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Original Article

Morphological and anatomical observations on *Ranunculus lingua* L. under flooding conditions in Vlasina Lake (SE Serbia)

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***Abstract*:**
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R. lingua lives in the peat bog shrubberies on Vlasina plateau. It can face pronounced anaerobic conditions when it grows in aquatic environments or nearby lakes. Anatomical analysis of leaf, stem and root were performed. Samples were prepared according to the standard methods that were modified due to the specific material. The total water deficit and water potential were determined. Water deficit was highest in September (50 %) and lowest in August and October. The mesophyll of leaves is differentiated into palisade and spongy tissue. The vascular tissue consists of closed collateral bundles and their number varies from 24-26. The vascular bundles of stem are collateral, closed and covered by sclerenchymatic cells. This study showed that *R. lingua* survives in intensely flooded habitat due to the structure and water relations.

Key words: *Ranunculus lingua*, flooded habitat, Vlasina, anatomy, water relation

Introduction

R. lingua is widespread in Europe, from Scandinavia to the South across Central Europe to the mountains of Southern Europe and Western Asia. In Southeastern Serbia, this species lives in the peat bog shrubberies, the peat islands and marshes in bays of the Vlasina Lake at an altitude of 1200 m on the Vlasina plateau. Temporary or continuous flooding occurs as a result of overflowing of the lake (Ranđelović, Zlatković, 2010; Atanackovic, 2007). The habitat's conditions seem to influence the survival of this species and could be a reason that *R. lingua* is represented with only one population. The species belongs to the category of critically endangered species (CR), (Stevanovic et al., 1999). Moreover, *R. lingua* is at extremely high risk of extinction at the regional scale and a decade later it is still recognized as a critically endangered variety (Ranđelovic et al., 2010). Despite the peat bog conditions, the species seems to have developed some mechanisms to overcome flooding and to exist in such habitat.

The mechanisms by which flood-tolerant plants survive water-logging are complex and involve interactions of morphological, anatomical, and physiological adaptations (Kozlowski, 1997; Stevens et al., 2002). Generally, adverse effects of flooding may include changes in shoot growth of many plants due to suppressed leaf formation and expansion of leaves and internodes. This may increase growth of stem diameter of some flood-tolerant plants (McKevlin et al., 1995). Flooding often increases the proportion of parenchymatous tissue in the xylem and phloem of both angiosperms and gymnosperms (Kozlowski, 1997). This may cause root decay, inhibit root branches growth and growth of the existing roots (DeBell et al. 1984; Lieffers, Rothwell, 1986a; Lieffers, Rothwell, 1986b). Certain terrestrial plant species respond to poor aeration in flooded areas and produce aerenchyma mainly in the cortical tissues of the stem and root (Jackson, Colmer, 2006; Stevens et al., 2002; Jung et al. 2008). Thus, flooded habitats may change the anatomical structure such as the cuticle layer and phloem in leaves, pith cavity area and vessel lumen in stem (Tao et al. 2009).

One major task of ecophysiological research is to determine the regulation of water relations for an efficient utilization of water resources. Relative water contents (RWC) is a measure of the relative cellular volume. The highest RWC is 100-90 % that occurs when the stomata pores on a leaf are closed; sometimes it is related to a reduction of cellular expansion and growth (Gonalez et al., 2003).

In this study, it was assumed that *R. lingua* adapted and survived in a temporal flooded peat bog habitat due to morphological, physiological and anatomical adjustments. Hence, our first goal was to investigate its morphological and anatomical features of leaf and stem and anatomical adaptations of root. The second goal was to research if the flooded habitat caused high water deficit.

Materials and methods

Sampling of material

Plant material were collected from the northeast coastal flooded area of Vlasina Lake at latitude 42° 41' 46 N and longitude 22° 22' 14 E. The variety was identified in the field and laboratory. Plant samples were fixed in 50% ethanol and later deposited and recorded at the Herbarium and Wet Collection of the Department of Ecology and Geography of Plants, Institute of Botany and Botanical garden "Jevremovac", Faculty of Biology, University of Belgrade.

Preparation of plant material for anatomical analysis

Anatomical analysis of leaf, stem and root were performed. Samples were prepared according to the standard methods described by Ruzin (1999), though slightly modified. The modified protocol was as follows; plant specimens were fixed in Navashin's solution for 24 hrs followed by 5 min rinse under running water. Then, specimens were dehydrated in 70% ethanol for an hour, 80% for an hour, 90% for at least 2 hrs and then 100% for 5 min. After dehydration, the tissues were treated in a mixture solution of alcohol: xylol at a 1:1 ratio for 15 min and then transferred into pure xylol (bio-grade; Bio-Optica, Milano, Italy) for 30 min. Specimens were immersed in a "Bio-plast" melted paraffin (Bio-Optica, Milano, Italy) and kept for 24 hrs at 63-65°C. After that, specimens embedded in paraffin blocks were prepared in chrome moulds where fine and heated tweezers were used to correctly orient specimens and friendly cut and shape the blocks. Specimens embedded in paraffin blocks were mounted onto plastic block-holders by "Bio-plast", cooled on ice for 5 min and then attached to a microtome (Reichert, USA).

Slices of 5-7 μm thick were cut, floated in a water bath, and later transferred onto glass slides. Slides contained samples were dried in a hot oven at 65°C for an hour, cooled at room temperature for 2 min and then the paraffin was removed by immersing slides into pure xylol for 15 min. Slides were hydrated into a series of ethanol; 100%, 90% and 70% for 2 min each, kept in pure water for 1 min and then stained in aqueous solutions of stains in staining cuvettes. Differential staining was performed in safranin for 2 min, transferred into ordinary water for 3 min, followed by haematoxylin for 2 min and then washed in water for 5 min. Slides were washed into 70%, 96% and 100% ethanol for 5 min each, and then placed in clean xylol for 1 minute. Cover glasses were mounted on slides after a drop of Canada balsam was added and dried for 2 min at room temperature.

Morphological observations and anatomical analysis under light microscope

Certain quantitative measures were carried out for leaf, stem and root of *R. lingua*. Morphometric measures were taken for leaves that included; leaf length (though only for upper leaves), leaf length from base to the widest part, leaf width, leaf perimeter, and leaf area. While those for the plant included; length of each of the first three internodes, stem length and total number of internodes. Measurements performed on the cross section of leaf included; leaf thickness, width and thickness of epidermal cells and surface, total thickness of mesophyll including thickness of spongy and palisade tissues and number of layers for both tissues. Cortex thickness and radius of pith cavity were measured from the stem cross section.

The diameter of primary cortex and central cylinder at the radius of root cross section were also measured.

Measurement of leaf relative water content (RWC)and water deficit (WD)

Leaves of *R. lingua* were collected in the field on 30 August, 23 September and 20 October 2001. Leaves were selected for measurement of water deficit due to the ability of *R. lingua* to adapt to conditions of frequent moisture variations in flooded habitats. This was determined by total water content in leaf tissue, which was expressed as the amount of water per unit fresh weight of leaf RWC (Turner, 1981; Sinzar-Sekulic, unpublished data). Fresh weight of leaves was measured on a balance (Denver Instrument XL-410, US). The leaves were cut and weighed immediately and recorded as fresh weight (FW), and then leaves were placed onto moist filter paper to reach full saturation. The leaves were removed after about 24 h, blotted dry, and re-weighed to obtain turgid fresh weight (TFW). It was then dried for 24 h at 105 °C and re-weighed to record dry weight (DW). The percentage of relative water content (RWC) was calculated based on the following formula:

$$RWC = \frac{FW - DW}{TFW - DW} x100 \quad (1)$$

Afterwards, water deficit WD, which represents the amount of water, expressed as a percentage that lacks in fully water-saturated plant organs and was calculated by the formula:

$$WD = 100 - RWC \quad (2)$$

Measurement of water pressure (ψp)

Water pressure was measured in a pressure chamber (PMS Instruments, USA). Cut off leaves with petioles were placed in steel chamber that is filled with nitrogen. Direct pressure of nitrogen shows the amount of water pressure which is expressed in MPa.

Temperature of water, air and pH of water

Water and air temperature were measured by portable thermometers and the pH of water was measured using a portable pH meter (HANNA Instruments, Italy). The speed of the wind was measured by one anemometer.

Statistical analysis

To increase the accuracy of data and statistical analysis, each character was measured on 25 samples of leaf, stem and root of individuals from the plant population. All measurements of quantitative characters were performed on an "Image Analyzer" software package (QWin, Leica Microsystems Imaging Solutions Ltd, Cambridge, UK), and then the measurement results were processed by the software SPSS 15 for Windows (SPSS 15.0., 2006). For each character, basic parameters were statistically analyzed such as mean, minimum value, maximum value and standard errors.

Results

R. lingua belongs to a life form of large plant, tall and emerged hidrogeophytes, with rhizomes and hemicryptophytes with erect stems (Meg and emer-Alt-Rhiz Hyd G H scap). *R. lingua* represents a part of the wetland vegetation communities in Vlasina peat bog.

Table 1. Morphological and anatomical characteristics of *R. Lingua* leaves

Characteristic	Mean ± SE
Leaf length (mm)	055.38 ± 0.50
Length of mid-leaf (mm)	039.90 ± 0.40
Leaf width (mm)	000.50 ± 6.11
Perimeter (mm)	123.25 ± 0.10
Leaf thickness (μm)	130.13 ± 4.30
Mesophyl thickness (μm)	108.73 ± 0.40
Palisade thickness (μm)	032.96 ± 0.20
Spongy tissue thickness (μm)	075.78 ± 0.40
Width of upper epidermis (μm)	023.54 ± 0.10
Thickness of upper epidermis (μm)	009.53 ± 0.50
Width of lower epidermis (μm)	022.63 ± 0.90
Thickness of lower epidermis(μm)	011.87 ± 0.50
No of layers of palisade tissue	001.12 ± 0.07
No of layers of spongy tissue	005.92 ± 0.10

[1] Mean value ± Standard Error

Morphological characteristics of leaves

Leaves of *R. lingua* are long, wide and linear in shape and petioles are absent or leaves are sessile and differ only in size. This variety was identified as *R. lingua* var. *hirsutus* due to the presence of trichomes on both sides and long hair on the leaf edge (Table 1).

Anatomical characteristics

The leaf

The upper and lower epidermis consists of one layer of cells (Fig. 1). The exterior walls of these cells are strongly thickened with cuticle. The mesophyll is differentiated into palisade and spongy tissue. The palisade tissue consists of one layer, while the spongy tissue composed of 4 -7 layers of cells. The palisade tissue cells are large and long with intercellular spaces. The spongy tissue consists of irregularly shaped cells with thick walls. The thickness of palisade layer is less than that of the spongy tissue (Table 1).

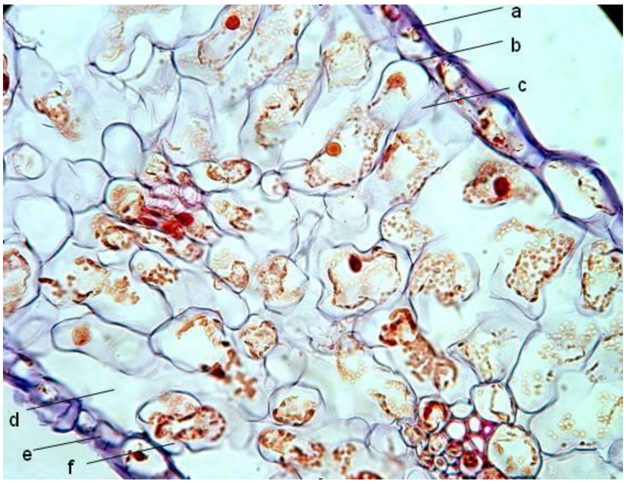

Figure 1. Cross section of *R. lingua* leaf: a. cuticule, b. epidermis, c. palisade tissue, d. intercelular space, e. stomata and f. spongy tissue.

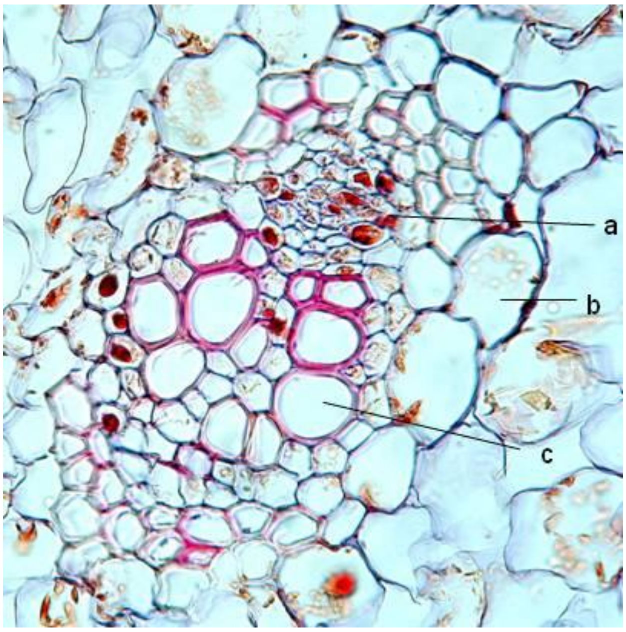

Figure 2. Cross section of *R. lingua* leaf vascular bundle: a. phloem, b. parenhymatic tissue and c. xylem.

The vascular tissue consists of closed collateral bundles, which are surrounded by mechanical tissue (Fig. 2). Number of vascular bundles varies from 24-26. Stomata were rarely observed.

The stem

The stem of this species is strong and right, very branched on its upper part, hairs are scarce, with many leaves and flowers. The stem height is up to 33 cm; with a large number of internodes and the first internode is the shortest (Table 2). The epidermis consists of one layer and another layer that is the outer layer of primary cortex. The cells of the primary cortex are arranged in radius with large intercellular spaces. The central cylinder contains a number of vascular bundles, and each vascular bundle is collateral, closed and covered by sclerenchymatic cells. In the center of the stem there is a rexigenous cavity (Fig. 3).

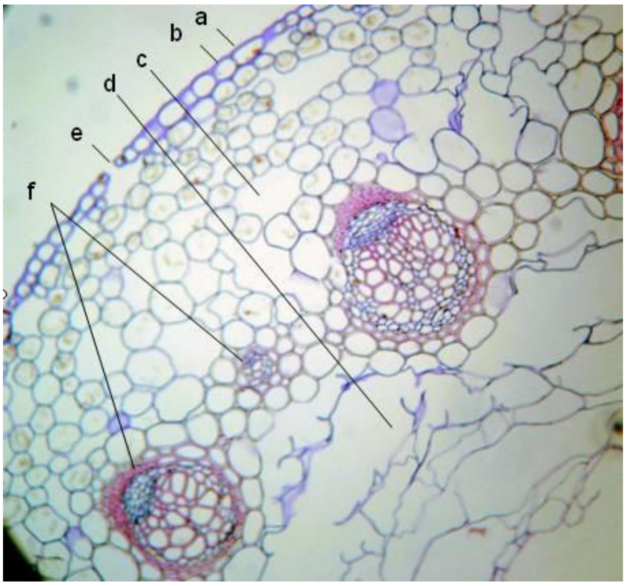

Figure 3. Cross section of *R. lingua* stem: a. cuticule, b.epidermis, c.intercelular space, d.rexigen cavity, e. stomata and f. vascular bundles.

Table 2. Morphological and anatomical characteristics of *R. lingua* stem

Characteristics	Mean with SE
Stem length (cm)	033.23 ± 0.30
Length of first internode (cm)	003.89 ± 0.30
Length of second internode (cm)	004.27 ± 0.03
Length of third internode (cm)	005.10 ± 0.05
Total number of internodes	008.08 ± 0.60
Diameter of stem cortex (μm)	711.18 ± 0.30
Radius of the cavity (μm)	995.17 ± 0.50

[1] Mean value ± Standard Error

The root

The root composed of the primary cortex and a central cylinder (Fig. 4). The epiblem with hairy cells replaced the epidermis. The first layer consists of small cells that are densely packed, but the rest of cells are larger. To the inside, the aerenchyma was observed, i.e. air filled-tissue that is characteristic of plants in aquatic habitats. Endodermis clearly forms the border of the primary cortex and central cylinder and made of large irregularly-shaped cells.

The central cylinder consists of a single or dual layer of pericycle and a radial vascular bundle. The xylem composed of large central bundles that are not well divided into 3-4 parts. The narrow parts of the bundles are made of trachieds and the central cylinder made of phloem. The primary cortex is highly developed (Table 3) and much wider than the central cylinder in the root (Fig. 4).

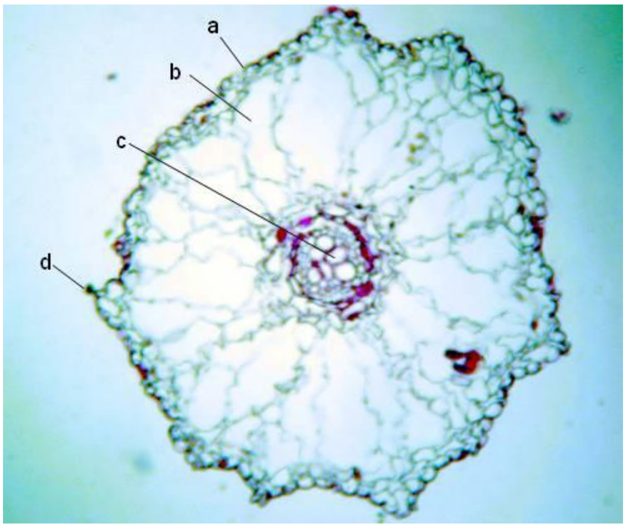

Figure 4. Cross section of *R. lingua* root: a. epiblem, b. intercelular space, c. xylem and d. hair cell.

Table 3. Anatomical characteristics of *R. lingua* root

Characteristics	Mean ±
Diameter of primary cortex (μm)	618.91 ± 0.3
Radius of central cylinder (μm)	191.73 ± 0.5

[1] Mean value ± Standard Error

Relative water contents (RWC), water deficit and water pressure

The relative water content was lowest in September (49 %) and increased during August (60%) and October (70%) (Fig. 5). Water deficit was highest in September due to the lower relative water content. Water pressure ranged between 0.3 and 1.2 MPa (Table 4).

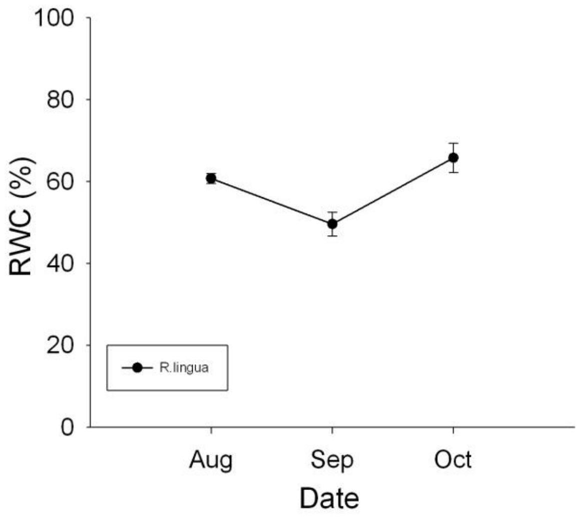

Figure 5. Relative water content (RWC) in leaves of *Ranunculus lingua* during August, September and October 2001. Bars represent standard errors.

Table 4. Water deficit in leaves of *R. lingua* during the period from August to October.

Date	Water deficit (%) Mean ± SE	Water pressure (MPa) Mean ± SE
30 August	39.24 ± 1.00	0.3 ± 0.2
23 September	50.37 ± 2.87	1.0 ± 0.2
20 October	34.22 ± 3.56	1.1 ± 0.1

[1] Mean value ± Standard Error

Weather and habitat conditions

Temperature of water decreased during the season, while air temperature was highest in September. The pH of water was neutral in August and higher in September and October. The speed of the wind ranged between 1.25 up and 3 m/s during the season (Table 5).

Table 5. Weather and water conditions from 30 August until 20 October in Murina dolina

Date	T. (C°) of water	T. (C°) of air	pH of water	Wind speed (m/s)
30-Aug	23.5	19.80	7.5	2.50
23-Sep	18.9	24.75	8.4	1.25
20-Oct	15.4	19.70	9.8	3.00

T; temperature

Discussion

The first goal of this study was to observe the morphological characteristics of leaves and stem and anatomical characteristics of leaves, stem and root of *R. lingua* in peat bog habitat, temporarily flooded in SE Serbia. Despite this terrestrial mode of life, *R. lingua* managed to adapt and survive during long or short periods of flood, when the root faces anaerobic conditions.

The flooded and disturbed habitat partially influenced the anatomical structure of leaves, stem and root. The leaves were long, wide and thick as an adaptation to protect leaves in an open habitat (Lakusic et. al. 2006). The epidermis composed of one layer of epidermal cells and the stomata were rarely observed. Low number of stomata and large intercellular spaces between cells in the palisade and spongy tissue could be an adaptation characteristic to wet habitats (i.e. Ciccarelli et al. 2009), that is in contrary to species from dry sites (Lakušic et al. 2006, Tao et al. 2009).

Length of the first internode of stem was shorter, which was in agreement with similar results of *Nyssa aquatica* L. that were reported by McKevlin et al. (1995). Regarding the anatomy, the stem showed typical structural features of the genus *Ranunculus* that consists of primary cortex, vascular tissue and cavity located at the centre. However, the influence of wet conditions was observed, too. The primary cortex composed of small cells separated by large intercellular spaces that are characteristics of a plant living in a wet habitat (Justin, Armostrong, 1987). Large cavity of the stem seems to be in relation to a strong stem elongation that develops in water, which causes cracks of parenchyma cells in the central cylinder during the development process (Tao et al. 2009). The central cylinder of stem consisted of many vascular bundles (see Results) possibly due to the emmersed conditions (Vasselati et al. 2001; Tao et al. 2009).

The root consisted of the primary cortex and central cylinder. The large intercellular spaces clearly show that the species adjusted to the frequent floods in habitat and the root mainly adapted to such water conditions (Raschio et al. 1999).

The second goal of this study was to determine water deficit and water pressure in order to investigate the adaptation of the species in the flooded habitat. The water deficit in leaves followed the changes in air temperature and reached its peak in September when air temperature was the highest (Table 5), and leaves were exposed to direct sun light that could have caused water loss through the few stomata that are present. Water deficit above 50 % seems critical for leaves under temperatures higher than 20˚C. According to Chaves et al. (2002), stomata may open in order to cool leaves under temperatures above 25˚C that cause intense evaporation. However, the low number of the present stomata seems to protect the leaves from the heat. It could be explained by wet conditions of the flooded habitat and water availability. In addition, the high number of vascular bundles in leaves (24-26) enables water conduction from other parts of the plant. The water deficit in August was around 40% despite the highest temperature of water in the lake that was recorded in this test, while that of October was around 35%, despite lower temperature of water recorded. Nevertheless, the water temperature seems not to affect water deficit. The air temperature influenced the difference in water deficit during the season. The growing period of *R. lingua* is during the mid of season when air temperature reaches its peak, which could lead to higher water deficit in large leaves. However, it is clear that *R. lingua* could withstand, thanks to an average of air temperature between 19˚C and 25˚C in that specific site, and water deficit was not higher than 50 %.

Conclusion

This study investigated *R. lingua* and its survivability in intensely flooded habitats. Low number of stomata and large intercellular spaces between cells indicated the influence of wet habitat. The morphological characteristics of stem were not influenced by the habitat except for the length of first internodes. The root was well adapted to water conditions. The growing period of plant is during the mid of season with higher air temperatures that could lead to higher water deficit up to around 50 %.

This study showed that the structure of plant organs are constant and contribute to species to cope with the habitat conditions. Water relations to the plant depended on flooding and temperature of the air and water in the lake. However, the highest water deficit was around 50 % and this was enough to allow *R. lingua* to overcome less water availability. *R. lingua* is able to survive in that small habitat in the Vlasina's peat bog. This species developed a root system typical of that of water plants and it was able to regulate its water deficit. In addition, the average air temperature was suitable for the species survival. However, this species is well adjusted for wet conditions and it is in a danger of longer disturbance of its habitat, such as drought that could severely decrease the population.

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