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ANATOMICAL AND MICROMORPHOLOGICAL PECULIARITIES OF *ADONIS VERNALIS* L. (*RANUNCULACEAE*)

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Abstract

Adonis vernalis is a pontic element and rare plant distributed in grassland communities in the South-East Europe steppe zone. Histo-anatomical and micromorphological investigations regarding root, stem and leaves was carried out in order to emphasize the adaptation of this species to the living environment. The root acquires early secondary structure by cambium activity; the exodermis shows Casparian bands. Root epidermis consists of cells with thickened walls; the absorbent hairs are absent. The stem has primary structure from the top to the basis. The vascular bundles, of different dimensions are arranged in a circle. Cortical bundles, of collateral and concentric types, were also observed. The sclerenchyma sheaths from the periphery of the vascular bundles become visible at the stem basis. The tector hairs are present only on young leaves. The anatomy of vegetative organs showed some xerophytic structures, but the majority of their features are those of typical mesophytes. These features are correlated with the plant life cycle.

Introduction

The ephemeroid *Adonis vernalis* L., is a pontic element (Akeroyd, 1993) distributed in the South-East Europe steppe zone and is characteristic for different xerothermic associations of continental-submediterranean type, belonging to *Festucetalia valesiaceae* (Denisow *et al*., 2008). Its main distribution area ranges from the Eastern part of middle Europe through East and South East Europe, Western Siberia to Eastern Siberia reaching the Jenissei region (Akeroyd, 1993).

Adonis vernalis, is a rare and protected plant (mentioned in the Convention on International Trade Endangered Species of Wild Flora and Fauna-CITES), a relict of the steppe flora, which occurs in the xerothermic grasslands. It prefers sunny places or at most semi-shaded ones, where the soil can warm up easily enabling the plant to emerge early in spring. *A. vernalis* stands are normally found on well-drained but sufficiently moistured soils, calcareous chernozems (black earth) (Melnik, 1998) or loess soil (Forycka *et al.*, 2004).

For medicinal purposes, mainly the aerial plant parts (stems, leaves, flowers, and fruits) of *A. vernalis* are used. It is a highly valuable medicinal plant with a cardiotropic effect (Poluyanova & Lyubarskii, 2008). The plant contains cardiac glycosides similar to those found in the *Digitalis purpurea* (Chevallier, 1996). It also has a sedative action; the herb is cardiotonic, diuretic, sedative and vasoconstrictor.

The environmental conditions have a strong influence on the plant's structural traits. Although they live in dry environments, ephemeroid perennial plants such as *A. vernalis* do not possess all the specific xerophytes features. They survive from the summer and winter as underground rhizomes or tubers. Most ephemeral and ephemeroid plants present marked mesophytic characteristics in physiology and anatomy (Qian *et al.*, 2008). Despite their relative mesophytic characteristics, *A. vernalis* require specific soil and water conditions (Hoffman, 1997). The ecology of seed germination was investigated by Poluyanova and Lyubarskii (2008): the seeds have a low germination rate (below 20%), drought in the

summer and of cold in the winter. Although there are physiological studies on perennial ephemeroids, histo-anatomical investigations regarding ephemeroid plants in order to emphasize the structural support of adaptation to the environment of life are scarce.

Whereas *A. vernalis* has been extensively investigated phytochemically and pharmacologically, the studies on the histology of this species are limited. Not much data concerning anatomical and micromorphological features of *A. vernalis* can be found in literature. In describing some representatives of the family *Ranunculaceae* only main anatomical characteristics of these species have been given (Metcalfe & Chalk, 1957). Poshkurlat and Milevskaia (1969) studied morphological and anatomical characteristics of vegetative organs of *Adonis turkestanicus*. In a previous paper we have investigated the morphological characters of the flower of *A. vernalis* using scanning electron microscopy (Gostin, 2009). Therefore, the purpose of this paper is to investigate the anatomical micromorphological properties of *A. vernalis* vegetative organs.

Material and Methods

The plant material was collected from the Valea lui David natural reservation, located close to the city of Iaşi (Romania) (47°11'31.5''N, 27°28'06.8''E and altitude 180 m) in April 2009. A voucher specimen is stored in the Herbarium of Faculty of Biology, Al. I. Cuza Iasi University, Romania.

For histo-anatomical analysis the plant material (10 whole plants in the anthesis phase) was fixed and conserved in 70% ethylic alcohol. For anatomical analysis, cross sections of root (middle, stem (top, middle and basis) and leaves were used. Free hand sections were performed using a razor blade. The sections were coloured with Iodine Green and Ruthenium Red.

For histochemical research fresh free-hand sections were used and were stained as follows: saturated Sudan III solution in 70% ethanol (Bronner, 1975) for lipids; phloroglucin and hydrochloric acid for lignin (Johansen, 1940), iodine potassium iodine (Jensen, 1962) for starch grains. For the detection of AM (arbuscular mycorrhizae) root colonization a modified method of Phillips and Hayman (1970) was use. Roots were washed in tap water and cleared with 10% KOH for over nigth at 70°C, placed in NaOCl 5% for bleaching, rinsed with acetic acid (2% solution), then stained with 0.05% lactic-glycerol-Trypan Blue for 20 min at room temperature.

Photographs were taken with an Olympus E-330 photo camera, using an Olympus BX51 research microscope. The measurements of the epidermis cells, stomata and assimilating parenchyma were made using the biometrical software from Nikon (NIR-Demonstration). Fifty measurements were made for each parameter.

Scanning electron microscopy (SEM) investigations: the investigated material consists of small leaf pieces. The plant material was fixed in FEA (formol: 70% ethanol: acetic acid –5:90:5) for 48 hours, washed with distilled water and stored in 70% ethanol. Some pieces were sectioned under the stereomicroscope with a razor blade. After dehydration in a graded ethanol series (80%, 90% and 100%) and acetone, the material was critical point dried with CO2 (using a EMS 850 Critical Point Dryer), sputter-coated with a thin layer of gold (30 nm) (using a EMS 550X Sputter Coater) and, finally, examined by scanning electron microscopy (Tescan Vega II SBH) at an acceleration voltage of 27.88 kV.

Results

Root anatomy: All roots are adventitious in origin, formed on the plant rhizome. In the primary structure the roots are of the triarch, tetrarch and pentarch type (Fig. 1A). They

have a unilayered epidermis, the cortical parenchyma is formed by isodiametric cells, and the endodermis shows Caspary strips (Fig. 1B). In the central cylinder the pericycle is unilayered. The root epidermis does not show absorbent hairs. The external walls of the cells are particularly thick (8.72±0.85μm), but cellulosic (Fig. 1C). The secondary structure is acquired only by cambium activity (Fig. 1D). The vessels from the secondary xylem are narrow (10.85±2.96μm), but have thick and intensely lignified walls (lignin identification was made with phloroglucin). The endodermis remains of primary structure even at the root basis. The exodermis is uniseriate with conspicuous Casparian bands; this feature could be observed can be observed throughout almost the entire length of root.

The histochemical tests showed large amounts from two types of storage substances in root parenchymatic cells (both in the cortex and in the central cylinder): lipids (stained in red with Sudan III) (Fig. 1E) and starch (stained in violet with Lugol solution). Arbuscular mycorrhizal fungi in cleared roots stained with trypan blue were rarely observed (not show); the hyphae were both septate and nonseptate. Neither arbuscules nor vesicles were observed.

Stem anatomy: *A. vernalis* is a perennial plant, with an underground sympodial rhizome. The lateral buds produce flowering and non-flowering stems. Both these stem types were investigated from an anatomical point of view. Cross sections from the basis, middle and top of the stem were performed.

Flowering stem: The stem is circular in cross sections, with ribs in their superior part (Fig. 2A). Epidermal cells are slightly radialy elongated; stomata are located at the same level with the epidermis; the tector hairs are rare, unicellular, presents only in the top of the stem. The collenchyma is present only in the stem ribs (4-5 layers in the top of the stem) (Fig. 2B) or is continuous under the epidermis (2-3 layers in the middle and basis of the stem) (Fig. 2C). The vascular bundles from the central cylinder are numerousness (25-32), of collaterally types, of different sizes, arranged in a ring; they are only primary structure (Fig. 2B). In the top of the stem, the vascular bundles are composed only by xylem, procambium and phloem; in the middle and in the basal part of the stem the vascular bundles are surrounded by a thick sclerenchyma sheath (Fig. 2D).

Cortical bundles, of different types, are also present. They could be: of collaterally type, similar with the bundles from the central cylinder, surrounded by sclerenchyma (Fig. 2C) or included into the sclerenchyma sheath of a regular bundle; amphicribral concentric vascular bundles (Fig. 2D), partially surrounded by sclerenchyma sheath; small bundles, without conducting elements, consisting only of xylem fibres. An intense lignification degree was identified (histochemical reaction with phluoroglucinol) both in the cell walls of xylem vessels and sclerenchyma fibres.

The pith is thick, consisting of round cells with thin walls and with large intercellular air spaces.

Non flowering stem: The flowering and non flowering non flowering stems showed similar anatomical pattern; however, some differences could be noticed (Fig. 3A, C). The bundles of the non-flowering stem are more numerous than those of the flowering stem (33-38). The sclerenchyma sheaths consist of cells with thinner walls than those of the flowering stems. The procambium activity is intense and numerous division walls are visible in this area (Fig. 3B, D). Some anatomical parameters measured in flowering and non-flowering stems are given in Table 1.

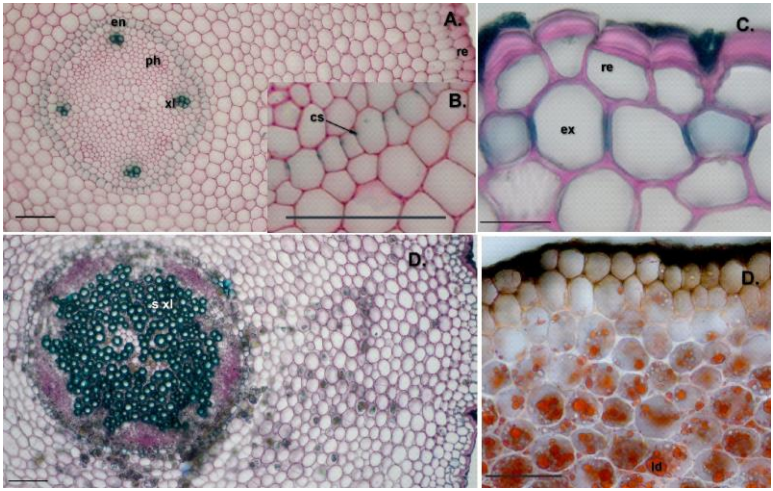

Fig. 1. Cross sections through the root: A- root with primary structure (bar = 100 µm), B- detail from the casparian endodermis, C- detail from the external part of the root (bars = 50 µm), D- root with secondary structure (bar = 100 µm), E- histochemical identification of the lipids with Sudan III (bar = 50 µm): cs- Casparian band, en- endodermis, ex- exodermis, lp- lipid droplet, ph- phloem bundle, re- root epidermis, s xl- secondary xylem, xl- xylem bundle.

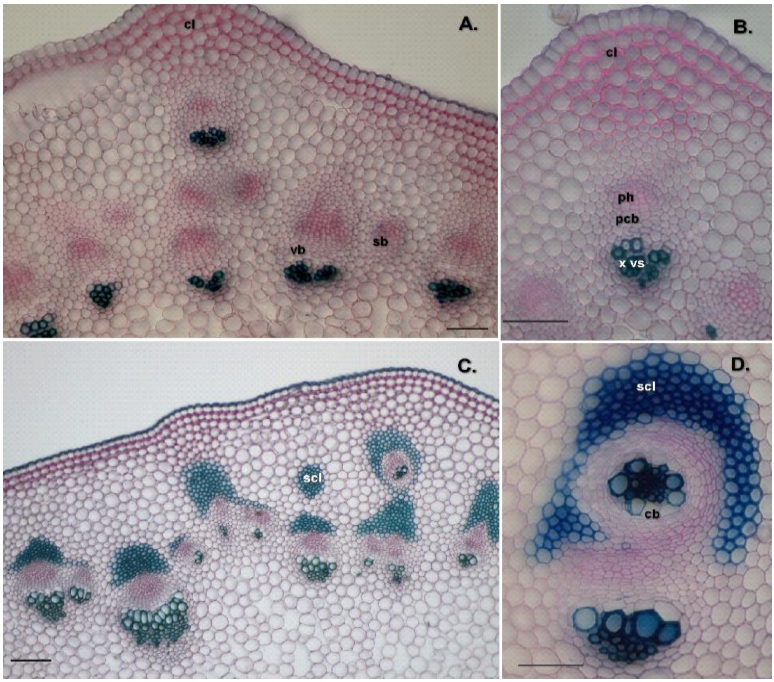

Fig. 2. Cross sections through the flowering stem: A- the top of the stem (bar = 100 µm), B- detail with a vascular (bar = 50 µm), C- stem basis (bar = 100 µm), D- detail with two vascular bundles (bar = 50 µm): cb- concentric bundle, cl- collenchyma, pcb- procambium, ph- phloem, sb- small vascular bundle without xylem vessels, scl- sclerenchyma sheath, vb- vascular bundle bundle, x vs- xylem vessels.

Table 1. Anatomical parameters of flowering and non-flowering stems.

	Flowering stem			Non-flowering stem		
	Top	**Middle**	**Base**	**Top**	**Middle**	**Base**
HEC	18.18±2.21	21.22±2.73	21.32±2.26	20.67±2.23	23.49±2.67	20.68±2.14
HEWC	1.88±0.27	3.03±0.37	5.05±0.86	1.88±0.38	2.75±0.45	3.2±0.29
MXD	7.65±1.84	14.52±3.56	16.4±4.98	8.55±1.77	18.29±4.72	20.33±3.34
VBA	0.0083±0.004	0.0287±0.002	0.0251±0.008	0.0162±0.005	0.0373±0.003	0.0368±0.002
XA	0.0038±0.001	0.0056±0.001	0.0079±0.002	0.0051±0.001	0.0079±0.003	0.0082±0.004
SA	-	0.0105±0.002	0.0108±0.003	-	0.0183±0.006	0.0148±0.005

HEC- Height of the epidermis cells (μm), HEWC- Height of the external wall of epidermis cell (μm), MXD- Diameter of the metaxylem vessels (μm), VBA- Vascular bundle area (mm^2), XA- Xylem area (mm^2), SA- Sclerenchyma sheath area (mm^2) (values are mean ± standard deviation)

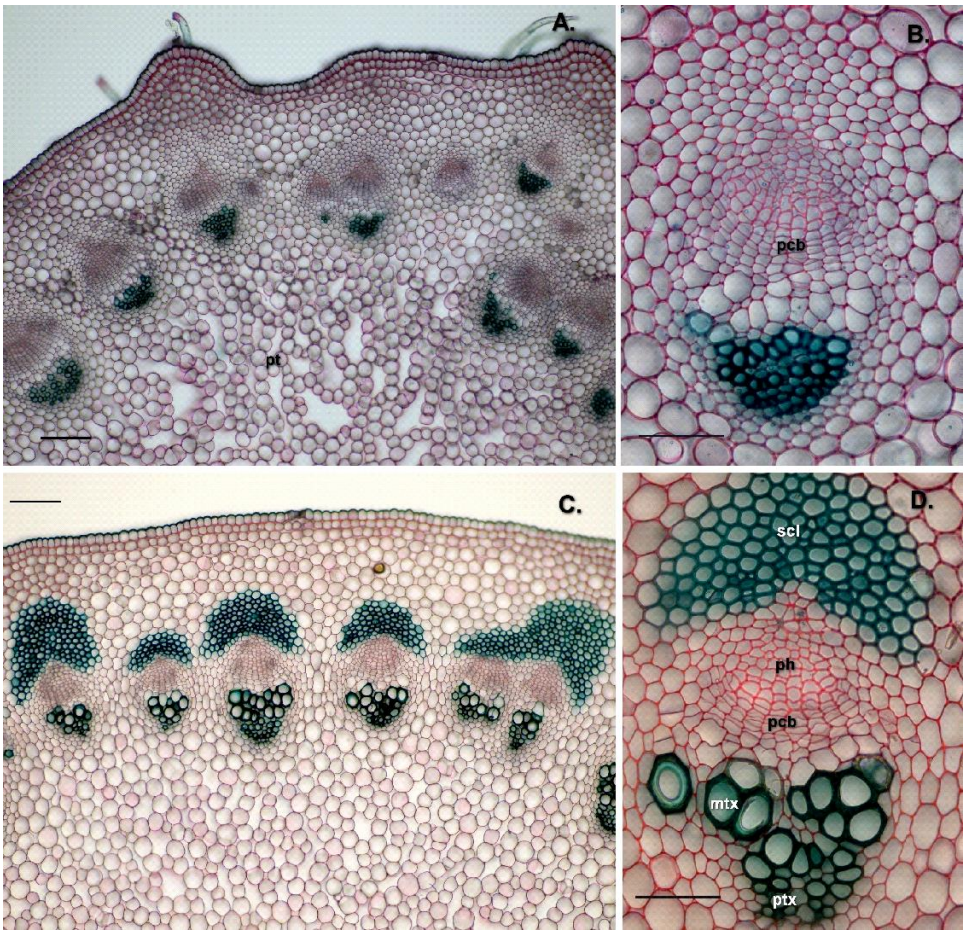

Fig. 3. Cross sections through the non flowering stem: A- the top of the stem (bar = 100 μm), B- detail with a vascular bundle (bar = 50 μm), C- stem basis (bar = 100 μm), D- detail with a vascular bundle (bar = 50 μm): mtx- metaxylem, pcb- procambium, pt- pith with air- cavities, vessels, pcb- procambium, ph- phloem, scl- sclerenchyma

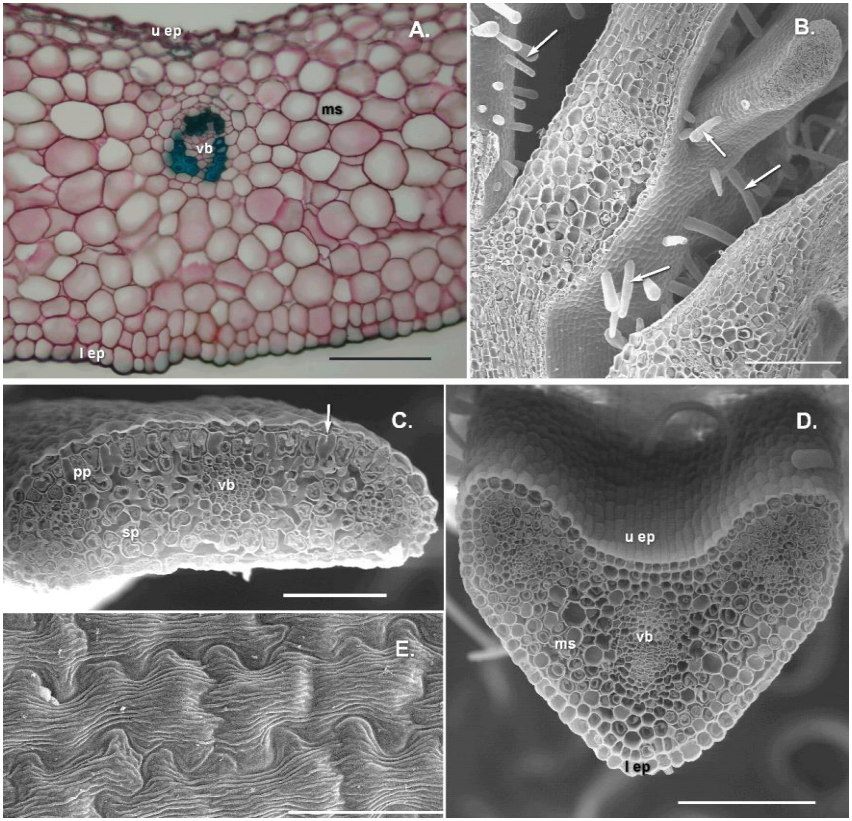

Fig. 4. A- Cross section through a basal leaf (scale) (bar = 100 μm), B- general view o some young leaves (SEM microphotography): arrows indicate the tector hairs (bar = 200 μm), C- cross section through a young leaf (SEM microphotography), arrow indicate an armed cell (bar = 200 μm), D- cross section through the midrib of an young leaf (SEM microphotography) (bar = 200 μm), E- upper epidermis of a young leaf with striate cuticle (bar = 20 μm): l ep- lower epidermis, ms- homogenous mesophyll, pp- palisade parenchyma, sp- spongy parenchyma, u ep- upper epidermis, vb- vascular bundle

Leaf structure: The basal leaves are scale like, sessile; caulinare leaves are short-petiolate to sessile; the lamina is 1-2-pinnati-sectate.

The basal leaves (scales) have a homogenous structure; the upper (internal) epidermis consists of flat cells, with thin external walls; the vascular bundles are small, with a few sclerenchyma fibres (Fig. 4A).

Very young leaves (4-8 internodes from the top of the stem) were analyzed using scanning electron microscopy. On the epidermis, numerous tector hairs were observed (Fig. 4 B). The hairs are unicellular, long (localized especially on upper epidermis) and short (localized especially on the leaf edges). These hairs are absent on mature leaves. In the first developmental stages, the mesophyll structure is still homogenous, with small vascular bundles (Fig. 4C). During the differentiation, the palisade parenchyma appears in the mesophyll (under the upper epidermis), with short, sometime armed cells. In the midrib region the mesophyll remains homogenous (Fig. 4D). The vascular bundles (3-5) are of collaterally type, without any sclerenchyma fibers.

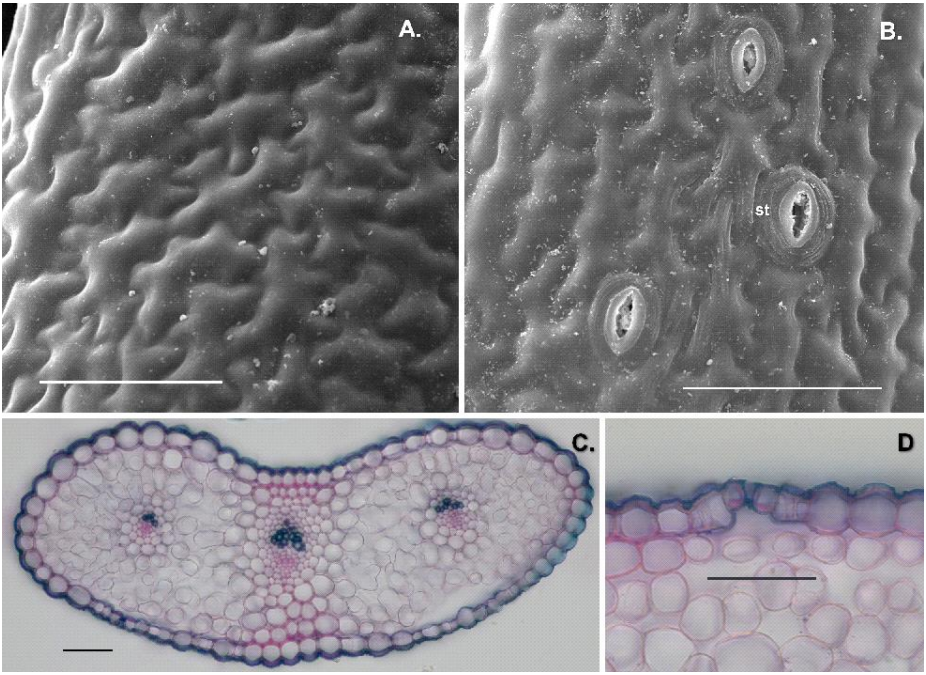

Fig. 5. A- Upper epidermis of a mature leaf (SEM microphotography) (bar = 100 μm), B- lower epidermis of a mature leaf (SEM microphotography) (bar = 100 μm), C- cross section through a mature leaf (bar = 100 μm), D- detail with a stomata (bar = 50 μm).

Under SEM observation, the upper and the lower epidermis cells are isodiametricaly and have undulated walls (Fig. 4E). Cuticular striations are prominent on the upper cell epidermis only in young leaves.

On the mature leaves the cuticle is smooth, both on upper and lower epidermis (Fig. 5A, B). The assimilatory cells are by pallisadic type (in the leaf edges) or by spongy type (Fig. 5C). The leaves are hypostomatous, with the guard cells situated at the same level with the epidermis cells (Fig. 5D). The stomata complexes are anomocytic (without subsidiary cells). The stomata size was (22.5-27.5) μm× (14.5-22.5) μm.

Discussions

Adonis vernalis is a rare plant, a relict of the steppe flora (Foryka *et al*, 2004) with specific requirements for the environmental conditions. Because it is considered to be a threatened species, it is included in most red data books of its range countries. In the synoptical red list for Central Europe the species is assessed as vulnerable by Schnittler & Gunther (1999). Despite growing in regions with low amounts of precipitation, the ephemeroid plants do not exhibit the specific characters of xerophytic plants. The anatomy of ephemeral plants vegetative organs showed that they have some xerophytic structures, but the majority of their features are those of typical mesophytes (Qian *et al.*, 2007). A detailed anatomical and micromorphological analysis shows several adaptative peculiarities in *A. vernalis* vegetative organs.

Our histological and histochemical data indicate a particular structure of the root. The unusually thick external wall (cellulosic) of the epidermis cells and the absence of the absorbent hairs may be related to the presence of the AM. According with Chilvers and Daft (1981), the plants showing a complete absence of the root hairs are substantialy dependent on mycorrhizas. It has been suggested that root hairs and mycorrhizal fungi were two alternative mechanisms for plant nutrient uptake (Baylis 1970; Koide & Mosse, 2004). The presence of the AM on *Ranunculaceae* species is a common feature. But the species that grows in nutrient-rich environments tend to be nonmycorrhizal and ephemerals from the *Ranunculaceae* family belong to this category (Wang, Qiu, 2006). The first report of the mycorrhizal status of *A. vernalis* was made by Zubek and Błaszkowski (2009) in a paper related to the medicinal plants as hosts of arbuscular mycorrhizal fungi. Our data confirm this research and correlate the AM presence with anatomical peculiarities in the root structure. The better growth responses of mycorrhizal plants are attributed to higher nutrients uptake and higher moisture absorption (Kung'u *et al.*, 2008).

The persistence of the first stage in exodermis development (with Casparian bands) is another particular feature of the *A. vernalis* root structure. In this case, even in old roots with secondary structure, the secondary stage (which suberin lamellae) is never achieved. It is demonstrated that the endodermis reacts very sensitively to various environmental stresses (Lux *et al.*, 2004); a water deficit increased the lignification of the exodermis in tomato roots (Nakano *et al.*, 2003) and environmental stresses induced intensive wall thickenings in the walls of endodermal cells (Degenhardt & Gimmler, 2000). The persistence of the Casparian structure can be interpreted as a reduced environmental stress at this level.

A. vernalis accumulate storage substances in root parenchyma (identified by histochemical methods). The accumulation of a considerable amount of lipid and starch in the underground organs during the previous vegetative period allow a rapid grow and development of the whole plant in the spring period (Abdurakhmanova *et al.*, 2001). The storage substances were not observed in the stem parenchyma, because they have a limited life.

The flowering and non flowering stems follow the general pattern described by Metcalfe and Chalk (1950). The vascular bundles are arranged in an irregular circle. The concentric vascular bundles are not characteristic for the *Ranunculaceae* species.

The presence of a well developed sclerenchyma, with fibers having thick and intense lignified walls is a xerophytic trait. The average area of the xylem, vascular bundles and sclerenchyma sheaths increase in flowering stem compared with that from the non flowering stem. Vascular bundle area seems to be directly related to efficient transport of water and nutrients from the soil (Ali *et al.*, 2009). Since the xylem and phloem are responsible for the uptake of ions and water and the transport of assimilates (Chen *et al.*, 2006), the increased needs for building and sustaining flowers justify the larger development of vascular and mechanical tissues in flowering stems.

On the young leaves, unicellular long and short tector hairs were observed, while the mature leaves are hairless. Young leaves are deficient in several aspects related to the ability to protect against water lost and irradiations (Bisba *et al.*, 1997); they have a thin cuticle and a thin external wall of the epidermis cells. At maturity, the leaves acquire different mechanisms for self protection (thicker epidermis cells walls, thicker cuticle).

Conclusions

The aerial organs of *A. vernalis* begin to develop in early spring (April, May), when the available water is relatively high. The biological cycle is complete until summer,

when the temperatures increase and the water reserves decrease. These explain the mesophytic characters of the vegetative organs of *A. vernalis*, which is correlated with a good growth rate in order to optimize its adaptation to the seasonal fluctuation of environmental conditions.

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