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Comparative study of the photosynthetic efficiency and leaf structure of four *Cotoneaster* species

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Plants belonging to the genus *Cotoneaster* can be valuable sources of phytochemicals with potential therapeutic properties. The natural habitats of most of these species are situated in Asia, Africa and southern Europe. Introducing them into other climatic conditions could expose them to abiotic and biotic stresses, affecting their bioactive properties. The aim of this study was to assess and compare the performance of four non-native *Cotoneaster* species (*C. roseus*, *C. hissaricus*, *C. hsingshangensis*, *C. nebrodensis*) grown in eastern Poland in terms of leaf morphology, anatomy, and efficiency of the photosynthetic apparatus in relation to lipid peroxidation level, being an indicator of oxidative stress. Photosynthetic pigments concentration and chlorophyll *a* fluorescence parameters were used to evaluate the photosynthetic capacity. The morphological and anatomical analysis of leaves did not show any anomalies or stress symptoms. *Cotoneaster roseus* was characterized by the highest chlorophyll concentration, viability index (Rfd) and a moderate lipid peroxidation level. On the other hand, the lowest values of photochemical quenching (qP), maximum fluorescence (F_m), Rfd, and quantum yield of the photosystem II (QY) observed for *C. nebrodensis* might indicate an inferior efficiency of the photosynthetic apparatus in this species; however, it demonstrated the lowest lipid peroxidation level. Nevertheless, the content and proportion of the photosynthetic pigments as well as overall chlorophyll *a* fluorescence parameters and lipid peroxidation levels indicate a good physiological condition of all examined plants. The observed differences between the species may be rather species specific and genetically established and not indicate their sensitivity to non-native growth conditions. This is the first report about the physiological parameters of the four *Cotoneaster* species, proving they are well adapted to growth in the climatic conditions of central Europe providing thus a raw material with potential for pharmaceutical applications.

During recent years, the interest in natural medicinal substances of plant origin has been increasing. Many plant species have been used in folk medicine for centuries, which advocates their curative properties. Nevertheless, the lack of reliable scientific characterization of such species impedes their large-scale application. Currently, modern analytical techniques facilitate a scientific and critical approach to investigate plants with potential therapeutic properties, which offers opportunities for the development of innovative natural medicinal products.

For a long time, plants from the genus *Cotoneaster* have been used in traditional Asian medicine, in particular to treat gastrointestinal, spasmolytic, hepatoprotective, astringent, expectorant, diuretic, and cardiotonic

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ailments[1]. Our phytochemical analyses and studies regarding the multidirectional biological activity of extracts from leaves and fruits of four species of the genus *Cotoneaster*, i.e. *C. hsingshangensis*, *C. hissaricus*, *C. roseus*, and *C. nebrodensis*, confirmed their activity against skin diseases and offered new possibilities for the treatment of acne[2,3]. The studies showed that the examined species exhibited considerable anti-inflammatory, antioxidant, and antibacterial activities against microorganisms involved in the etiopathogenesis of acne (*Cutibacterium acnes*, *C. granulosum*, and *Staphylococcus epidermidis*) and had a high therapeutic index[2,3]. It was found that, among the four investigated species, leaf extracts of *C. hsingshangensis* had the highest content of phenolic compounds and the highest biological activities, i.e. anti-inflammatory, antioxidant, and antibacterial effects. Furthermore, it inhibited lipoxygenase and hyaluronidase to the greatest extent. The LC-MS analysis revealed high contents of valuable phenolic compounds with therapeutic properties (e.g. chlorogenic acid and quercitrin); among the four species examined, the largest concentrations of these compounds were detected in the leaves of *C. hsingshangensis*. In turn, the highest concentrations of rutin, scopoletin, isoquercitrin, and hyperoside were found in the leaves of *C. hissaricus*, while *C. roseus* leaves had the highest contents of flavonoids that rarely occur in nature, i.e. 5-methylgenistein-4′-O-glucoside and sissotrin[2,3]. All these studies contributed to a reliable assessment of extracts from *C. roseus*, *C. hissaricus*, *C. hsingshangensis*, and *C. nebrodensis* as potential therapeutic raw materials for treatment of skin diseases. Noteworthy, the polyphenols found in these extracts are known to play important roles in plant responses to a variety of biotic and abiotic stress factors, and their synthesis is induced under harmful environmental conditions, including drought, extreme temperatures, UV radiation, salinity, and metal pollution[4–6]. Most of them possess strong antioxidant properties (e.g. naringenin, luteolin, apigenin, quercetin, epicatechin) combatting oxidative stress in plant cells. Moreover, some, like salicylic acid, are considered as plant hormones regulating plant metabolic processes under physiological and stressful conditions. These findings inspired our further investigation of these valuable species.

The natural habitats of species of the genus *Cotoneaster* are predominantly located in continental Eurasia, north-west Africa, southern India, and Taiwan. In recent decades, the distribution area of some species of this genus has extended into central Europe, where they are used as ground cover plants, hedges, or ornamental shrubs[7]. As reported in the literature, only four species of the genus *Cotoneaster* i.e. *C. integerrimus* Medik., *C. tomentosus* Lindl., *C. lucidus* Schltdl., and *C. melanocarpus* Lodd. ex C.K. Schneid, are found in Poland, predominantly in the south-east of the country[8]. *Cotoneaster roseus*, *C. hissaricus*, and *C. hsingshangensis* occur naturally in Asia, while *C. nebrodensis* is native in Italy and central Europe[7,9–12].

As a continuation of the investigations on these four species of the genus *Cotoneaster* (*C. roseus*, *C. hissaricus*, *C. hsingshangensis*, *C. nebrodensis*), our study was carried out to explore the biology of growing these plants in the climatic conditions of central and eastern Europe. Changing the growth habitat can be a source of biotic and abiotic stresses, and stress conditions may induce changes in the anatomical structure of leaves and reduce the efficiency of photosynthesis or eventually block the process[13,14], which obviously leads to impairment of plant growth and development (e.g. lower biomass production). Inappropriate growth conditions may not only result in disruption of the photosynthesis process (both "light" and "dark" reactions) but also cause damage to photosynthetic membranes at various levels, e.g. modification of the ultrastructure of chloroplasts, proteins, lipids, and pigments[15]. Additionally, quantitative changes in photosynthetic pigments lead to substantial structural and functional modifications in the photosynthetic apparatus and constitute one of the protective mechanisms of plant survival[16]. Stressful environmental conditions also strongly influence the levels and composition of bioactive compounds, including phenolic compounds, which is of pivotal importance in the case of species with potential medicinal application, such as *Cotoneaster*[17,18]. Since the areas of central and eastern Europe do not belong to the natural habitats of *C. roseus*, *C. hissaricus*, *C. nebrodensis*, and *C. hsingshangensis*, the objective of the study was to investigate the morphological and anatomical structure of leaves and to assess the photosynthetic apparatus efficiency in combination with the levels of oxidative stress in these *Cotoneaster* species. Photosynthetic performance was estimated based on the content of photosynthetic pigments (chlorophyll *a*, chlorophyll *b*, carotenoids) and chlorophyll *a* fluorescence parameters. Furthermore, in order to estimate oxidative stress in plant tissues, lipid peroxidation levels were determined.

The present comparative studies of the morphological and anatomical structure of leaves combined with the analysis of photochemical activity and oxidative stress, which is correlated with the physiological state of plants, were performed to assess the possibility of effective growth and development of *Cotoneaster* species in the climatic conditions of central and eastern Europe. We tested the research hypothesis, that the four non-native *Cotoneaster* species, namely *C. roseus*, *C. hissaricus*, *C. nebrodensis*, and *C. hsingshangensis*, are well adapted to growth in easter Poland, out of their natural habitats, and do not show significant stress symptoms. Such study is extremely important in terms of the extraction of biologically active molecules from this raw material.

Materials and methods
Plant material
Fresh leaves of four *Cotoneaster* species growing in the collection of the Botanical Garden of Maria Curie-Skłodowska University in Lublin (Poland) were sampled, namely *Cotoneaster roseus* Edgew. (number 2886), *Cotoneaster hissaricus* Pojark. (2843), *Cotoneaster nebrodensis* (Guss.) K. Koch (127), and *Cotoneaster hsingshangensis* Fryer et B. Hylmö (121). The Botanical Garden is situated in the eastern part of Poland within the borders of Lublin Upland (altitude: 181.2 m a.s.l., coordinates 51°15′46″ N; 22°30′51″ E) on brown soils prevailing in this area (Index Seminum 2014 Hortus Botanicus Universitatis Marie Curie-Skłodowska)[19]. According to the Köppen-Geiger climate classification, one of the most widely used climate classification systems, this area is characterized by a warm-summer humid continental climate (Dfb). The mean yearly temperature is 10.54 °C. The warmest month is August with the average temperature 25.36 °C, while the coldest month is January (-4.3 °C). The average annual rainfall is about 91.46 mm, with the highest precipitation in July (148.19 mm) and the lowest in November (56.71 mm). The number of rainy days (with rainfall ≥ 1.0 mm) over

the year is 168.91, i.e. 46.28% of the year, against 196.09 (53.72%) rainless days. The lowest relative humidity of the year is 77.75%. The snow period lasts 4.6 months, from November to March, with snowfall of approximately 25 mm in 31 days. The month with the highest snowfall in Lublin is February, with an average of 60 mm. The windiest month of the year is January and the calmest is July, with an average hourly wind speed of 19.0 and 13.6 km h⁻¹, respectively[20–22].

Each of the investigated species was growing in similar sun/shade conditions at a 2–50 m distance from each other. The research material was collected at the beginning of July 2022. The leaves taken for all analyses were fully developed and were in the same developmental stage – they originated from the same location (middle part) of the branches exposed to the sun and were in very good condition without any damage caused by pathogens, insects of other abiotic factors. Throughout the experimental period, no herbicides, fungicides, or any chemicals were applied. The tested species were only subjected to manual weeding. All macroscopic images of the leaves presented in various analyses were taken with a Nikon D300 photographic camera equipped with a 60 mm AF MICRO NIKKOR lens.

Morphological and anatomical analyses
Light microscopy (LM)
Manual transverse cross-sections of fragments from the middle part of leaves were analysed. Additionally, hand-made peel preparations of the leaf epidermis were made. Samples were placed in a drop of distilled water for adequate visualisation. A Nikon Eclipse N*i* light microscope was employed. The digital camera and NIS-Elements BR 4.30.00 software with the Extended Depth of Focus (EDF) module (Nikon Instruments Inc., www.microscope.healthcare.nikon.com/products/software/nis-elements/viewer) were used.

Fluorescence microscopy (FM)
Manual transverse cross-sections were made to visualise the autofluorescence of substances contained in the leaf tissues. The material was placed in a drop of distilled water. A fluorescence microscope Nikon Eclipse N*i* was used to analyse the slides at an excitation wavelength of 330–380 nm allowing visualisation of chlorophyll and lignin autofluorescence: red colour corresponds to autofluorescence of chlorophyll contained in chloroplasts (emission wavelength at 570–700 nm), blue colour corresponds to autofluorescence of lignin contained in lignified sclerenchyma and xylem cell walls (emission wavelength at 400–700 nm)[23]. Photographic documentation was performed using a digital camera and NIS-Elements BR 4.30.00 software with EDF module, as described above.

Physiological analyses
Photosynthetic pigment content
Fresh leaf samples (0.2 g F.W.) were stored at −80 °C until analysis. Frozen leaf tissues were homogenised in 80% acetone and centrifuged at 15,000 rpm for 5 min at 4 °C. The supernatant was read spectrophotometrically at 663 nm, 646 nm, and 470 nm for determination of chlorophyll *a*, chlorophyll *b*, and carotenoids, respectively, as described in[24]. The mean values were averaged from five independent replicates for each plant species.

Chlorophyll a fluorescence measurement
A closed chlorophyll fluorescence imaging system FluorCam 800MF (Photon System Instruments, Brno, Czech Republic, https://fluorcams.psi.cz/products/closed-fluorcam/#info) was employed to determine chlorophyll *a* fluorescence in fresh leaves of four *Cotoneaster* species in vivo after 30-min dark-adaptation. Quenching protocol, consisting of a modulated light of known wavelength, was used for all chlorophyll fluorescence measurements, to measure the Kautsky effect in the pulse-amplitude modulated (PAM) mode as described in[25,26] with some modifications. Briefly, the values of the minimal (F_0) and maximal fluorescence (F_m) were recorded using respectively a photon fluence rate of 0.15 μmol m⁻² s⁻¹ and saturating light pulse (SLP) – 4000 μmol m⁻² s⁻¹ for 0.8 s. After 40 s dark relaxation, the leaves were exposed for 20 s to actinic light with intensity of 1200 μmol m⁻² s⁻¹, similar to that experienced by plants in the field. Then, saturation flash was applied with the same intensity and duration as on dark-adapted leaves to determine the rise in peak fluorescence (F_p) during the initial phase of the Kautsky effect. Next steps in the protocol included ten consecutive light periods (20 s each) followed by saturating pulses until reaching of steady stage fluorescence (F_t), allowing also to read F_m'. A far-red pulse was applied immediately after the last actinic light switching off, allowing us to measure minimum fluorescence in the light-adapted state (F_0'). Based on the applied protocol, the chlorophyll fluorescence parameters, given in Table 1, were calculated. Fluorescence parameters were evaluated from five independent biological repetitions, which yielded the average fluorescence parameter values.

Anthocyanin content
The assay was conducted using the modified method provided by Hawrylak-Nowak and Mancinelli et al.[27,28]. Frozen leaf samples (0.2 g F.W.) were ground in a mortar and extracted with 2 mL methanol acidified by 1% HCl in darkness at 4 °C for 24 h. After this time, the samples were centrifuged at 15,000 rpm for 7 min. The anthocyanin content was estimated spectrophotometrically at 530 and 657 nm, and expressed as absorbance units at 530 nm per 1 g F.W., corrected by the absorbance measurement at 657 nm. The equation used was as follows: $A_{530}/1$ g F.W. $= A_{530} - 0.25 \times A_{657}$. The quantification data were the mean of five replicates for each species of the tested plant.

Estimation of the lipid peroxidation level
The level of lipid peroxides in leaf tissue was estimated using the TBA (thiobarbituric acid) reaction according to[29,30]. Fresh leaves (0.5 g) were homogenised in 5 mL of 0.1% w/v TCA (trichloroacetic acid) and centrifuged at 10,000 rpm for 10 min. Next, 2 mL of 20% TCA (w/v) containing 0.5% of TBA (w/v) was added to 2 mL

Parameter	Name	Formula
F_0	Minimal fluorescence in the dark-adapted state	Measured
F_m	Maximal fluorescence in the dark-adapted state	Measured
F_0' (F_0_Lss)	Steady-state minimum fluorescence in light	Measured
F_m' (F_m_Lss)	Steady-state maximum fluorescence in light	Measured
F_t	Steady-state fluorescence in light	Measured
F_p	Peak fluorescence during the initial phase of the Kautsky effect	Measured
Fv/Fm	Maximum PSII quantum yield in dark-adapted state	$(F_m - F_0)/F_m$
qP	Coefficient of photochemical quenching in steady-state	$(F_m' - F_t)/(F_m' - F_0')$
qN	Coefficient of non-photochemical quenching in steady-state	$1 - (F_m' - F_0') / (F_m - F_0)$
Rfd	Chlorophyll fluorescence decrease from maximum to steady state fluorescence	$(F_p - F_t)/F_t$
QY	Steady-state PSII quantum yield	$(F_m' - F_t)/F_m'$

Table 1. Chlorophyll fluorescence parameters used in this study [[25] – Supplemental Data 1].

of the supernatant. The solutions were heated at 95 °C for 30 min using a block heater and quickly cooled on ice afterwards. The mixture was re-centrifuged at 10,000 rpm for 10 min. The absorbance of the supernatant was measured at 532 and 600 nm. 0.25% TBA in 10% TCA was used as a blank. The amount of TBA reactive substances was assessed using an extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as nmol g^{-1} F.W. The following equation was used to determine the content of TBARS: $\Delta E \times V0/\varepsilon \times V \times g$.

ΔE - $E_{532} - E_{600}$

V0 – total volume of supernatant.

ε – molar absorbance coefficient – 155 mM^{-1} cm^{-1}.

V – volume of supernatant added to the reaction mixture.

g – fresh weight of leaves.

Statistical analysis
Statistics were performed using Origin Statistica 14.0.0.15 (StatSoft, Cracow, Poland). Mean values were calculated from five independent replicates. Statistically significant differences among the means were determined using one-way ANOVA. Before performing ANOVA, the assumptions for normality of data distribution were checked using Shapiro-Wilk's tests. Additionally, Levene's test was employed to check the homogeneity of variances. Post-hoc Tukey test was carried out to estimate statistically significant differences among the means. Means (±SD) were considered statistically different at $p < 0.05$. The principal component analysis (PCA) was performed to obtain a holistic view of the results and to evaluate some independent trends in the variations among the investigated data.

Results and discussion
Morphological and anatomical leaf analyses
The four species from the genus *Cotoneaster* examined in this study are perennial shrubs, reaching a height up to 2 m[31]. To assess the possible effects of the climatic conditions of central and eastern Europe on their growth and development, the morphological and anatomical structure of their leaves was investigated. The leaf blades of all the species were dark green and had an entire margin, but their shapes and sizes exhibited slight differences (Fig. 1a-d). The leaves of *C. roseus* were oval and had smaller sizes ranging from 1.5 cm to 3.5 cm long and from 1.0 cm to 2.0 cm wide. The leaves of *C. hissaricus* had an ovoid blade with a tapered apex, and the leaf blade was 3.0 to 6.0 cm long and 2.5 to 4.0 cm wide. The leaf blade in *C. nebrodensis* was broadly ovoid, from 4.5 to 6.0 cm long, and from 3.5 to 5.0 cm wide. In turn, the leaves of *C. hsingshangensis* differed in both their shape and size from the leaves of the other species, as they were clearly lanceolate and had the largest sizes in the range from 3.5 cm to 9.5 cm (length) and from 1.5 cm to 4.0 cm (width). The morphological structure of the leaves of the studied species is consistent with those in the few studies published before[32,33].

The microscopic analysis of the cellular structure of the upper (adaxial) leaf epidermis performed using peel sections revealed that all the species had hypostomatic leaves (absence of stomata in the upper epidermis). Additionally, the cells of the upper epidermis had approximately the same size with undulating edges (Fig. 1e-h). However, the lower (abaxial) epidermis of all the examined species had numerous densely arranged anomocytic stomata, and the epidermis cells had strongly undulating edges (Fig. 1i-l). Both the upper and lower leaf blades of the four species possessed unicellular elongated and pointed trichomes (Fig. 1i-l, small pictures). There were clear differences in the numbers and location of the trichomes, i.e. they were mainly present on the leaf margins and on the lower leaf blade (Fig. 1m-p). The *C. nebrodensis* leaves showed the greatest number of trichomes, which also formed a characteristic tomentum on the abaxial side of the leaf blade (Fig. 1o). Regardless of the differences in the position and density of trichomes, in all tested species trichomes were always bent at the base and adhered to the leaf blade, which is characteristic for all *Cotoneaster* species[32].

Next, histological analyses of the leaves were performed using a bright-field microscope and a fluorescence microscope. The cross-sections through the leaf blade of the four species showed a single-layered upper and lower epidermis, a palisade mesophyll usually composed of one row of slightly elongated and closely adjacent cells, and a spongy mesophyll composed of loosely arranged oval cells with large intercellular spaces (Fig. 2a, c, e, g). Vascular bundles were visible at the border of the palisade and spongy mesophyll (Fig. 2a, g, white arrows);
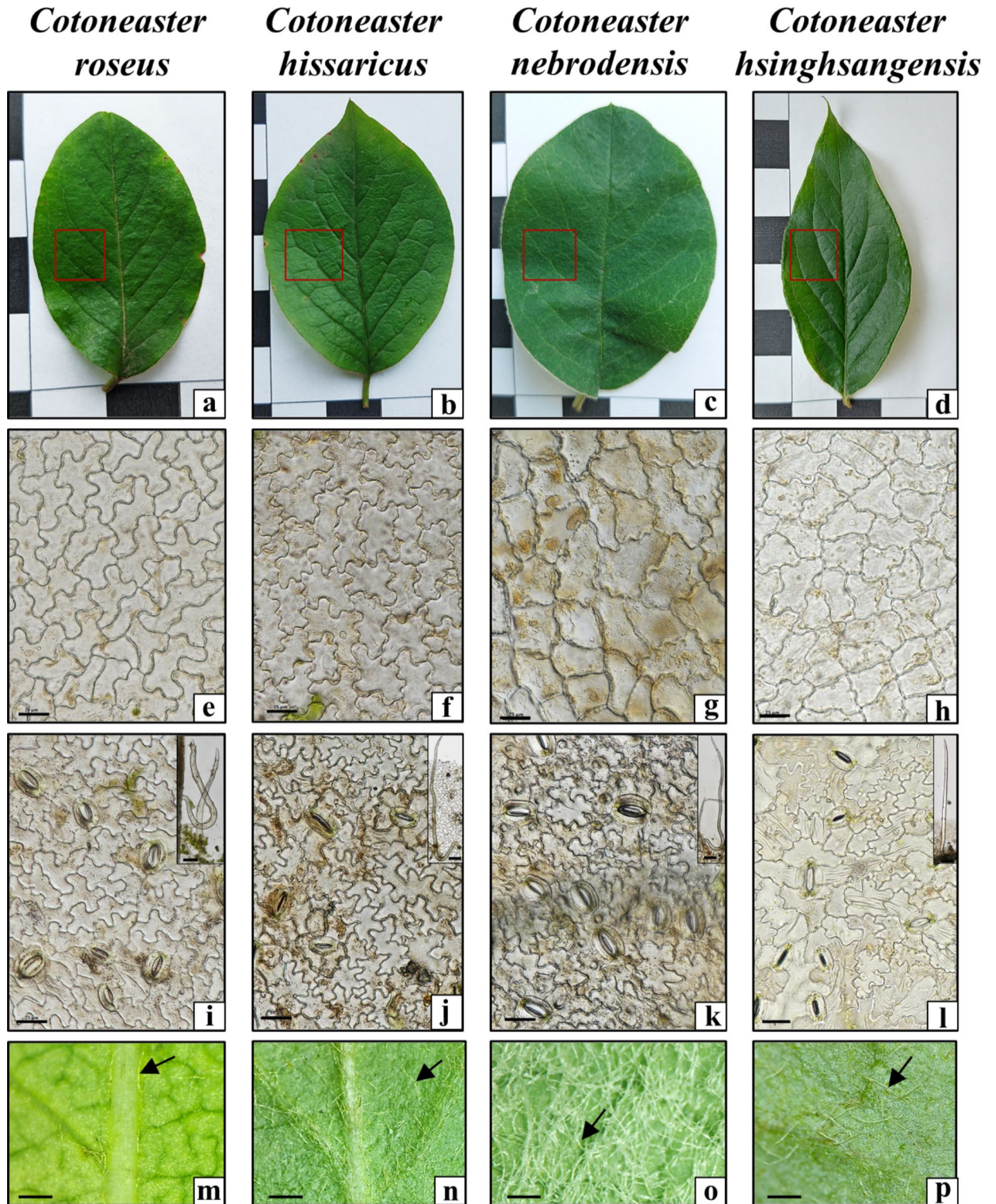
Fig. 1. Morphology of *Cotoneaster roseus*, *C. hissaricus*, *C. nebrodensis*, and *C. hsingshangensis* leaves: (**a-d**) macroscopic view (red square – analyzed fragments); (**e-h**) upper leaf epidermis; i-l lower leaf epidermis (trichome – small pictures); **m-p** location of trichomes (arrows) on the lower epidermis; **e-l** LM. Scale bars: **a-d** – 1 cm; **e-l** – 25 μm; **m-p** – 5 mm.

the latter exhibited the presence of calcium oxalate crystals (CaOx) (Fig. 2a, c, e, black arrows). Calcium oxalate crystals are ubiquitously present in numerous plant species belonging to over 215 different families[34,35]. Based on their morphology, they are classified into five categories: crystal sand, raphides (needle-shaped), styloids (column-shaped), prismatic (box-like), and druses (spherical clusters). The crystals observed in our study
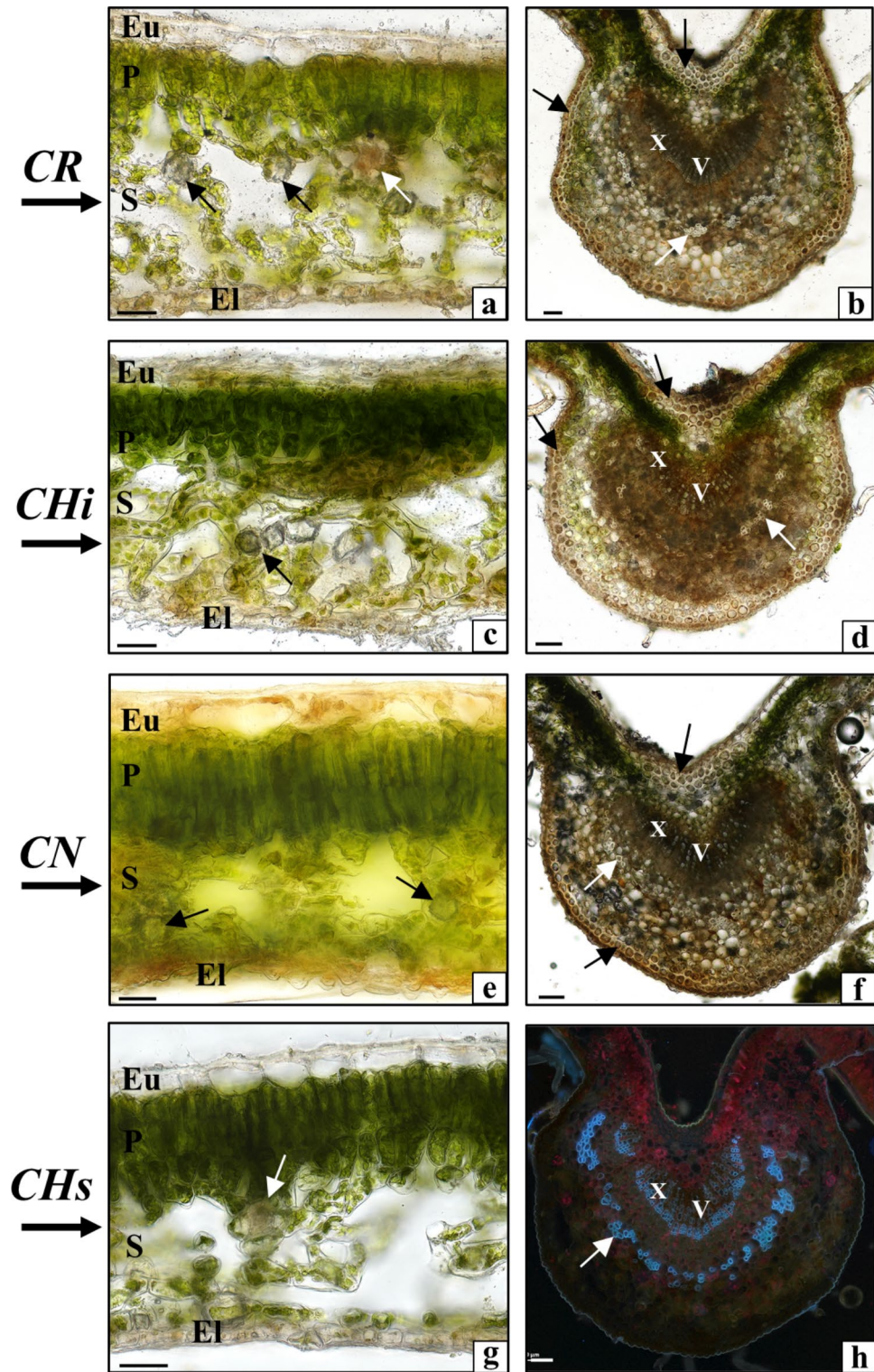
Fig. 2. Histology of *Cotoneaster roseus* (CR), *C. hissaricus* (CHi), *C. nebrodensis* (CN), and *C. hsingshangensis* (CHs) leaves: **a**, **c**, **e**, **g** cross-section through the leaf blade: upper epidermis (Eu), palisade mesophyll (P), spongy mesophyll (S), vascular bundle (white arrow), calcium oxalate crystal (black arrow), lower epidermis (El); **b**, **d**, **f**, **h** cross-section through the midrib: collenchyma (black arrow); sclerenchyma (white arrow), vascular bundle (V), xylem (X); **a**-**g** LM, **h** FM. Scale bars: **a**, **c**, **e**, **g** – 25 μm; **b**, **d**, **f**, **h** – 50 μm.

represent druses. Calcium oxalate crystals are found in a variety of plant tissues, including leaves, stems, roots, seeds, and fruits, where they play multiple roles, of which the regulation of tissue calcium level and homeostasis, protection against herbivory, or metal detoxification seem to be the most important ones[34,35]. In the midrib of the leaves, a single layer of collenchyma cells (supportive tissue) was observed under the single-layer epidermis. On the adaxial surface of the leaf, the collenchyma formed several layers. A large phloem-xylem bundle was located in the central part (Fig. 2b, d, f, h). On the abaxial side of the leaf, the vascular bundle was surrounded by sclerenchyma cells (strengthening tissue), which was confirmed by the fluorescence microscopy image showing blue autofluorescence of lignified cell walls of sclerenchymatous fibres and vessels of the xylem (Fig. 2h). The presence of both collenchyma and sclerenchyma in the leaf tissues and tomentum-forming trichomes indicates that these species are adapted to dry and hot habitat conditions[36].

Remarkably, to the best of our knowledge, such morphological and anatomical analyses have never been published before. Based on these investigations, it can be concluded that the leaves of the four examined species show relatively few differences in their morphological and anatomical structure. Moreover, all these traits do not differ from those of the typical leaves of other species belonging to the genus *Cotoneaster* grown in the climatic conditions of Central Europe[32].

Physiological analyses
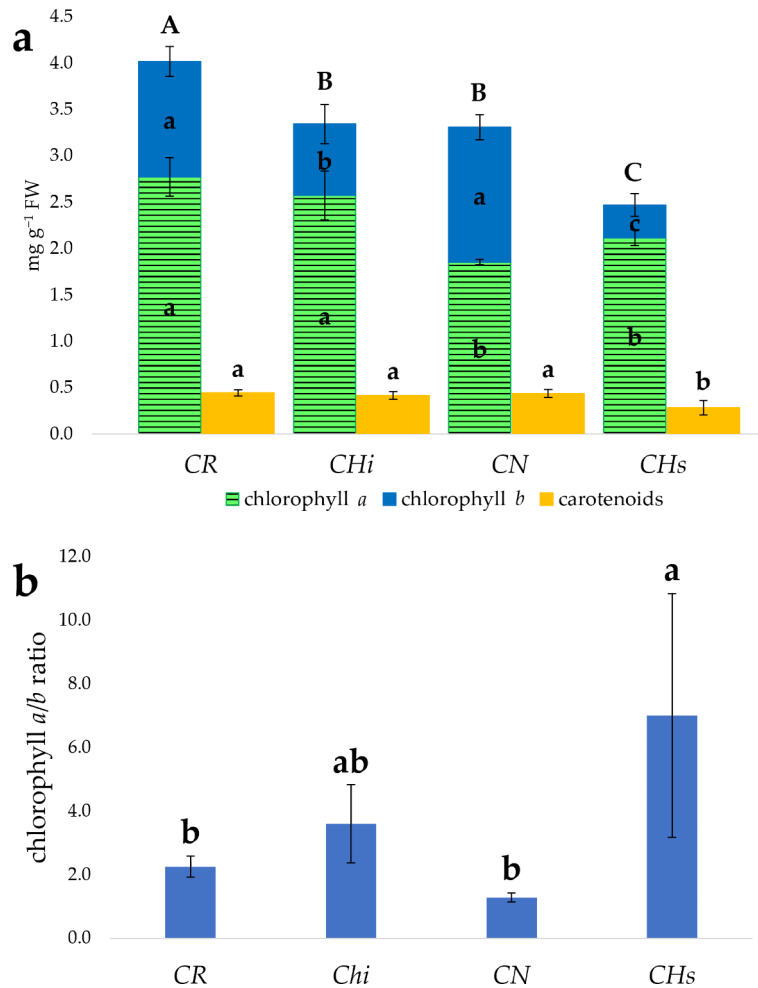
Photosynthetic pigment content

Photosynthetic pigments (chlorophylls and carotenoids) play a key role in the photosynthesis process, as they absorb energy from light with specific wavelengths. Very important for the evaluation of the physiological state of a plant is the fact that the quantitative and qualitative composition of pigments changes not only with plant age but also in response to environmental conditions. It has been found, for example, that the relative chlorophyll level decreases while the content of carotenoids increases in unfavorable environmental conditions, resulting in diminished photosynthesis and, in consequence, impaired plant growth[37]. On the other hand, the leaves of trees developing during strong water deficit are smaller but have more densely packed mesophyll cells and smaller aerial spaces; therefore, they may exhibit higher chlorophyll concentrations in comparison to leaves developed under optimal water supply[38].

Among the investigated species, *C. roseus* was characterized by the highest total chlorophyll content in the leaves, whereas the lowest level was found in *C. hsingshangensis* (Fig. 3a). At the same time, the chlorophyll *a*/*b* ratio was more or less similar in *C. nebrodensis*, *C. roseus*, and *C. hissaricus*, but was significantly higher in *C. hsingshangensis* (Fig. 3b). Such differences are often observed between sunlit and shaded leaves with higher chlorophyll *b* contribution in the shaded ones[16,39]. However, since the studied plants were exposed to the sun in an equal degree, the observed differences are rather unlikely to result from different light conditions and may be rather species specific or even be caused by individual variability. Nevertheless, the calculated chlorophyl *a*/*b* values are normal for plants with healthy physiological condition[40]. *Cotoneaster hsingshangensis* also had a significantly lower concentration of carotenoids than the other species examined. The highest value of chlorophyll $a + b$ /carotenoids ratio was determined in *C. roseus* (5.33 ± 0.29), and the lowest one in *C. hsingshangensis* (3.37 ± 0.57). Nevertheless, these values indicate that the concentrations of the pigments in all the examined species were optimal for the appropriate photosynthetic performance and there were no stress factors causing premature aging of leaves[39,40].

Chlorophyll a fluorescence

Solar radiation in the range of 400–700 nm is absorbed by photosynthetic pigment molecules (chlorophylls and carotenoids). It is used in the photosynthesis process, and the excess energy is lost as heat or is emitted as chlorophyll fluorescence[41]. The chlorophyll fluorescence induction curve (called the Hirsch curve) shows an inverse relationship between the intensity of fluorescence and the intensity of photosynthesis. Fluorimetric methods used for examination of fluorescence intensity facilitate precise determination of the efficiency of the radiation energy conversion process in the light-phase photosynthetic reactions, which decreases under the influence of environmental stresses[42,43]. Therefore, chlorophyll fluorescence analysis is one of the most powerful and widely adopted techniques to assess the effect of stresses on the photosynthetic efficiency displaying the physiological condition of plants[41,43,44]. The available literature provides no reports on this type of analyses in the *Cotoneaster* species studied here; hence, to the best of our knowledge, this type of research has been conducted for the first time.

F_0 and F_m belong to the basic technical parameters for describing the condition of thylakoid membranes and especially that of photosystem II (PSII), which is considered to be the most sensitive site of the photosynthetic apparatus under abiotic stress[41]. These parameters are assessed under darkness and in saturated light pulse conditions, when all reaction centres of photosystem II are open or closed, respectively. Rise in F_0 indicates the loss of excitation energy during its transmission from the light harvesting antennas to the PSII reaction centre, which could be due to a physical dissociation of light harvesting complex from the PSII core[45]. F_m provides information about the maximal possible value for fluorescence in the absence of any photochemical quenching, and its value is higher when the more excitable PSII reaction centres (more fluorescence-triggering sources) are present, and therefore when the PSII is potentially more efficient[46]. Increased values of F_0 or decreased values of F_m may evidence the occurrence of abiotic stresses, such as high temperature, drought or salinity[47–49]. The values of both parameters were similar in *C. roseus*, *C. hissaricus*, and *C. hsingshangensis* but significantly lower in *C. nebrodensis*; nevertheless, the values of the F_v/F_m parameter [$(F_m - F_0)/F_m$] were similar in all species (Fig. 4a, b, c). The F_v/F_m values were close to 0.7, which is within the range typically found in plants in normal physiological conditions (usually between 0.75 and 0.85)[43,50]. The available literature provides no information concerning the F_v/F_m parameter in the studied species, but it has been determined in other species of the genus *Cotoneaster*. As reported by Swoczyna et al.[44], depending on the month of plant material collection, the value of this parameter

Fig. 3. Concentrations of photosynthetic pigments (chlorophyll *a*, chlorophyll *b*, carotenoids) (**a**) and chlorophyl *a/b* ratio (**b**) in the leaves of *Cotoneaster roseus* (CR), *C. hissaricus* (CHi), *C. nebrodensis* (CN), and *C. hsingshangensis* (CHs). Different letters indicate statistically significant differences. Capital letters refer to the total ($a + b$) chlorophyll concentration.

was 0.590–0.774 in *C. dammeri* and 0.588–0.790 in *C. dammeri* 'Eichholz'. Moreover, the authors also mentioned the following values of the F_v/F_m parameter in other species from the family Rosaceae: 0.528–0.774 in *Potentilla fruticosa* and 0.608–0.746 in *Fragaria vesca*. The F_v/F_m ratio is a key indicator of plant photosynthetic performance, with its decrease indicating PSII photoinhibition caused by a stress factor[39,40]. Decreases in F_v/F_m may be related to the development of non-radiative dissipation of excitation energy and damage to the PSII reaction centres, both of which reduce the maximum quantum efficiency of PSII photochemistry, or its functional activity[51]. Low F_v/F_m values (approx. 0.2–0.3) indicate irreversible changes in the PSII structure[52].

Non-radiative dissipation of excitation energy (non-photochemical quenching, qN) represents rapid and reversible thermal dissipation of absorbed light energy in the PSII antenna. It involves structural changes connected with the xanthophyll cycle and slight modifications in pH within both sides of thylakoid membranes[53]. The value of this parameter fluctuates from 0 to 1. On the other hand, the coefficient of photochemical quenching qP indicates the proportion of open PSII reaction centres and is expressed as the number of photons absorbed by PSII divided by the number of photons used by the open centres during photosynthesis[54]. Both coefficients were the lowest in *C. nebrodensis* (Fig. 4e, f). Also, QY (PSII quantum yield) indicating the effective quantum efficiency of electron transport in PSII as a function of the number of photons emitted divided by the number of photons absorbed had the lowest value in *C. nebrodensis* (Fig. 4g).

Rfd is considered as a plant vitality index used to diagnose the physiological condition of plants. This parameter is an indicator of the photosynthetic capacity of leaves in specific light conditions[50,55]. Very similar and simultaneously the highest Rfd values were obtained in the case of *C. roseus* and *C. hsingshangensis* (Fig. 4d). The values of this parameter were significantly lower in *C. hissaricus* and *C. nebrodensis*. Although the decrease in the Rfd parameter value indicates impaired photosynthetic capacity and disruptions within thylakoids related to photochemical reactions, the Rfd value of all the investigated species were within the range reported in the literature for plants performing photosynthesis in stress-free conditions[56]. The statistically significantly lower value found for the *C. nebrodensis* leaves may indicate a lower efficiency of the photosynthetic apparatus than
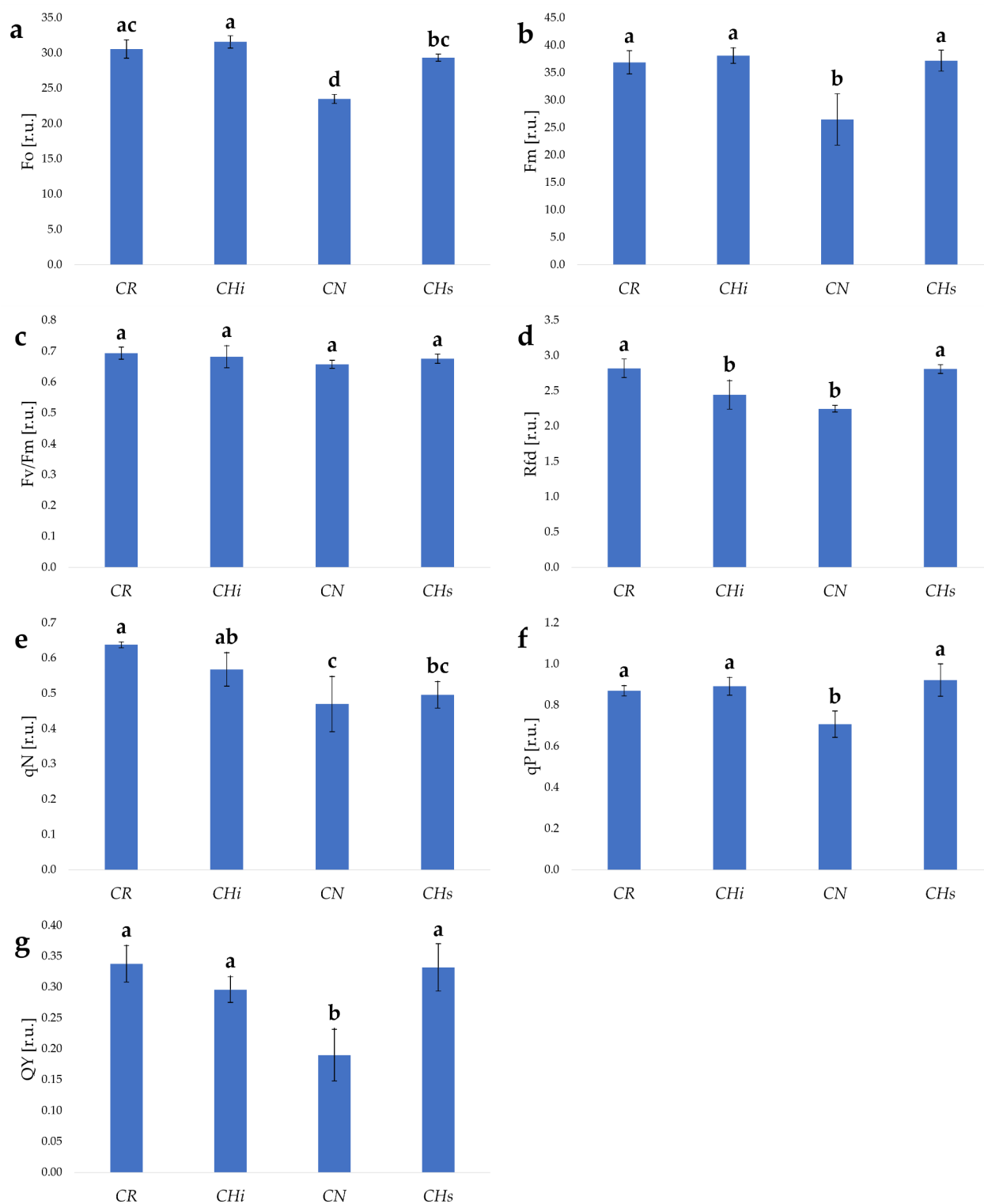
Fig. 4. Chlorophyll *a* fluorescence parameters: (**a**) F_0 (minimum fluorescence in the dark-adapted state); (**b**) F_m (maximum fluorescence in the dark-adapted state); (**c**) F_v/F_m (maximum efficiency of PSII photochemistry); (**d**) Rfd (chlorophyll fluorescence decrease from maximum to steady state fluorescence); (**e**) qN (non-photochemical quenching); (**f**) qP (coefficient of photochemical quenching); and (**g**) QY (PSII quantum yield)) in the leaves of *Cotoneaster roseus* (CR), *C. hissaricus* (CHi), *C. nebrodensis* (CN), and *C. hsingshangensis* (CHs), expressed as relative units (r.u.). Different letters indicate statistically significant differences.

in the other examined species. Nevertheless, these parameters are consistent with the standards reported in the scientific literature and indicate a good physiological condition of the examined plants.

Lipid peroxidation

Lipid peroxidation is a physiological indicator of both biotic and abiotic stress responses in plants[57]. Both types of stress result in direct or indirect generation of excessive reactive oxygen species (ROS) that attack unsaturated lipids in membranes leading to their peroxidation. This affects membrane properties, causing lipid and protein degradation, loss of ion transport, enzyme activity, and protein cross–linking. Usually, membrane lipid peroxidation is detected by measuring the level of thiobarbituric acid reactive substances (TBARS), which is a widely used marker of oxidative lipid injury caused by environmental stress in plants[58,59]. The highest level of TBARS was detected in the leaves of *C. hsingshangensis*, while the lowest content was determined in *C. nebrodensis* and *C. hissaricus* (Fig. 5). This result might suggest that *C. hsingshangensis* experienced higher stress than the other species. Interestingly, our earlier study showed that the leaf extract of *C. hsingshangensis* was the most efficient in inhibiting lipoxygenase (LOX) activity[3]. LOXs catalyse the oxidation of polyunsaturated fatty acids into fatty acid hydroperoxides[58,60]. These apparently inconsistent results require further investigation.

Anthocyanin content

Anthocyanins, one of the most conspicuous classes of flavonoids, accumulate in the vacuoles of cells in both vegetative and reproductive organs, protecting them against a variety of abiotic stress factors, such as drought, high salinity, metals, excess light or UV radiation, cold, wounding, and pathogen infections, where they often correlate with enhanced stress tolerance[61–63]. Anthocyanins, and especially the most frequently occurring cyanidin (3-glucoside), are responsible for the purple-red pigmentation of leaf tissues. Visually, the four *Cotoneaster* species did not differ in the anthocyanin accumulation in their leaves (Fig. 1). However, the biochemical analysis revealed that the average concentration of anthocyanins in *C. roseus* was about two times higher than in the other species (Fig. 6).

Based on these results, it cannot be concluded unequivocally that this is indicative of increased stress or stress resistance in *C. roseus* leaves, as the anthocyanin level can be also species specific. Moreover, many studies indicate that anthocyanins are not an unequivocal marker of stress in plants[64]. On the other hand, the elevated anthocyanin content in the *C. roseus* leaves is interesting in view of its potential application as a raw material with medicinal or health-promoting properties[2]. Health benefits of anthocyanins have been widely described, especially due to their antioxidative activities. Anthocyanin isolates and anthocyanin-rich mixtures of flavonoids may provide protection from DNA cleavage, estrogenic activity, enzyme inhibition, boosting production of cytokines, lipid peroxidation, decreasing capillary permeability and fragility[65,66]. Anthocyanins have been used to reduce inflammation and prevent cardiovascular and neurodegenerative diseases, liver dysfunction, hypertension, microbial infections, diarrhoea, and vision disorders[67,68]. Furthermore, recent evidence suggests that the health-promoting effects attributed to anthocyanins may also be related to modulation of gut microbiome[69]. The increasing interest in the anti-inflammatory and anti-acne effects of anthocyanin-rich plant extracts is also worth noting[70].
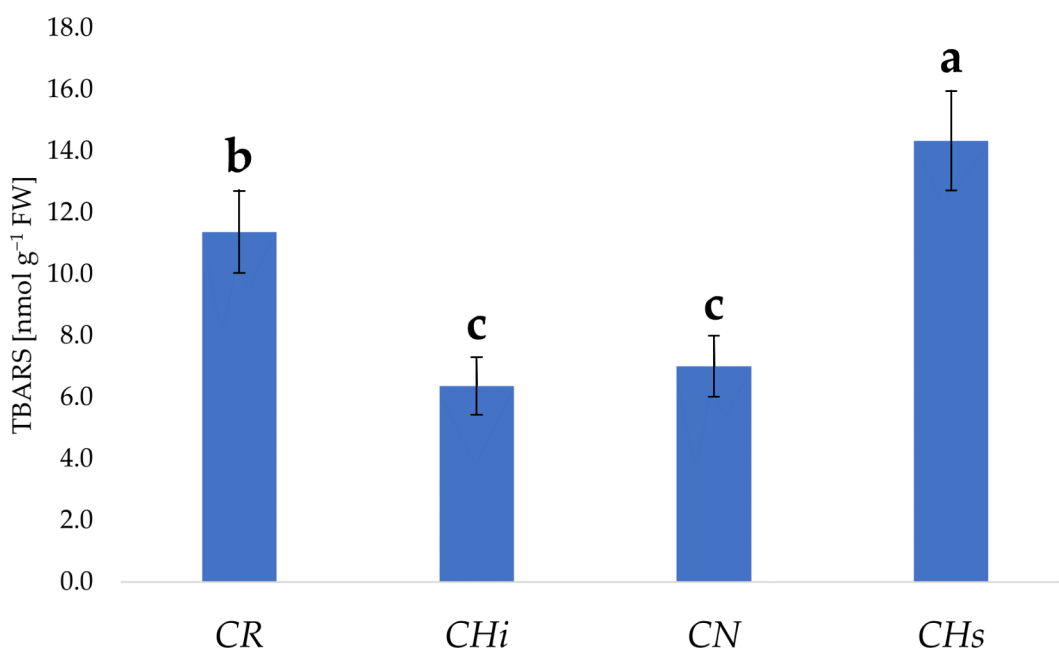
Fig. 5. Lipid peroxidation level expressed in terms of the concentration of thiobarbituric acid reactive substances (TBARS) in the leaves of *Cotoneaster roseus* (CR), *C. hissaricus* (CHi), *C. nebrodensis* (CN), and *C. hsingshangensis* (CHs). Different letters indicate statistically significant differences.
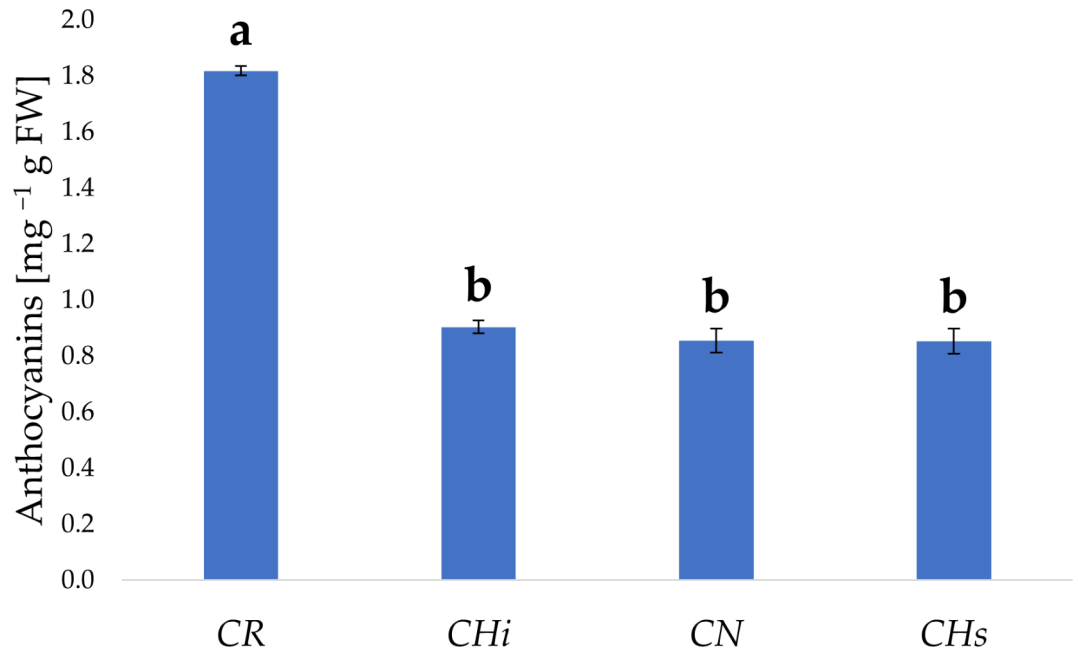
Fig. 6. Concentrations of anthocyanins in the leaves of *Cotoneaster roseus* (CR), *C. hissaricus* (CHi), *C. nebrodensis* (CN), and *C. hsingshangensis* (CHs). Different letters indicate statistically significant differences.

Principal component analysis

The principal component analysis (PCA) of the selected physiological parameters related to photosynthetic efficiency, lipid peroxidation levels, and pigment concentrations in the leaves of the four *Cotoneaster* species distinguished four groups corresponding to the species (Fig. 7a, b). The first component explained 43.45% of the total variance, and it was most strongly and negatively correlated with lipid peroxidation, Rfd, QY, and F_m. Chlorophyll $a+b$, carotenoids, and anthocyanins were positively correlated with the second component, which explained 29.65% of the variance (Fig. 7a). Overall, the two principal components explained 73% of the variance of the original variables. PC 1 facilitated the separation of *C. nebrodensis*, characterised by the lowest lipid peroxidation level, Rfd, and QY, from *C. roseus* and *C. hsingshangensis*, showing the highest values of these parameters. *Cotoneaster roseus* was characterized by the highest contents of all pigments in contrast to *C. hsingshangensis*, as determined by the PC2. However, *C. hissaricus* was hardly influenced by these factors, as it showed the most average values of all the parameters.

Conclusions

The morphological and anatomical analyses showed that the leaves of the four species studied were fairly similar to each other, typical for the genus *Cotoneaster*, and did not show any anomalies or stress symptoms. The photosynthetic efficiency had the relatively lowest value in *C. nebrodensis* but this was definitely not due to increased oxidative stress. On the other hand, *C. hsingshangensis* exhibited a significantly higher lipid peroxidation level and lower photosynthetic pigments concentrations than the other species; however, it did not negatively influence its photosynthetic capacity as confirmed by relatively highest values of PSII quantum yield and vitality index. Since the four examined *Cotoneaster* species were growing outdoor in the same site and no experimental stressful conditions were applied during the course of the experiment to any of them, it is not possible to state unequivocally whether they respond similarly to changing environmental conditions, climate anomalies etc. Further research on the influence of different factors at supraoptimal concentration/intensity and conducted under controlled conditions should be performed on these species in order to compare their stress response at the cellular and molecular level. Our results could be used as a reference for these studies. The observed by us differences between the studied species may be species specific and genetically established and not indicate their sensitivity to non-native growth conditions. On the contrary, leaf structure and markers of biotic and abiotic stress, such as photosynthetic pigment concentrations along with the parameters of chlorophyll *a* fluorescence, level of lipid peroxidation and anthocyanin accumulation clearly showed a good physiological state of the studied plants confirming thus that they are well adapted to growth out of their natural habitats. Therefore, all the four *Cotoneaster* species studied can be grown in the climatic conditions of central and eastern Europe, which highlights their potential to be used for pharmaceutical purposes.
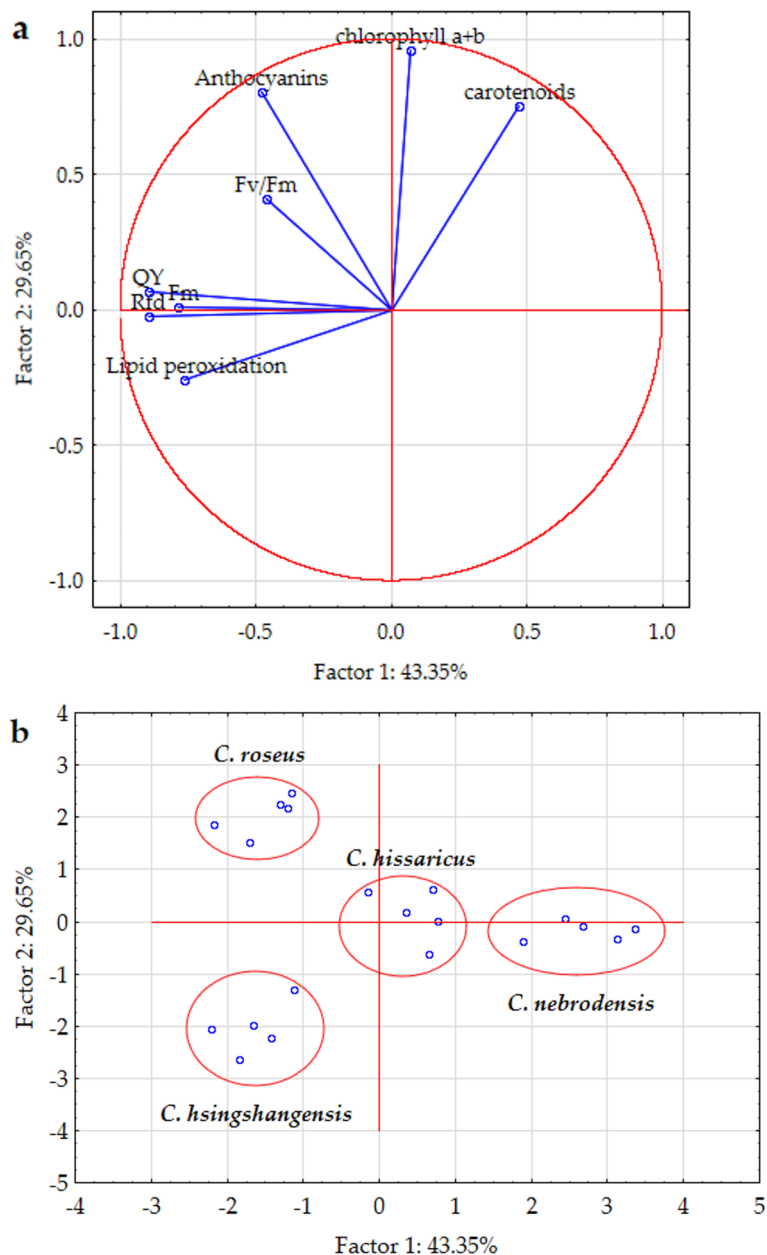
Fig. 7. Principal component analysis of physiological parameters determined in the leaves of *Cotoneaster roseus*, *C. hissaricus*, *C. nebrodensis*, and *C. hsingshangensi*s. (**a**) Projection of variables on the factor plane; (**b**) projection of cases on the factor plane.

Data availability

Data is provided within the manuscript, any further data available from M.W. upon request.

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Author contributions

Conceptualization: B.K., I.B., K.D.S.S., M.W.; Methodology: B.K., I.B., D.T., M.W.; Formal analysis: M.M.; Investigation: B.K. collected the plant material; B.K. and D.T. performed morphological and histochemical analyses; B.K. and I.B. performed physiological analyses; B.K., D.T., and M.W wrote the main manuscript text; K.D.S.S., A.V. and J.V. reviewed the manuscript; B.K., D.T., and M.M. prepared figures. All authors read and approved the final manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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