The regeneration of phenoxyl radicals can be carried out via ascorbate-glutathione cycle (Szőllősi 2014). However, the expected correlation between phenols, glutathione and cysteine was not statistically important, as the coefficient between the phenols and thiols was - 0.204 and -0.010. respectively. Correlation between proteins and phenols was not expected.

CONCLUSION

The presented metabolite screening in leaves of old local mulberry varieties (*M. alba)* from Gorizia region showed that some evaluated genotypes expressed high contents of metabolites. Samples 21, 15 and 22 contained the highest protein concentrations, samples 17, 11, 19, 18 and 7 contained the most phenols, sample 15 contained the highest rate of cysteine, and samples 5 and 3 contained the highest reduced glutathione concentrations. The nutritively richer varieties could be propagated as highly recommendable feed

Table 2: Correlation between studied biochemical components

		GSH [nmol/g]	Proteins	CYS [nmol/g]	Phenols
GSH [nmol/g]	Pearson Correlation	1	,293*	,707**	-,204
	Sig. (2-tailed)		,037	,000	,151
Proteins (mg/g)	Pearson Correlation	,293*	1	,241	-,032
	Sig. (2-tailed)	,037		,089	,772
CYS [nmol/g]	Pearson Correlation	,707**	,241	1	-,010
	Sig. (2-tailed)	,000	,089		,942
Phenols (mg/g)	Pearson Correlation	-,204	-,032	-,010	1
	Sig. (2-tailed)	,151	,772	,942	

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

for silkworm and other animals. These conclusions may be a stepping stone for selecting cultivars with desired nutrient profiles for breeding.

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Spremenljivost vsebnosti skupnih proteinov, fenolov in tiolov, v listih starih genotipov murv v Goriški regiji

IZVLEČEK

Ohranjanje genskih virov rodu *Morus* je nezadostno, čeprav je nedvomno bistvenega pomena za ohranjanje svetovne zapuščine za naše naslednike. Ta raziskava temelji na določitvi ključnih metabolitov (proteinov, fenolov in tiolov) v listih starih genotipovmurv iz Goriške regije v Sloveniji z namenom proučitve antioksidativne aktivnosti in hranilne vrednosti. Skupni proteini in fenoli so bili določeni spektrofotometrično s pomočjo Bradfordove in Folin-Ciocalteu metode, tioli so bili določeni s pomočjo HPLC po predhodnem markiranju s fluorescenčnim barvilom monobromobimane. Rezultati določitve metabolitov kažejo, da imajo nekateri genotipi visoko vsebnost glutationa,v primerjavi z rezultati drugih avtorjev so superiorni v vsebnosti proteinov in fenolov in bodov nadaljnjih raziskavah razmnoženi z namenom uporabe listov za krmo sviloprejk in ostalih živali.