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Research Article

Rubus plicatus Weihe & Nees: resilience to pollution caused by stone quarries

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Abstract

This study aimed to analyze the effect of pollution caused by stone quarries on the morpho-anatomy, biochemistry, and physiology of a medicinal wild bramble *Rubus plicatus* Weihe & Nees. Samples were collected from two natural protected areas: Iron Gates Natural Park and Jiu Gorge National Park, both located in the southwestern part of Romania, and two unpolluted areas from these parks as background sites. We carried out the following analyses on the collected leaves of this taxon: morphology, micromorphology, anatomy, assimilating pigments, heavy metals (Pb, Ni, Cr, Fe), dry mass, bioactive compounds (total phenols and flavonoids), and antioxidant capacity. The results showed more stomata, higher amounts of assimilating pigments, higher amounts of heavy metals (especially lead), less dry mass, less phenols, and more flavonoids in *Rubus plicatus* leaves from polluted areas compared to areas without sources of pollution. The increased number of stomata and the amounts of assimilator pigments revealed the mechanisms developed by this species in order to survive in polluted conditions.

Key words: Chlorophyll, dust, flavonoids, metals, micromorphology, phenols, stomata, structure

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Introduction

Plants have been widely used in the food industry as well as in the pharmaceutical industry, with consumers and researchers showing an increasing interest in these products because they are an important source of flavonoids (Brodowska 2017; Bjørklund et al. 2018; Konieczynski et al. 2021). However, the quality and quantity of these plant chemical metabolites are influenced by a multitude of factors, most of them environmental (Ncube et al. 2012; Perrino et al. 2023). Plants constantly and rapidly interact with potentially harmful external environmental factors (Ncube et al. 2012). Being immobile organisms, throughout their evolution, plants had to develop alternative defense strategies, among which we now mention the biochemical ones, respectively secondary metabolites, which are important in helping plants adapt to the environment (Holopainen and Gershenzon 2010). On the other hand, these secondary metabolites offer a diverse range of benefits to humans, which include, among others, medicinal properties (Casella et al. 2023). Plant responses to environmental cues are specific (Ncube et al. 2012). A multidisciplinary analysis of this topic, combining ecology, biochemistry, and molecular physiology, would have great potential to reveal the extent to which plant-environment interactions contribute to phytomedicine. The medicinal quality of plants is determined in the field (Ncube et al. 2012). Heavy metals, in addition to direct their effects on the plant communities (Perrino et al. 2014), have the potential to induce the production of secondary metabolites in medicinal plants, however, high levels of this can suppress it (Nasim and Dhir 2010). So, heavy metals induce the production of secondary metabolites in medicinal plants. Metal contamination can change the chemical composition of plants, and modify the quantity, quality, safety, and efficacy of natural plant extract (Murch et al. 2003; Lajayer et al. 2017; Nobahar et al. 2021; Pandey et al. 2023), including the genus *Rubus* (Micu et al. 2016).

On the other hand, the biodiversity of medicinal plants is suffering a great loss globally, along with other animal and plant species (Halder and Jha 2023). Another alternative source to the collection from wild flora, which could potentially ensure the uniform supply of phytotherapy quality, would be their cultivation in vitro, in cell cultures, and in bioreactors, where the manipulation and maintenance of environmental factors are controlled (Bruce and West 1989; Ncube et al. 2011).

Chromium (Cr), iron (Fe), nickel (Ni), and other 14 trace elements are essential for the plants (Kabata-Pendias and Pendias 2001), while lead (Pb), cadmium (Cd), mercury (Hg), and arsenic (As) are non-essential, with unknown biological functions and are toxic to plants and human even at low concentrations (Shahid et al. 2017). Their presence can be caused by natural sources, but a dramatic increase in these elements is linked to anthropogenic activity (Sarma et al. 2012), such as transportation (De Lurdes Dinis and Fiúza 2011), and illegal waste dumping (Perrino et al. 2014). In the food chain, heavy metals from air, water, and soil can be absorbed by plants and cause their metabolism to malfunction, as primary producers, but also are a risk, already present in unmonitored territories, to human health as consumers (Shahid et al. 2017). Plants, including medicinal plants, use different strategies to deal with heavy metals that enter their cells, and the tolerance to a particular heavy metal is controlled by a complex interdependent of morphological, physiological, biochemical, and genetic mechanisms (Maleki et al. 2017), with some plants that have specialized in absorbing the heavy metals as the species belonging to the Brassicaceae family (choose reference). The most frequently highlighted and earliest result of heavy metal stress in plant cells is the excessive generation of ROS (Reactive Oxygen Species) (Schutzendübel and Polle 2002; Das and Roychoudhury 2014). Unlike physiologically non-redox-active heavy metals such as As, Cd, Co (Cobalt), Hg, Mn (Magnesium), Ni, Pb, and Zn (Zinc), free redox-active metals such as Cr, Cu (Cooper), and Fe directly increase ROS production (Kovacik and Backor 2008). To eliminate ROS overproduced by heavy metal stress, plants use specific mechanisms, including the activation of antioxidant enzymes (Blokhina et al. 2003), and non-enzymatic antioxidants such as carotenoids (Mittler 2002). Specifically, Cr represents a heavy metal that is very dangerous to humans when is linked to oxygen. Through the oxidation states of peroxo Cr(V) intermediates or through the action of ROS oxidative DNA damage occurs (Bokare and Choi 2011). Many phenolic compounds, including flavonoids and anthocyanins, are well known for their role in increasing tolerance to heavy metals (Keilig and Ludwig-Müller 2009).

Rubus plicatus Weihe & Nees (Rosaceae family) leaves are rich in tannins and also contain a notable amount of flavonoids, phenolic acids, but also triterpenes, mineral salts, and vitamin C (Gudej and Tomczyk 2004; Zia- Ul-Haq et al. 2014; Abu-Shandi et al. 2015; Oszmiański et al. 2015; Bhatt et al. 2023). This taxon has often been studied and recommended for biomonitoring of contaminated areas (Nujkić et al. 2016), but it accumulates Pb poorly (Steingräber et al. 2022). Air pollution is widespread in a consumerist society. The main air pollutants are gases such as carbon dioxide (CO_2), sulfur dioxide (SO_2), ammonia (NH_3), ozone (O_3), volatile organic compounds, and carbon monoxide, or heavy metals such as Cd, Cr, Fe, Mn, Ni, Pb, etc. (Sawarkar et al. 2023). The increase in demand for quality construction stones and sand has led to the intensive mining of hard rock in many parts of the world (Ukpong 2012; Vandana et. al. 2020). Size and crushed stones are the final output of such an industry, and they are used for various purposes, such as the construction of new roads and highways (Nartey et al. 2012). Many species of *Rubus*, including the one studied here, are present in different types of human impact: railways, roads, and quarries (Sargent 1984; Dehaan et al. 2007; Gentili et al. 2011). Unfortunately, these exploitations harm the environment because they involve the presence of heavy machines and explosives, exhaust gases, and dust; these activities are associated with air pollution, noise pollution, biodiversity damage, and habitat destruction (Lameed and Ayodele 2010). Sayara et al. (2016) concluded that it is recommended to develop a green belt in the surroundings of the exploitation site, using pollution-tolerant trees (usually with broad leaves) to limit the spread of quarry dust by intercepting, filtering, and absorbing pollutants. The high cost of instrumental monitoring methods and difficulties in sampling limited the studies on atmospheric contamination. These are the reasons why there is more interest in the use of indirect monitoring methods such as the use of bioaccumulators organisms, as higher plants (Bargagli 1998). The use of plant leaves, primarily as accumulators and biomonitors of trace metal pollution, has gained great ecological importance (Mulgrew and Williams 2000).

When stone quarries are near or within protected areas, protecting these ecosystems is as important as protecting humans from pollution (Ukpong 2012; Vandana et. al. 2020). This is the reason why in the present study we proposed and analyzed various parameters of the plants, to identify the impact of the pollution caused by the presence of stone quarries on them. The hypothesis we started from was that stone quarries affect the structure and growth of plants from the genus *Rubus*, with the possibility of developing morpho-functional resilience to survive.

Materials and methods

Plant material

The plant material consisted of *Rubus plicatus* leaves, taken in September 2023, from two protected areas in Romania, Jiu Gorge National Park (**JGNP**) and Iron Gates Natural Park (**IGNP**), both from unpolluted areas: Bratcu

Valley (DDM: 45°13.6933'N, 23°21.5983'E), respectively Sirinia Valley (DDM: 44°39.105'N, 22°4.3233'E), as well as around stone quarries in these parks: Meri Quarry (DDM: 45°13.08'N, 23°22.5967'E), and Eșelnița Quarry (DDM: 44°43.9883'N, 22°20.8767'E). We used Google Maps (2023) to take the GPS coordinates in the WGS84 geographic system. The leaves were taken from the middle of the stem of five shrubs at each collection point. They were kept cold in polyethylene bags during transport and storage in the laboratory. The analyses of morphology and assimilating pigments were carried out within a maximum of 48 hours from the sampling. For microstructure and anatomy analyses, leaf fragments were preserved in 70% ethyl alcohol. For quantification of bioactive compounds and antioxidant capacity, fresh leaves were dried at 40 °C for 7 days.

Samples for morphological, micromorphological, and anatomical study

The technique of imprinting the epidermis with a collodion film was used to determine stomatal density, and the counting was carried out under 200× magnification. The morphology of the leaf was analyzed with an Olympus SDF PLAPO 1XPF stereomicroscope with an Olympus UC 50 camera, and the morphology of the epidermis and the structure of the leaf limb were studied with an Olympus IX73 Inverted LED microscope with an Olympus UC50 camera. In addition, scanning electron microscope (SEM) observations for micromorphology were performed at the median level of the leaves, as follows: after dehydration, up to 96% alcohol concentration, leaf samples mounted on hubs, covered with 2 mm gold, in a sputter coater and examined at 8.00 KV, at SEM LEO 436VP. Transverse sectioning was done manually, at the median level of the leaf limb, in the direction of the main rib; 30 sections were analyzed for each sample. The abbreviations for morphological measurements are: **AdECL** adaxial epidermis cell length; **AdECW** adaxial epidermis cell width; **AdESN** adaxial epidermis stomata number; **AbECL** abaxial epidermis cell length; **AbECW** abaxial epidermis cell width; **AbESN** abaxial epidermis stomata number; **AbESL** abaxial epidermis stomata length; **AbESW** abaxial epidermis stomata width.

Assimilating pigments analysis

Fresh leaves were cut into small pieces, 2.5 mg weighted, and homogenized with 5 ml DMF (N, N-dimethylformamide). The mixture was kept at 4 °C for 72 h. At the time of the reading, a 10× dilution was also made with DMF. Measurements were performed in three replications with a T60 UV-VIS spectrophotometer.

For quantifying chlorophylls (Chl), namely Chl *a*, Chl *b*, or total Chl, the equations necessary were: Chl $a = 12.70A_{664.5} - 2.79A_{647}$; Chl $b = 20.70\ A_{647} - 4.62A_{664.5}$; total chlorophyll pigments Chl $a{+}b = 17.90A_{647} + 8.08A_{664.5}$, where in 1.00-centimeter cuvettes and Chl = chlorophyll in milligrams per liter (Inskeep and Bloom 1985). Total carotenoids (Car) were calculated with the formula: Car = $1000A_{480} - 0.89$ Chl a – 52.02 Chl $b/245$ (Wellburn 1994). The extinction coefficients necessary for the quantification of Chl *a*, *b*, and total Chl were determined by the Moran (1982) method.

Heavy metals

The dried leaf samples were mineralized with a Speedway Xpert microwave digestion system in 10% nitric acid solution. The metals were analyzed by atomic absorption spectrometry (AAS), Cr (χ - 357 nm), Ni (χ - 232 nm), and Pb (χ - 217 nm), by GF-AAS (graphite furnace), and Fe (χ - 248 nm) by FL-AAS (air-acetylene flame), using ZEEnit 700P spectrometer. To plot the calibration curve, standard solutions of concentrations were prepared: for Pb - 3, 12, 30, 45, and 60 µg/L; for Ni: 2, 5, 10, 15, and 20 µg/L; for Cr: 2.5; 5; 7.5; 10 µg/L; for Fe 0.1; 0.5; 1; 2; 3 mg/L).

Total biomass

The dry biomass of the *Rubus* leaves was weighed after 2 g of fresh leaves from each category of leaves was desiccated.

Determination of bioactive compounds. The total phenols content (TPC), total flavonoids content (TFC), and antioxidant capacity (ferric reducing antioxidant power - FRAP and free radical scavenging capacity - DPPH)

The powder of each leaf sample (10 mg) was suspended for 48 h in ethanol 70%. 10 ml of this mixture was centrifuged for 20 minutes at 5000 rpm, and the resulting supernatant was collected for analysis. The **TPC** was determined using the Folin-Ciocalteu method (Singleton et al. 1999), with some modifications. Briefly, 100 µL diluted samples (1:20, v/v) were combined with 1700 µL of distilled water. Subsequently, 200 µL Folin-Ciocalteu reagent (freshly diluted 1:10, v/v) was added and mixed using a homogenizer. Then, 1000 µL of 7.5% Na_2CO_3 solution was mixed again. The mixture was incubated at room temperature, in the dark, for 2 h. The absorbance measured at 765 nm was noted in mg gallic acid equivalents (GAE)/g of dry weight (DW), based on the calibration curve. For **TFC** the aluminum chloride colorimetric method was used. Summarily, 1000 µL diluted extract sample, 4000 µL distilled water, and 300 µL $NaNO_2$ 5% were combined. After 5 minutes, 2000 µL of 1 M NaOH, and after another 6 minutes, 300 of µL 10% $AlCl_3$ were added. Lastly, distilled water was used to complete the volumetric flask to bring the volume up to 10 ml (Pekal and Pyrzynska 2014). The absorbance values measured at 510 nm were reported as mg quercetin equivalents (QE)/g DW. **FRAP** was performed according to Benzie and Strain (1996) with some modifications. Therefore, 100 µL diluted samples (1:20, v/v) were mixed with 2000 µL distilled water and 500 µL FRAP reagent. The mixture was held for 1 h in the dark, and after that the absorbance was measured at 595 nm. The **DPPH** (2.2-diphenyl-1-picrylhydrazyl) was performed according to Brand-Williams et al. (1995) with some modifications. A portion of the 100 µL diluted sample was mixed with 2.8 ml DPPH solution (80 µM) and kept for 30 minutes in the dark. The percentage of the DPPH scavenging effect of samples was calculated using formula 1.

$$\text{The percentage of DPPH scavenging effect (\%)} = [(A_0\text{-}A_s) \times 100]/A_0 \qquad (1)$$

where A_0 is the absorbance of the blank, and A_S is the sample absorbance, both monitored at 517 nm. All absorbances were measured using a spectrophotometer Shimadzu UV mini-1240 UV-VIS.

Statistical analysis

The raw data were processed mathematically and statistically, calculating the mean and the standard deviation for three replications. The significance between polluted and unpolluted areas was determined by a Two-Sample *t*-test by MICROSOFT OFFICE16\EXCEL software.

Results

Morphologically, the stereomicroscope observations revealed major changes between the leaves taken from the unpolluted area and those from the quarry-polluted area (Fig. 1). Thus, compared to the control leaves (Fig. 1A, B, E, F), the leaves from the polluted areas, respectively the Meri quarry (Fig. 1C, D) or the Eșelnița quarry (Fig. 1G, H) showed lesions and necrosis. In Meri and Eșelnița quarries but also in Sirinia Valley, deposits of insect droppings were identified on the abaxial side of the leaves (Fig. 1D).

The upper epidermis is devoid of stomata, and these and numerous tector hairs are arranged on the abaxial surface (hypostomatic) (Fig. 2).

Following biometry of epidermal cells in the leaves of the blackberry bushes from both operating stone quarries from the two parks, smaller epidermal cells were observed, compared to those of the leaves from the unpolluted areas, both located on the adaxial and abaxial faces; some differences were significant from a statistical point of view (Table 1). On the other hand, in the polluted sites, the number of stomata per unit area was significantly higher compared to the control sites (Table 1).

In Sirinia Valley, the blackberry leaves had about 5 times larger epidermal cells than those grown in Bratcu Valley, and the number of stomata per unit area was lower (Table 1). All stomata were closed at all sampling points. The lesions were observed macroscopically and morphologically (Fig. 3). If in the unpolluted sites of the two valleys, the leaf tissues have a normal structural appearance, in the lamina sections of the leaves from plants grown in the quarries area, the parenchyma lesions were either subepidermal (Fig. 3B) or periphloemic (Fig. 3D). Also, in the quarry area, dust particles were caught (Fig. 3E) on the abaxial side of the leaf. On the upper part of the leaf, the dust particles were larger and fewer than on the lower part of the leaf, where they were smaller but numerous. Tector hairs were rare on the adaxial side and more numerous on the abaxial side of the leaves. Comparatively, between the sample of control leaves and those from the quarries, the tector hairs were more numerous in the latter, especially on the lower epidermis.

The amount of assimilating pigments was increased in the polluted areas compared to the unpolluted areas (Table 2), but in the Iron Gates Natural Park, at both points, it was higher than in similar areas in the Jiu Gorge National Park (Table 2). The amount of chlorophyll *a* was higher in all cases than the amount of chlorophyll *b* (Table 2), and the ratio between the two is relatively lower in both quarriers, versus the control (Table 2). We notice a more intense increase in the Chl/car ratio, of 2.75, in Sirinia Valley, caused by the low values of carotenoids, while in the Eșelnița quarry, we measured the highest value of carotenoids (Table 2).

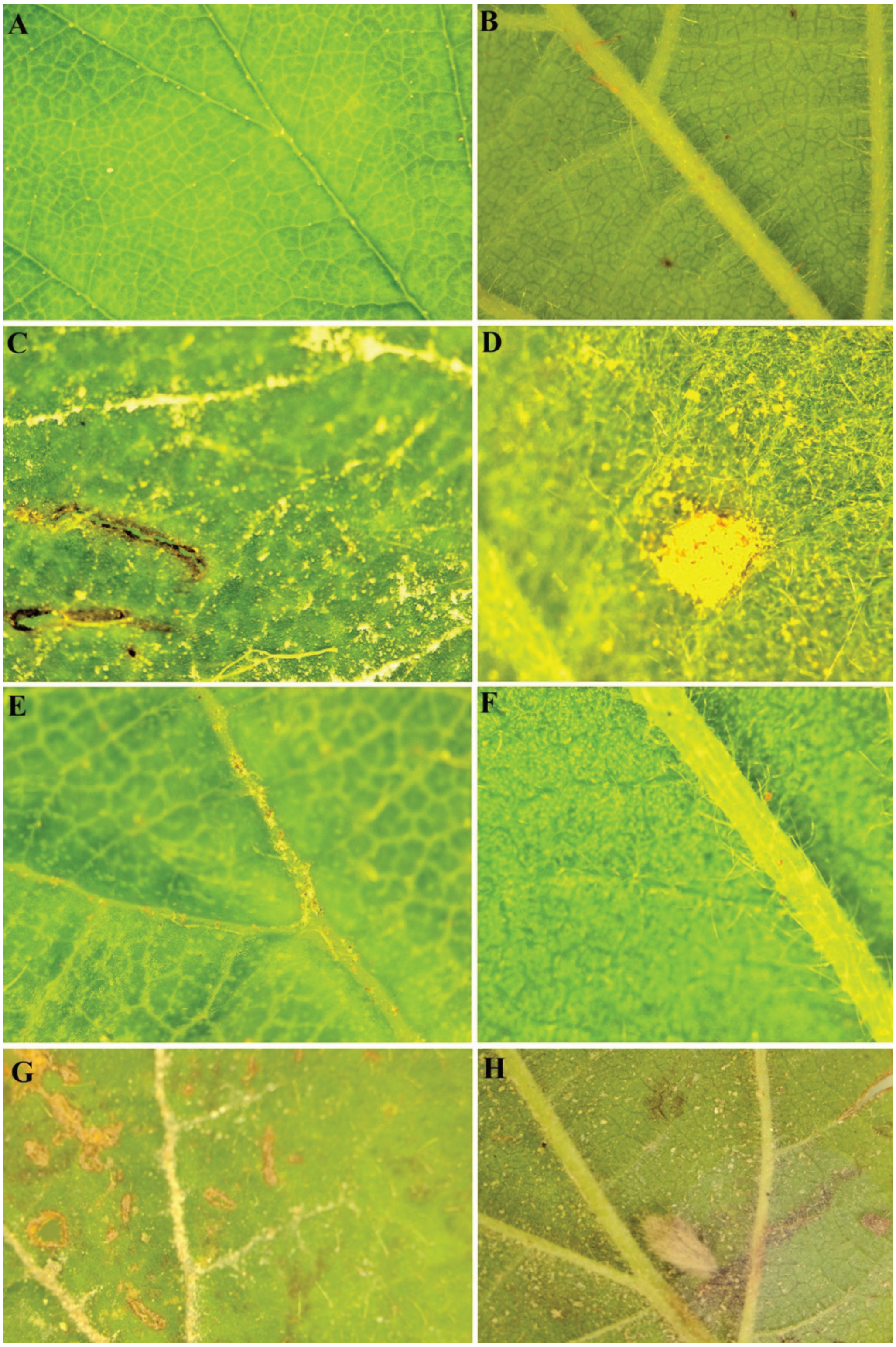

Figure 1. The morphology of the blackberry leaf (*Rubus plicatus Weihe & Nees*) captured with the stereomicroscope, depending on the sampling point. Jiu Gorge Natural Park: Bratcu Valley (control) **A** adaxial surface (4×) **B** abaxial surface (2×); Meri quarry **C** adaxial surface, with lesions (4×) **D** abaxial surface, with insect egg deposits (4×); Iron Gates National Park: Sirinia Valley (control) **E** adaxial surface (2×) **F** the abaxial surface (4×); Eșelnița quarry **G** the adaxial surface, with necrosis (2×), **H** abaxial surfaceface, with necrosis, insects and dust (1×).

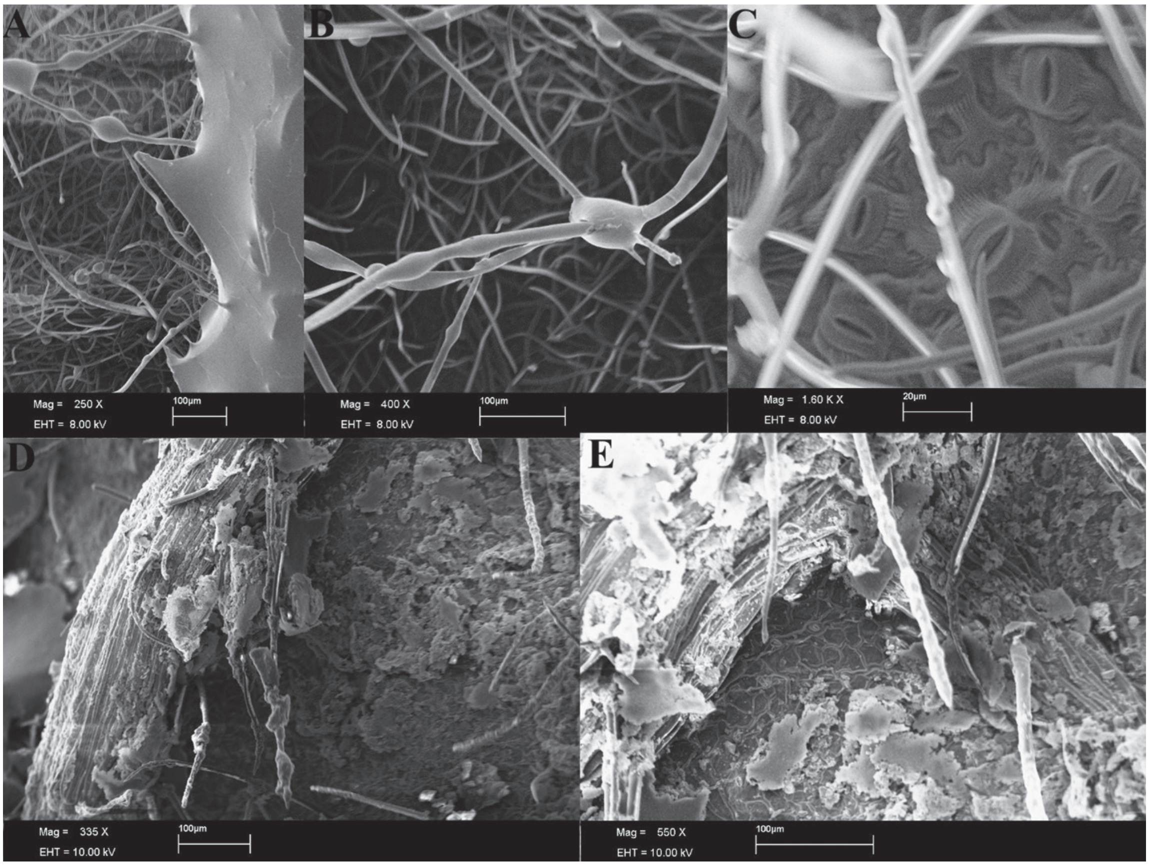

Figure 2. Photomicrographs with epidermal formations in the blackberry (*Rubus plicatus* Weihe & Nees). Unpolluted area **A** thorns (250×) **B** tector brushes (400×) **C** stomata on the abaxial epidermis (1.60 K×), and polluted area **D** epidermis with particle deposits in the leaves from the Eșelnița quarry (335×) and **E** Meri quarry (E – 550×). Scale bars: 100 µm (**A, B, D, E**); 20 µm (**C**).

Table 1. Parameters of the foliar lamina (AdECL adaxial epidermis cell length; AdECW adaxial epidermis cell width; AdESN adaxial epidermis stomata number; AbECL abaxial epidermis cell length; AbECW abaxial epidermis cell width; AbESN abaxial epidermis stomata number; AbESL abaxial epidermis stomata length; AbESW abaxial epidermis stomata width) (average ± standard deviation).

Protected area	Sample	AdECL (µm)	AdECW (µm)	AdESN	AbECL (µm)	AbECW (µm)	AbESN	AbESL (µm)	AbESW (µm)
Jiu Gorge	Bratcu Valley	33.37±1.15	21.99±2.21	0.00±0.00	20.302±2.45	14.05±2.75	19.1±0.55	21.62±2.23	17.11±2.91
	Meristone quarry	30.0±0.71	18.2±1.48	0.00±0.00	19.1±0.89	12.80±1.30	22.8±0.84	22.2±1.10	18.20±1.53
	t -test	0.001	0.01	-	0.34	0.39	0.0002	0.62	0.48
Iron Gates	Sirinia Valley	164.00±6.57	85.29±9.20	0.00±0.00	17.66±1.82	13.18±0.26	17.2±1.30	22.8±1.64	18.96±0.57
	Eșelnița stone quarry	155.0±0.71	72.8±1.48	0.00±0.00	16.4±1.34	12.2±1.10	23.6±0.89	23.6±1.14	20.26±1.24
	t-test	0.295	0.036	-	0.251	0.116	0.000	0.400	0.080

Note: p<0.01 – statistically very significant; p<0.05 – distinctly significant; p<0.1 – significant (s); p>0.1 ns– non-significant. The values measured in unpolluted areas were the reference (control).

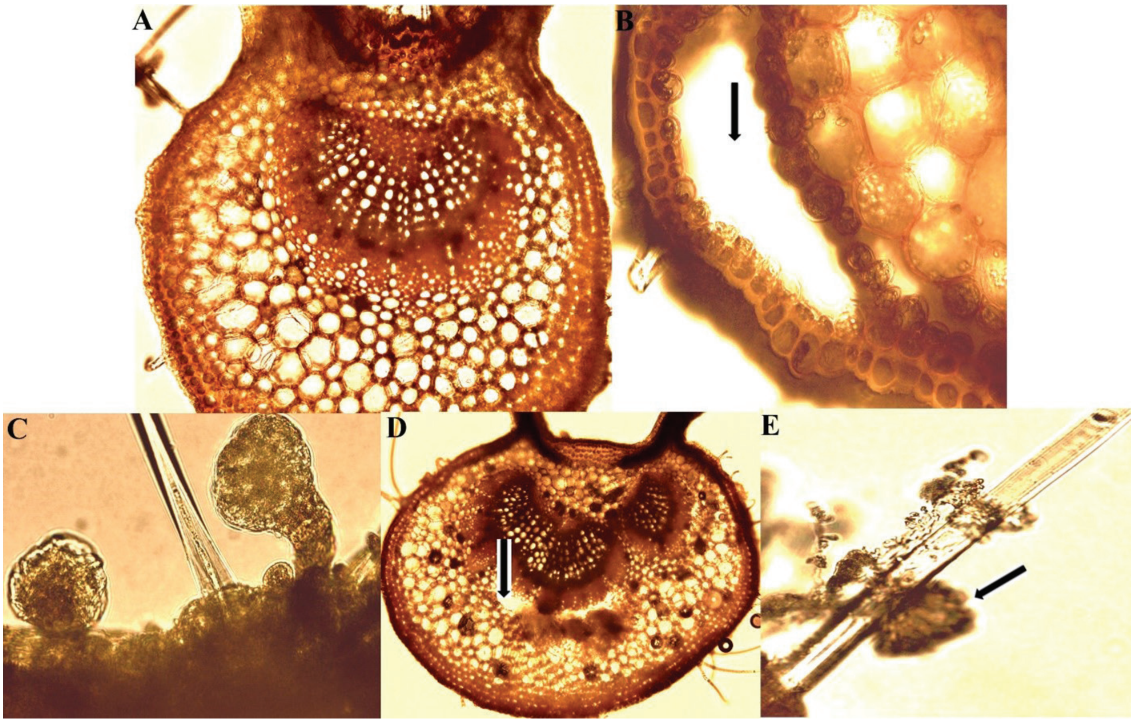

Figure 3. Blackberry leaves structure (*Rubus plicatus* Weihe & Nees). Provided from Jiu Gorge National Park **A** normal aspects in Bratcu Valley (200×) **B** with parenchyma lesions (row) from Jiu Gorge, Meri stone quarry (600×); from Iron Gates Natural Park **C** without dust in Sirinia Valley (1000×) **D** with lesions in the periphloem parenchyma (row) (100×), and **E** dust (row) on tector hair (1000×) in Eșelnița stone quarry.

Table 2. The assimilating pigment content of *Rubus plicatus* Weihe & Nees leaves from unpolluted and polluted areas (stone quarry) (average ± standard deviation).

Protected area	Sample	Chl *a* (mg L^{-1})	Chl *b* (mg L^{-1})	Car (mg L^{-1})	Chla/*b*	Chl *a*+Chl *b* (mg L^{-1})	Chl/Car
Jiu Gorge	Bratcu Valley	1.92±0.5	1.45±0.7	2.55±0.3	1.33	3.37±0.4	1.32
	Meri stone quarry	3.21***±0.6	2.51**±0.9	3.66**±0.6	1.28	5.72***±0.5	1.56
Iron Gates	Sirinia Valley	7.32±1.2	6.02±1.1	4.84±1.2	1.22	13.33±0.9	2.75
	Eșelnița stone quarry	9.38***±0.9	9.09***±1.4	9.26***±2.1	1.03	18.46***±1.2	1.99

Note: p<0.01 – ***statistically very significant; p<0.05 – **distinctly significant; p<0.1 – *significant (s); p>0.1 ns– non-significant. The values measured at unpolluted areas were the reference (control).

The anthropogenic activity in stone quarries increased the concentration of all heavy metals measured by us (Pb, Ni, Cr, Fe) in the vegetation and decreased the dry mass (Table 3). Among non-essential heavy metals, lead was in higher quantities in Iron Gates Natural Park, especially in the area of the stone quarry (Table 3).

The blackberry leaves from the Meri quarry contained higher amounts of flavonoids and DPPH than those of the control group (Table 4). The lowest amount of flavonoids was recorded in the Eșelnița quarry (Table 4).

Table 3. Median concentration (mg/100g dry leaf) of Pb, Ni, Cr, Fe in *Rubus plicatus* Weihe & Nees leaves (average ± standard deviation).

Protected area	Sample	Pb	Ni	Cr	Fe	Dry weight
Jiu Gorge	Bratcu Valley	0.015±0.1	0.009±1.1	0.026±0.1	6.72±1.2	1.003±0.02
	Meri stone quarry	0.079***±0.3	0.046***±1.4	0.065**±0.2	23.35***±1.1	0.68***±0.03
Iron Gates	Sirinia Valley	0.024±0.2	0.015±0.7	0.03±0.1	12.09±1.5	0.76±0.02
	Eșelnița stone quarry	0.096***±0.4	0.165***±0.6	0.228***±0.7	86.56***±2.1	0.62**±0.08

Note: p<0.01 – ***statistically very significant; p<0.05 – **distinctly significant; p<0.1 – *significant (s); p>0.1 ns– non-significant. The values measured at unpolluted areas were the reference (control).

Table 4. The total phenols content, total flavonoids, and antioxidant capacity (average ± standard deviation).

Natural protected area	Sample	TPC (mg GAE/ g)	FRAP (mmol TE/g)	TFC (mg QE/g)	DPPH (mmol TE/g)
Jiu Gorge	Bratcu Valley	85.48±3.89	0.36±0.007	84.72±8.45	326.08±8.97
	Meri stone quarry	75.71±1.13	0.37±0.001	189.97±5.32	346.95±1.45
	t -test	0.15	0.56	0.01	0.18
Iron Gates	Sirinia Valley	62.54±1.33	0.36±0.007	47.59±47.5910	337.74±3.47
	Eșelnița stone quarry	32.00±2.35	0.29±0.011	31.86±1.70	330.58±6.66
	t -test	0.010	0.024	0.057	0.355

Note: p<0.01 – statistically very significant; p<0.05 – distinctly significant; p<0.1 – significant (s); p>0.1 ns– non-significant. The values measured at unpolluted areas were the reference (control).

Discussion

Morpho-anatomy of the leaves

Lesions, necrosis, and insect attacks on shrub leaves signal the plants' suffering, even at a macroscopic level. Moreover, they suggest cellular and functional stress of the affected organisms. Some species, such as blackberry, manage to survive even in less favorable conditions. The blackberry succeeds in occupying different types of habitats through the adaptations it has, accumulated throughout evolution, such as hypostomatic leaves, tector hairs, and thorns, but also through the ability to adapt short term to environmental conditions, such as identified in the present study. One adaptation was manifested by the increase of the number of stomata on the leaves of plants located in the particulate matter (PM) pollution area to support the supply of gases in vital processes, such as respiration and photosynthesis. However, all types of air pollutants, especially PM, have potentially harmful impacts on morphological, physiological, and biochemical parameters, which can further reduce plant growth and development, e.g., they can cause direct chronic injury (chlorosis) and productivity losses (premature leaf death, reduced height growth) (Rai 2016). In contrast, many plants show no visible changes because, much more often, plant changes occur at the anatomical and biochemical level (Rai 2016).

In our case, the fact that the epidermal cells of the leaves were smaller in polluted areas compared to unpolluted ones, led to an increase in the density of stomata. This increase can also be explained by the fact that the leaves are covered by impurities, which cause stomata to be blocked, and their basic functions to suffer, which led to the adaptation to increasing their number, as already observed in many species of wild plants (Chaudhary and Rathore 2018). It is natural for the density of stomata to increase if the epidermal cell size decreases. PM can be deposited in stomatal openings and disrupt the intensity of respiration and transpiration or even enter stomatal pores and disrupt mesophyll function (Burkhardt and Grantz 2016; Grantz et al. 2018). Long-term changes in stomatal behavior (e.g., stomatal size and stomatal density) Long-term changes of stomatal behavior (e.g., stomatal size and stomatal density) determined by the environment may co-occur across species and genotypes (Reid et al. 2003). Over time, stomata developed adaptative mechanisms to respond to different environmental factors to balance the conflict between the two fundamental processes: photosynthesis and transpiration (Mansfield 1998). Air quality influences morphology (structure of stomata, number of stomata, number of leaves, leaf surface, etc.), along with biochemical influences (ascorbic acid, pigments, enzymes, proteins, and sugar content) and physiological influences (pH and relative water content) (Sharma et al. 2017; Kaur and Nagpal 2017). Moreover, there are plant species that, because they adapt to pollution, can also improve air quality (Łukowski et al. 2018). One such species from the genus *Rubus* is *R. ellipticus* Sm., recommended for the development of green belts due to its high tolerance to air pollution (Sharma et al. 2019).

We also measured cuticle thicknesses in all leaf categories, and the results showed that on the surface of leaves grown in a polluted environment, the cuticle layer was increased, which is another form of adaptation of the cuticle, along with its main role of protection against dehydration, a secondary function of physical protection, by self-cleaning, against dust or pathogens (Yeats and Rose 2013). Thus, the adaxial surface presented a layer of epicuticular wax with dimensions of 3050 μm - 3700 μm (for the leaves from the stone quarries), while in the control samples this layer was 1200 μm - 1670 μm. On the abaxial side, the cuticle was thicker, but much thicker in the leaves from the polluted areas (4170 μm - 4780 μm) compared to those from the unpolluted areas (2100 μm - 2200 μm). The structural aspects we identified confirm the morphologically highlighted damages. The two valleys, even if they are similar from the point of view of pollution, have different climatic conditions, but the fact that in both points of the IGNP the same trend was observed leads to the conclusion that other common environmental factors have influenced this growth.

We observed that the blackberry leaves had fewer hairs on the adaxial epidermis, compared to the abaxial one, which allowed the wind to blow the dust from this level, and prevent its accumulation, which would have led to even stronger shading of the leaf. Only larger dust particles remained on the adaxial side. The capacity of leaves to retain particles from the atmosphere depends on the particle's interactions and their surfaces (Moradi et al. 2017; Soheili et al. 2023), on the species or the shape of the leaf, the presence of hairs allowed a significantly higher accumulation PM (Leonard et al. 2016). Atmospheric dust induces stress on plants similar to drought. Leaves with high tector hair content can retain more dust, causing chlorosis and necrosis on the leaves. Fine particles up to 2.5 μm can penetrate inside the leaf tissues through stomata, leading to the degradation of chloroplasts and assimilating pigments (Soheili et al. 2023). However, in the polluted areas, both on the adaxial and abaxial sides, the density of the tector hairs was higher than in the leaves from the unpolluted areas, as a stress adaptation factor, but leaf tector hairs influence biophysical processes such as light reflectance (Ehleringer et al. 1976).

Assimilating pigments

A similar condition was recorded in terms of the amount of assimilating pigments, which is much higher in the case of plants grown in polluted areas, although air pollution leading to a decrease in the amount of chlorophyll was also reported (Prusty et al. 2005). It would seem that PM mainly decreases plant vigor through a shading effect, whereby accumulated PM absorbs and scatters light rays, preventing them from freely accessing chloroplasts. This is reflected in a decrease in photosynthetic efficiency (Przybysz et al. 2014; Saadullah et al. 2014) and an additional increase or decrease in leaf temperature (Eller 1977), due to albedo-type effects (Sharma et al. 2015). We believe that the shading caused by dust can also lead to an adaptation response of the individuals of some species through the quantitative increase of chlorophyll, just as it happens in shaded plants or transferred to the shade (Brand 1997). Chlorophyll *a* and chlorophyll *b* absorb slightly different wavelengths of light, sun leaves have a higher Chl *a + b* content and higher values for the ratio Chl *a/b* (Lichtenthaler et al. 1981). Plants that are shade-adapted will optimize their growth to suit the conditions, so while plants in the sun will tend to produce more chlorophyll *a*, those in the shade will produce more chlorophyll *b*. Leaves from the more shaded parts will contain more chlorophyll *b* compared to chlorophyll *a* (i.e., the Chl *a/b* ratio will be lower) (Lichtenthaler 2007). The change in the Chl *a/b* ratio is a simple parameter to reveal changes quickly and roughly in light-harvesting complexes (LHCs) (Chazaux et al. 2022). The Chl *a/b* ratio also indicates the quality of the chloroplasts (Lichtenthaler et al. 1981). Thus, the high quality of the granule membranes is indicated by a ratio of 2 whereas a higher value will indicate a degradation of the granule membranes (Danielsson and Albertsson 2008). Carotenoids are essential for photosynthesis and photoprotection and their derivatives are signaling molecules in response to environmental conditions (Sun et al. 2022).

The impact of PM can be far-reaching because their accumulation has significant effects not only on the physiological processes of the whole plant but also on organisms at higher trophic levels, such as folivore insects (Khan et al. 2013; Łukowski et al. 2018).

Heavy metals and antioxidants

It is demonstrated that the effects and toxicity of dust depend on the origin source and quantity (Łukowski et al. 2020). As the leaves are the primary receptors of pollutants (Rai 2016), they are generally used for analysis. Thus, leaves are heavy metal accumulators, just like in the case of Babić et al. (2022), where high accumulations of Pb in *Mentha piperita* L. plants were reported, especially in roots and leaves (up to 1.26 mg kg-1), less in stems, and also in leaves, chromium 0.23 mg kg^{-1}, cadmium 0.12 mg $kg^{1,}$ and manganese 49.88 mg kg^{-1}. In our case, the only variable between the control and tested samples, from the perspective of heavy metal accumulation, is the quarrying activity, which is solely responsible for increasing the concentration of heavy metals in blackberry leaves. Lead, one of the non-essential heavy metals associated with poisoning, was in higher quantities in the Eșelnița quarry in the Iron Gates Natural Park compared to the Meri quarry in the Jiu Gorge National Park.

Absorption of ingested lead can induce, in adults, high blood pressure and cardiovascular disease, fetal neurodevelopmental effects, and reduced learning ability in children (WHO Report 2011). Provisional Tolerable Weekly Intake (PTWI) recommendations are 0.025 mg/kg (25 μg/kg) body weight, i.e., 1.75 mg/week (1750 μg/week) for a person weighing 70 kg (SCOOP 3.2.11. 2004). Maximum levels of lead in vegetable mg/kg, as defined in Commission Regulation (EC) No 466/2001 are between 0.027 mg/kg and 0.1 mg/kg, depending on the European state (SCOOP 3.2.11. 2004).

Heavy metals accumulated in some plants increase the level of antioxidant potential and total phenolic content (Márquez-García et al. 2012; Makuch-Pietraś et al. 2023), but in another species, the increase of heavy metal concentrations (Cu, Ni, Zn) led to the decrease in phenol concentrations (Kulbat-Warycha et al. 2020). The lowest amount of flavonoids was recorded in the Eşelniţa quarry, possibly due to the high amount of Pb, which is identified here as the highest. Thus, in Ukraine (Konieczynski et al. 2021), probably due to the anthropogenic factor, the order of the amount of tested metals in mg/kg dry weight was: Cu < Mn < Zn < Fe. Total flavonoids were found in the range of 7.30–251.60 mg/g dry weight (Konieczynski et al. 2021). The relevant analyses were the level of Zn Mn, and TFC (Konieczynski et al. 2021).

Metals on the leaves associated with vehicle presence were chromium, copper, and manganese found in PM (Leonard et al. 2016).

When plants are exposed to pollutants, reactive oxygen species (ROS) increase, which first leads to oxidative stress and finally causes the cell death procedure (Shahid et al. 2014). Oxidative stress can increase antioxidant production (Williams et al. 2004). In our case, only the total content of flavonoids from the Meri quarry was higher. In plants, flavonoids can increase metal chelation, which leads to a decrease in levels of hydroxyl radicals (Mira et al. 2002; Williams et al. 2004). On the other hand, flavonoids function as a defense mechanism against herbivorous insects and mammals, which is why they contribute to resistance to diseases as constitutive antifungal agents or as phytoalexins in plants (Harborne and Williams 1992). We identified insect eggs on leaves from the Meri quarry. A similar situation was described with the leaf extracts collected both from the cultivated area of a former sodium factory, inactive for 25 years, or from the calamine waste area or the settling pond area formed as a result of material removed during the processing of processes of zinc ores and lead, where a significantly higher DPPH free radical scavenging activity was identified (Czaja et al. 2015).

Conclusions

Blackberry is a species that manages to adapt to the dust pollution of the quarries to survive, through an increased number of stomata, and higher amounts of assimilating pigments in leaves.

Stone quarries are a polluting factor for plant species, even for the most resistant ones, located in the immediate vicinity, consequently, the amount of heavy metals found in blackberry leaves is high. Lead, as a non-essential heavy metal, was identified in much higher quantities in blackberry leaves in the vicinity of stone quarries.

Dust pollution decreased the amounts of phenolics in the leaves of blackberry, a medicinal plant, and increased the total flavonoid content and antioxidant capacity.

To protect the human species from various sources of air pollution, buffer zones, and plant curtains are sometimes established. We also recommend the introduction of such procedures if the source of pollution is in a protected area to reduce the impact between the polluter and the plant and animal species of interest in the protected area. Quarries should be obliged to ensure a buffer space around them.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

All authors contributed to the study's conception and design. AP-V, DNP, DC, FNS, A-RD: material preparation and data collection; AP-V and DNP: morpho-anatomical and physiological analysis; SIV, OS and DNP: biochemical analysis; TOC and DNP: SEM micromorphology; DC: revised it critically for important intellectual content and approved the version to be published. AP-V wrote the first draft of the manuscript. All authors read, commented, and approved the previous and final versions of the manuscript.

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Data availability

All of the data that support the findings of this study are available in the main.

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