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# MORPHO-ANATOMICAL BASIS OF DISCRIMINATION BETWEEN THREE *VIOLA* SPECIES

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**Abstract:** Species in the *Viola* genus form a complex taxonomical group, with several species overlapping in morphological traits. Moreover, some species exhibit significant phenological plasticity, further complicating correct identification. Molecular and micromorphological analyses are used to complement traditional means of identification of the species, but the latter still offer valuable taxonomical information. The current paper assesses in a comparative manner some of the more frequently used morphological and anatomical characteristics of 3 *Viola* species (*V. declinata, V. dacica, V. canina*), for which such data are scant, to offer a basis for further comparison and identification of taxa. Morphological differences, including those assessed through PCA, were recorded mainly for leaves and stipules parameters.

**Keywords:** morphometry, *Viola* sp., endemic species, taxonomy.

## INTRODUCTION

The genus *Viola* L. comprises about 600 species, mostly cosmopolitan to the temperate regions of the world. Most of these species are perennial herbaceous plants, rarely annual herbs or shrubs (Ballard *et al*., 1999; Yockteng *et al.*, 2003; Marcussen *et al.*, 2022). In Romania the *Viola* genus comprises around 30 species (Sârbu *et al*., 2013). The most known species for their use in Romanian traditional medicine are *V. tricolor* L., *V. arvensis* Murray and *V. odorata* L., but also for their potential use in the modern pharmacology, as various studies highlight this (Roberts, 2000; Pană *et al.*, 2012; Muhammad *et al*., 2012; Sârbu *et al*., 2013).

The species of this genus are known for their taxonomic complexity due to the phenotypic plasticity, regional differentiation and interspecific hybridization (Marcussen and Borgen 2000; Mereďa *et al*., 2008; Hodálová *et al.*, 2008). In the differentiation between closely related species, molecular studies are increasingly used, bringing valuable data, but morphological analyses are still the main tool for taxonomical identification, as they can be performed in the field (Oja and Paal, 2005). This is also true for the species of *Viola* genus, which is a very large taxonomical group, and a number of species are closely related and morphologically similar (Migdałek *et al*., 2013). The use of a limited number of morphological characters to delimit taxa and their morphological plasticity has led to wrong biogeographical signalling of taxa that can lead to difficulties in plant conservation. For example, *V. declinata* and *V. dacica* are species that are often confused with each other, due to the fact that their habitat area overlaps, and both species are endemic to the Carpathian Mountain area, so there are ambiguities regarding the systematic classification due to gaps in the scientific literature (Hurdu *et al*., 2012).

The morphological characters that are mainly used to identify species of the genus *Viola* are: the presence or absence of stolons, stipule shape and fimbriation, leaf shape, petals sizes and colour (Scoppola and Lattanzi, 2012; Sârbu *et al*., 2013), pollen morphology (Słomka *et al.*, 2013) and, more recently, micromorphological characters of the leaf surface (Chen *et al*., 2010). When confusion arises between morphological elements it is recommended to use anatomical characters for correct identification of taxa (Yousefi *et al.*, 2012).

*V. declinata* and *V. dacica* are endemic for Carpathians and protected in Romania (Coldea *et al*., 2009; Burescu, 2015). These species are common in the spruce meadows to juniper floor, from 800 to 2000 m altitude and they are characteristic for pastures with *Nardus stricta* L., which grows on siliceous substrate, and together with other species contributes to the quality of mountain pasture (Doniță *et al.*, 2005; Hurdu *et al.* 2012, Togor & Burescu 2013). In Romania, the range of these species overlaps with that of *V. canina*. Due to the high variety of inhabited habitats, *V. canina* may have stipules and leaves of different sizes and different general shape, or flowers of various colors (Beldie *et al.*, 1955; Jonsell *et al.*, 2009; Espeut, 2020).

The morphological and anatomical research on *V. dacica*, *V. declinata* and *V. canina* are scarce, the most comprehensive informations are found in Beldie (1955), Tutin *et al*. (1968) and Metcalfe *et al*. (1972). Through this study we want to bring a few notes on the morphology of these three species. We conducted the first anatomical study for the *V. declinata* and *V. dacica*.

## MATERIALS AND METHODS

The vegetal material from the investigated species was collected from wild populations from Suceava County, North-East of Romanian Carpathians (Table 1). The analysed plants were chosen randomly from different populations found at several altitudes, for each specie, at anthesis, with the majority of flowers opened. The identities of the species were determined according to Sârbu *et al.*,2013.

Shoot length, peduncle height, bracteole length, flower length, sepal length, sepal width, petiole length, stipel length and stipel width were determined using a

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calliper. Leaf area, leaf perimeter, lamina length, lamina width and leaf apex angle, as well as petal total and pedicle length, petal width, petal distance base-maximum width point, spur length for anterior, posterior and lateral petals (Hodálová *et al.*, 2008) were measured on photographs using ImageJ software (Figure 1).

**Table 1.**

The collecting locations for the plant material

| Species | Collecting area | Habitat type |
|---|---|---|
| *V. canina* | Suhard Mountains group - Ouşoru Mountain, 900-1000 m altitude | Meadow |
| | Bucovina Mountains group - Rarău Mountains, 1000-1400 m altitude | Coniferous forest |
| *V. declinata* | Gura Haitii, Călimani Mountains, Dorna Depression, 900-950 m altitude | Meadow |
| | Neagra Șarului, Călimani Mountains, Dorna Depression, 900-950 m altitude | Bank creek vegetation |
| *V.dacica* | Călimani Mountains, 1700 m altitude | Alpine meadow |
| | Călimani Mountains, 1500 m altitude | Alpine meadow with juniper shrubs |

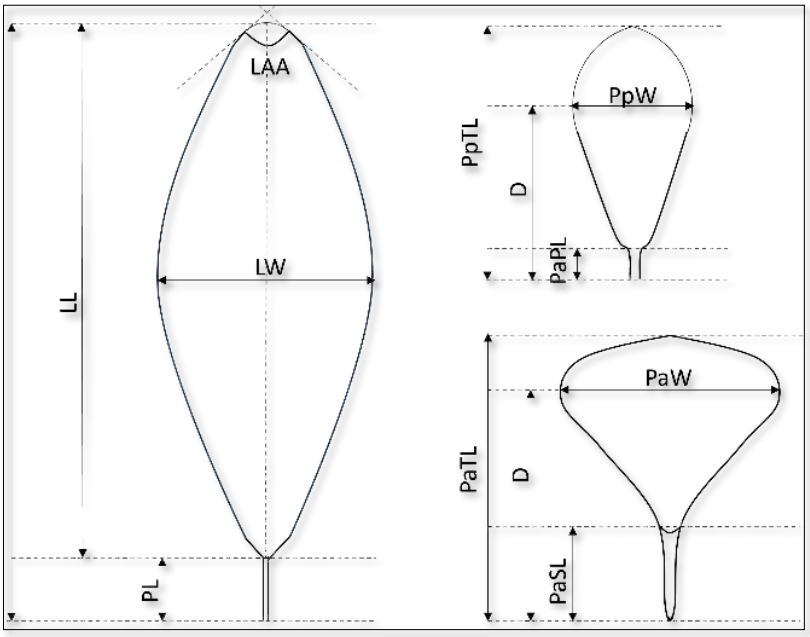


**Fig. 1.** Morphological characters used in morphometric assessments of leaves and petals (LL – Lamina Length, LW - Lamina Width, LAA - Leaf Apex Angle, PL- Pedicle Length, PpTL - Posterior Petal Total Length, PpPL - Posterior Petal Pedicle Length, PpW - Posterior Petal Width, D - Distance petal base-maximum width point, PaTL - Anterior Petal Total Length, PaSL - Anterior Petal Spur Length, PaW - Anterior Petal Width).

The anatomical analyses were conducted on fixed and preserved in 70% alcohol plant material. Transversal sections were made using a botanical razor and a hand microtome. The sections were stained with ruthenium red and iodine green. The samples were embedded in glycero-gelatin, to obtain permanent slides. The anatomical investigations were performed on cross-sections of the root, rhizome, stem, petiole and leaf blade.

The statistical differences among species were determined by conducting analysis of variance (ANOVA), post-hoc Dunnett tests and Principal Component Analysis, using the Multibase package (Numerical Dynamics, Japan) software.

## RESULTS AND DISCUSSIONS

### Morphometry

Among the 3 analyzed *Viola* species, a series of morphological parameters have similar values, while others are statistically different, concerning mainly shoots and leaves (Table 2, a-b) as well as flowers (Table 3).

**Table 2.**

Morphological parameters of *Viola* species for shoot and leaves (a) and leaf apex angle (b) (n=20).

| Parameter | *V. canina* | *V. dacica* | *V. declinata* |
|---|---|---|---|
| *a) Shoot and leaves* | | | |
| Stem height (SH) | $33^{a} \pm 2$ | $72^{b} \pm 9.03$ | $129^{c} \pm 3.67$ |



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| Parameter | *V. canina* | *V. dacica* | *V. declinata* |
|---|---|---|---|
| Leaf area (LA) | $171.9^{a} \pm 8.71$ | $85.75^{b} \pm 7.05$ | $142.2^{a,c} \pm 17.48$ |
| Leaf perimeter (Lpe) | $49.63^{a} \pm 5.07$ | $46.13^{a} \pm 4.08$ | $55.29^{a} \pm 5.48$ |
| Lamina length (LL) | $12.98^{a} \pm 0.81$ | $7.25^{b} \pm 0.73$ | $10.26^{a,b} \pm 0.83$ |
| Lamina width (LW) | $24.44^{a} \pm 3.21$ | $21.89^{a} \pm 3.85$ | $27.42^{a} \pm 1.87$ |
| Petiole length (PL) | $12.4^{a} \pm 0.8$ | $3.6^{b} \pm 0.29$ | $2.5^{b,c} \pm 0.35$ |
| Stipel main lobe length | $4.4^{a} \pm 0.24$ | $5.3^{b} \pm 0.12$ | $1^{c} \pm 0$ |
| Stipel main lobe width | $1.07^{a} \pm 0.05$ | $1.08^{a,b} \pm 0.03$ | $4.9^{c} \pm 0.1$ |
| *b) Leaf apex angle° (LAA)* | | | |
| Basal leaf | - | $89.86^{a} \pm 4.76$ | $66.16^{b} \pm 2.96$ |
| Upper leaf | $82.06 \pm 1.89$ | $42.65^{a} \pm 2.88$ | $48.38^{b} \pm 2.98$ |

Different letters within the same row represent significant statistical differences for $p < 0.05$; all parameters are expressed in mm, except for leaf area, expressed in $mm^2$.

**Table 3.**

Morphological parameters of *Viola* species for flowers (n=20).

| Parameter | *V. canina* | *V. dacica* | *V. declinata* |
|---|---|---|---|
| Flower colour | purple, white | purple | purple |
| Peduncle height | $30^{a,b} \pm 2.74$ | $35^{a} \pm 2.74$ | $51^{c} \pm 1.5$ |
| Bracteole length | $1^{a} \pm 0$ | $1^{a} \pm 0$ | $1^{a} \pm 0$ |
| Flower length | $14^{a} \pm 0.35$ | $14.9^{a} \pm 0.1$ | $14.4^{a} \pm 0.24$ |
| Sepal length | $7.3^{b} \pm 0.2$ | $1.85^{a} \pm 0.06$ | $1.39^{a,c} \pm 0.07$ |
| Sepal width | $1.2^{a,b} \pm 0.09$ | $1^{a} \pm 0$ | $1.5^{b,c} \pm 0.16$ |
| Posterior petal total length (PpTL) | $11.33^{b} \pm 1.42$ | $13.95^{a} \pm 1.55$ | $13.82^{a,c} \pm 1.62$ |
| Posterior petal pedicle length (PpPL) | $2.34^{b} \pm 1.11$ | $1.2^{a} \pm 0.37$ | $1.58^{a,c} \pm 0.48$ |
| Posterior petal width (PpW) | $4.12^{b} \pm 0.75$ | $6.61^{a} \pm 1.05$ | $6.31^{a,c} \pm 1.09$ |
| Distance posterior petal base-maximum width point (D) | $8.03^{b} \pm 1.07$ | $9.96^{a} \pm 1.81$ | $10.26^{a,c} \pm 1.59$ |
| Anterior petal total length (PaTL) | $15.93^{a,b} \pm 1.85$ | $16.94^{a,c} \pm 2.02$ | $17.52^{a,c} \pm 1.72$ |
| Anterior petal spur length (PaSL) | $6.53^{a} \pm 1.22$ | $6.45^{a} \pm 1.2$ | $7.22^{a} \pm 0.92$ |
| Anterior petal width (PaW) | $5.58^{b} \pm 1.09$ | $10.19^{a} \pm 2.75$ | $10.56^{a,c} \pm 2.2$ |
| Distance anterior petal base-maximum width point (D) | $12.4^{b} \pm 1.48$ | $14.09^{a} \pm 1.62$ | $14.44^{a,c} \pm 1.53$ |
| Lateral petal total length | $11.64^{b} \pm 1.66$ | $13.55^{a} \pm 1.83$ | $13.3^{a,c} \pm 1.31$ |
| Lateral petal pedicle length | $1.91^{b} \pm 0.53$ | $2.86^{a} \pm 0.82$ | $3^{a,c} \pm 0.46$ |
| Lateral petal width | $4.02^{b} \pm 1.01$ | $5.91^{a} \pm 1.61$ | $5.94^{a,c} \pm 0.85$ |
| Distance lateral petal base-maximum width point | $8.43^{b} \pm 1.39$ | $10.68^{a} \pm 1.33$ | $10.46^{a,c} \pm 0.96$ |

Different letters within the same row represent significant statistical differences for $p < 0.05$.

According to the current literature, *V. dacica* and *V. declinata* are morphologically similar, with little differences at the level of leaves and stipules (Beldie,1955; Tutin *et al*., 1968; Sârbu *et al*., 2013). However, the differences concerning lamina shape are difficult to identify. In the literature it is specified that the upper leaves may be spear shaped in both species, sometimes ovate in *V. dacica*. Our results show that the two species are very similar in both lamina length and width, with no significant differences.

The parameter that allows separation of the two species is the leaf apex angle, for basal leaves as well as for upper leaves (Table 2, b). This angle in *V. declinata* is broader for the upper leaves and is narrower for the basal leaves. Differences between the two types of leaves are more obvious for *V. dacica*, where the basal leaves are rounder than the upper leaves, compared to *V. declinata*, where the two types are similar.

Also, the results confirm the differences at stipules level, which are longer in *V. dacica* than in *V. declinata*

and are broader in *V. declinata*. Another leaf parameter that shows differences between these two species is the size, which is larger in *V. declinata* (Table 2, a).

Concerning morphometrical indices of the flowers, *V. dacica* and *V. declinata* share similar values for almost all parameters. One exception was recorded in the case of the height of the peduncle, which is significantly longer in *V. declinata* (Table 2, c).

Another species of the *Viola* genus that occurs in similar habitat with *V. dacica* and *V. declinata* is *V. canina*. It is morphologically distinct from the latter species, with main differences found in stipules and leaves. Our results show that, indeed, *V. canina* displays the largest leaf area of the three species as well as the longest petiole (Table 2, a). In this species, there is only one type of leaves present having an ovate and cordate base shape. The stipules are leaf-like in *V. canina* and lobate in the other two species.

Significant differences between *V. canina* and its two other analysed congeners were found at the level of petals (Table 3). Although flower length is similar among species, petals lengths and widths are generally smaller in *V. canina* except for the posterior petal pedicle length, which is longer in this species.

The Principal Component Analysis showed that differentiation of the species may be performed based on some morphometrical indices. As such, *V. declinata* is mainly different from the other two species with respect to shoot height, stipule width, stipule main lobe width and peduncle height, *V. dacica* is clearly individualised based on posterior petal pedicle length, while *V. canina* records distinct values of petiole length, upper leaf apex angle, leaf area and lamina length.

In general, morphological discrimination among *Viola* species is based on several characters related to the presence or absence of stolons, leaves widths, lengths and angles, stipules length and width, spur length and colour (Marcussen and Nordal, 1998; Hodálová *et al*., 2008). For the analysed species, the characters which allowed statistical differentiation were leaf area, leaf apex angle, peduncle height and stipule sizes.

The Principal Component Analysis was conducted for a subset of the analysed morphometrical indices, which were not strongly correlated. Indices that presented correlations were leaf perimeter, bracteole length, flower length, posterior petal total length, distance posterior petal base-maximum width point, anterior petal total length, anterior petal spur length, lateral petal total length, distance lateral petal base-maximum width point. The analysis results are presented in Table 4 while variable loadings within the principal components is presented in Table 5.

**Table 4.**

Principal Component Analysis report for morphometrical indices of analysed *Viola* species.

| | **Comp 1** | **Comp 2** |
|---|---|---|
| **R2** | 63% | 37% |
| **R2(cum)** | 63% | 100% |
| **Eigenvalue** | 1.89 | 1.11 |
| **Q2** | 63% | 71% |
| **Q2(cum)** | 63% | 89% |

**Table 5.**

Principal Component Analysis variable loadings for morphometrical indices of analysed *Viola* species.

| **Variables** | **Comp 1** | **Comp 2** |
|---|---|---|
| Stem height (SH) | -0.09 | 0.38 |
| Leaf area (LA) | -0.26 | -0.21 |
| Lamina length (LL) | -0.24 | -0.25 |
| Lamina width (LW) | -0.29 | 0.14 |
| Petiole length (PL) | -0.07 | -0.39 |
| Stipel main lobe length (SLL) | 0.24 | -0.24 |
| Stipel main lobe width (SLW) | -0.20 | 0.30 |
| Basal leaf (BL) | 0.27 | -0.20 |
| Upper leaf (UL) | -0.14 | -0.36 |
| Peduncle height (PH) | -0.25 | 0.23 |
| Sepal length (SL) | 0.30 | 0.05 |
| Sepal width (SW) | -0.09 | 0.38 |
| Posterior petal pedicle length (PpPL) | 0.26 | 0.20 |
| Posterior petal width (PpW) | -0.29 | -0.12 |
| Anterior petal width (PaW) | -0.30 | -0.05 |
| Lateral petal pedicle length (LppL) | -0.30 | -0.03 |
| Lateral petal width (LpW) | -0.30 | -0.07 |

Results show that the loadings of the variables vary in absolute values between 0.21 and 0.39 on the two principal components (Fig. 2), the main contributors to species individualisation being stem height (SH), leaf area (LA), lamina length (LL), petiole length (PL), stipel main lobe length (SLL), stipel main lobe width (SLW), upper leaf apex angle (UL), peduncle height (PH), sepal width (SW), posterior petal pedicle length (PpPL).

As a result, the three species are clearly differentiated on the basis of considered parameters (Fig. 3).



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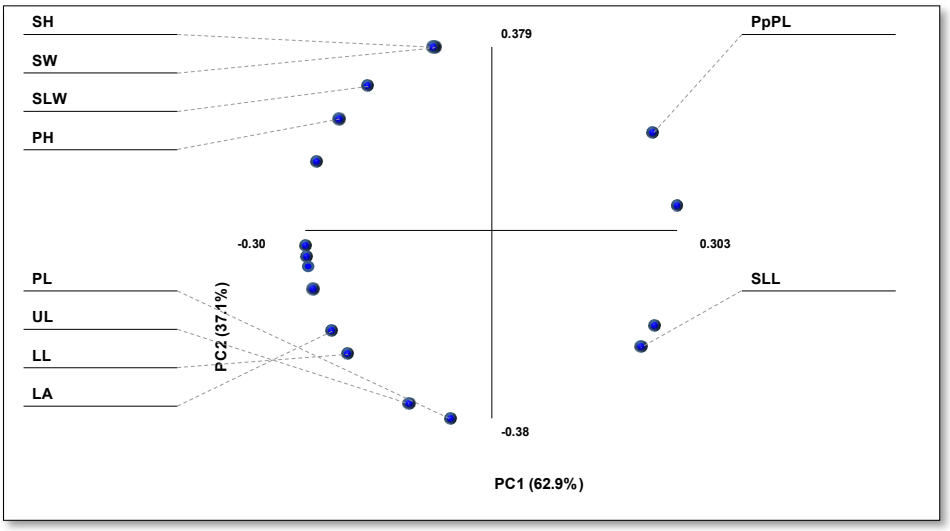


**Fig. 2.** Principal Component Analysis components loadings for morphometrical indices for the *Viola* species.

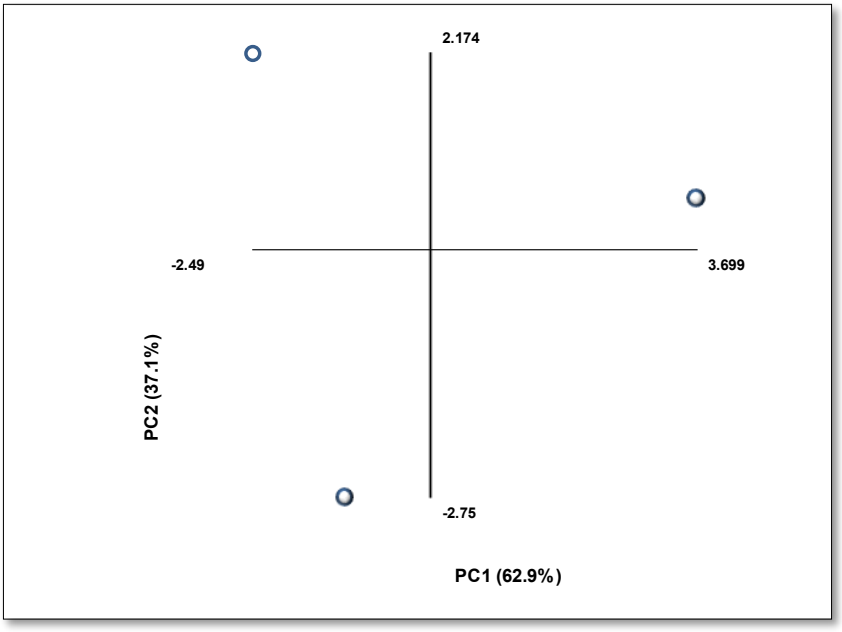


**Fig. 3.** Principal Component Analysis scores for the *Viola* species that were analysed.

## Anatomy

The **root** cross-section contour is circular for all three species (Table 6, Figure 2 - A, B and C). In *V. canina* and *V. declinata*, the secondary root structure results from the meristematic activity of both cambium and phellogen. The phellogen is generated within the primary cortex, producing several layers of suber towards outside and only a few phelloderm layers towards inside. These three tissues form a thin periderm. In *V. dacica*, the secondary root structure has resulted only from the cambium activity. In all species, between the cortex and the central cylinder a Casparian type endodermis is present.

The central cylinder is composed of a thick xylem ring and a thinner phloem ring, the latter having cells arranged radially. The pith region is completely filled with secondary xylem, except for *V. declinata*, where the pith still contains parenchymatic cells embedded with secondary xylem, with vessels of different sizes and few woody fibers. The xylem contains numerous libriform layers. Compared to *V. declinata*, in *V. dacica* the number of libriform layers is reduced.

The root cross-sections did not provide any systematically significant characters. The variability among species at the root level can be noted by the presence in the pith of the secondary xylem arranged in radial rows, also found in *V. behboudiana*, *V. cinerea* and *V. stocksii* (Mehrvarz *et al*., 2013), while in other species, such as *V. sintenisii* and *V. reichenbachiana* the pith region is parenchymatous (Yousefi *et al*., 2012).


**Fig. 4.** Cross-sections: (A, B, C) - root of *V. dacica, V. declinata, V. canina*; (D, E, F) – rhizome of *V. dacica, V. declinata, V. canina*; (G, H, I) – stem of *V. dacica, V. declinata, V. canina*; (J, K, L) – petiole of *V. dacica, V. declinata, V. canina*; (M, N, O) – lamina of *V. dacica, V. declinata, V. canina*; Cu – cuticle, H – trichomes, E – epidermis, Es – superior epidermis, Ei – inferior epidermis, En – endodermis, Co – cortex, Cl – collenchyma, P – parenchyma, Pp – palisade parenchyma, Sp – spongy parenchyma, D – druses, Ph – phloem, Xy – xylem, Pi – pith, Lvb – lateral vascular bundle, Ri – ridge, MR – main rib, LR – lateral rib.

The **rhizome** has a secondary structure in all three species (Table 5, Figure 2 - D, E and F). The epidermis and most of the cortex are exfoliated. In *V. canina* and *V. dacica*, the central cylinder is composed of a phloem ring, having a starchy parenchyma and a compact xylem ring. The pith is interspersed with short layers of primary xylem vessels. In *V. declinata*, 2-3 bundles of different sizes can be observed, with few phloem layers towards the exterior and numerous xylem layers.

In *V. dacica* and *V. declinata* the annual rings are present and distinct, similar to *V. calcarata*, *V. arborescens* and *V. reichenbachiana* (Schweingruber *et al.*, 2011). The ring boundaries are marked by a zone


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with flat, thick-walled fibres. In all species the vessels are arranged in radial rows.

The **stem** presents a primary structure (Table 5, Figure 2 - G, H and I). The epidermis presents isodiametric cells and a thin cuticle. Single-celled trichomes can be observed especially on adaxial ridges. In *V. dacica* the trichomes are shorter than in *V. canina*. In *V. canina* there is a bundle of trichomes opposite to the ridges and druses are visible within parenchyma cells. The collenchyma is composed of circular cells and intercellular spaces. In *V. dacica* there are 1-2 layers beneath epidermis, increasing to 3-4 layers at the corners of the ridges. In *V. declinata* the collenchyma consists of 2-3 layers, increasing to 3-4 layers at the corners of the ridges whereas in *V. canina* there are generally 3 layers of cells, but 4-5 layers in the ridges.

The central cylinder is very thick in all three species, comprising collateral opens vascular bundles.

The major differences between species at the stem level is the number of vascular bundles, greatest in *V. dacica*. Similar differences between species, for example, between *V. rupestris*, *V. reichenbachiana*, *V. tricolor*, and *V. caspia*, where the number ranges between 7 and 12 bundles (Yousefi *et al*., 2012). Calcium oxalate crystals are present in all analysed species and are druse shaped. The crystals shape and frequency are considered a taxonomical character (Edeoga and Okoli, 1995; Mehrvarz *et al*., 2013; Raman *et al*., 2014). However, in *V. declinata* and *V. dacica*, the shape and frequency of the crystals are very similar and therefore, cannot constitute a differentiation character. In *V. canina* the calcium oxalate crystals are large and numerous in the pith and fewer in the cortex.

At the leaf level, the main differences between analysed species in the petiole are the contour, the presence of trichomes, the positioning of the collenchyma and number of the vascular bundles.

The fundamental parenchyma of the **petiole** has calcium oxalate crystals present and visible intercellular spaces (Table 6, Figure 2 - J, K and L). The vascular tissue is composed of several collateral bundles, the central one being the largest, with mechanical elements present facing both xylem and phloem in *V. canina* and facing only the xylem in *V. dacica* and *V. declinata*.

**Table 6.**

Anatomical differences between cross-sections of the vegetative organs of *V. canina*, *V. dacica* and *V. declinata.*

| Vegetative organs | | *Viola canina* | *Viola dacica* | *Viola declinata* |
|---|---|---|---|---|
| **Root** | | - thick<br>- the rhizodermis is exfoliated<br>- calcium oxalate crystals are missing<br>- a thick phloem ring is present, where parenchyma cells predominate, arranged in radial rows | - thinner<br>- the rhizodermis is exfoliated<br>- air pockets can be observed in the cortical parenchyma<br>- calcium oxalate crystals are scarce<br>- the phloem ring is thin, with few phloem parenchyma cells | - thick<br>- the rhizodermis still persists, with absorbant hairs present<br>- starch cells can be observed, belonging to the cortical parenchyma<br>- calcium oxalate crystals are missing<br>- thick phloem ring with tangentially elongated cells, disposed in radial rows |
| **Rhizome** | | - asymmetrical secondary structure<br>- some cortex cells have calcium oxalate crystals<br>- annual rings<br>- parenchyma-type pith | - asymmetrical secondary structure<br>- calcium oxalate crystals are missing<br>- annual rings<br>- parenchyma-type pith | - asymmetrical secondary structure, lacking differentiation between the anatomical regions,<br>- the pith is not visible, instead parenchymatic areas are present, originating from the secondary xylem. |
| **Stem** | | - semi-circular contour with two shallow adaxial ridges and a flat adaxial surface<br>- thick cortex with 8-10 cell layers<br>- central cylinder composed of 7-8 bundles<br>- the medullary rays between vascular bundles are very narrow<br>- the pith is thick, with calcium oxalate crystals present | - elliptical contour, with two prominent, opposed ridges<br>- thin cortex<br>- central cylinder composed of 10 bundles<br>- the medullary rays between vascular bundles are heavily sclerified and lignified<br>- the pith is replaced by a large cavity surrounded by 2-3 layers of isodiametric flattened cells | - elliptical contour, with two prominent, opposed ridges<br>- thin cortex<br>- central cylinder composed of 7 bundles<br>- the medullary rays between vascular bundles are lightly sclerified and lignified<br>- the pith is replaced by a large cavity surrounded by 1-2 layers of flattened cells |
| **Leaf** | Petiole | - elliptical contour, with two divergent latero-adaxial ridges<br>- epidermis with isodiametric cells and a striated cuticle<br>- trichomes missing | - triangular contour with two large adaxial wings with foliar lamina structure<br>- epidermis with cells of different sizes and a thin, straight cuticle | - semi-elliptical contour with two latero-adaxial ridges<br>- epidermis with isodiametric cells and a striated cuticle<br>- missing trichomes |

| Vegetative organs | | *Viola canina* | *Viola dacica* | *Viola declinata* |
|---|---|---|---|---|
| | | - collenchyma present especially in the abaxial side and on adaxial ridges<br>- at the adaxial side of ridges, cells acquire palisade shape<br>- vascular tissue composed of 3-4 bundles | - trichomes short and frequent, often needle-shaped<br>- collenchyma missing<br>- calcium oxalate crystals frequent<br>- the parenchyma of the wing differentiates in a single-layered palisade tissue beneath the inner epidermis and a lacunar tissue underneath the outer epidermis<br>- vascular tissue composed of 7 bundles, with mechanical elements | - discontinuous collenchyma present only in the abaxial side<br>- calcium oxalate crystals infrequent<br>- at the adaxial side of ridges, cells acquire palisade shape<br>- vascular tissue composed of 3 bundles, with mechanical elements facing only the xylem |
| | Lamina | - cells of upper epidermis larger than those of lower epidermis<br>- the mesophyll is thick, with the palisade tissue organized in 2-3 layers, having tall cells, of approximately 50% the thickness of the mesophyll | - the epidermis cells are of different sizes<br>- the mesophyll is thinner with single-layered palisade tissue, containing calcium oxalate crystals<br>- the vascular bundles outside veins are not well delimited | - the epidermis cells are generally isodiametric<br>- the mesophyll has bi-layered palisade tissue with frequent calcium oxalate crystals<br>- the vascular bundles outside veins are surrounded by a layer of smaller, rounded cells |

Also, the lamina differs regarding the epidermis cells, the breadth of the palisade tissue and delimitation of vascular bundles (Table 6, Figure 2 - M, N and O). The mesophyll is differentiated into palisade tissue beneath the upper epidermis and spongy tissue above the lower epidermis. The midrib is prominent at both sides of the lamina, while 1st order lateral veins are visible only on the lower face of the lamina. Stomatae are present on both sides of the lamina. Unicellular trichomes are short, frequent in *V. canina* and *V. dacica* and rare in *V. declinata*.

Variability among species at the petiole and lamina level in *Viola* genus were already reported in characters such as the width of the collenchyma, number and disposition of wings and presence and number of calcium oxalate crystals (Yousefi *et al.,* 2012; Mehrvarz *et al*., 2013). The collenchyma width is similar among the analysed species, 1-2 layered under epidermis, increasing to 4-5 layers at the corners of the ridges, similar to *V. kizildaghensis* (Dinç *et al*., 2007) and *V. arvensis*, *V. oculta*, *V. tricolor*, but different than in *V. spathulata* (Yousefi *et al.*, 2012). Calcium oxalate crystals are reported in some *Viola* species (Toiu *et al.,* 2010; Pilberg *at al.*, 2016), while are missing in others and can be used as taxonomical character (Yousefi *et al*., 2012). In *Viola* species, the calcium oxalate crystals present in leaves are cubic, such as in *V. behboudiana* or stellate, such as in *V. cinerea* and *V. stocksii* (Mehrvarz *et al.*, 2013).

## CONCLUSION

From a morphological point of view, the characters that may allow differentiation between analysed species were stem height, lamina shape, leaf apex angle, leaf area, stipules shape and length. The root and rhizome cross-sections did not show any significant differences between species. The main anatomical dissimilarities observed were in stem and petiole. The characters that could be considered are the contour, the number of vascular bundles, the shape and frequency of the calcium oxalate crystals and the collenchyma width. At the lamina level, the main differences were the size and shape of the epidermis shape and palisade tissue.

The mentioned characters may serve for discrimination especially between *V. declinata* and *V. dacica*, species that morphologically are similar. The anatomical and morphological traits analysed expand the current knowledge on the three considered species and constitute a basis for further comparisons.

## AUTHORS CONTRIBUTIONS

Conceptualization, E.R., A.L., I.B. and M.M.Z.; methodology, E.R., A.L., I.B. and M.M.Z.; data collection, E.R., A.L., I.B. and M.M.Z.; data validation, E.R., A.L., I.B. and M.M.Z.; data processing E.R., A.L., I.B. and M.M.Z.; writing—review and editing, E.R., A.L., I.B. and M.M.Z.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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