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THE EFFECT OF TOXIC ELEMENTS ON THE MICROANATOMY OF THE LEAVES OF THE *SALIX ALBA* L.

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ABSTRACT
The effects of a heavy metal containing wastewater sediment on two cultivars of white willow were investigated in a pot experiment. Our aim was to examine the effect of toxic elements on the microanatomical parameters of the leaves of the tested plant. We examined the following parameters: stomatal density, stoma width and length, lamina thickness, adaxial and abaxial epidermis thickness, mesophyll thickness, palisad and spongy parenchyma thickness, main vein width and length. The experiment had the following results: with the presence of toxic elements, the thickness of the lamina increased, within this, there was a significant growth in the thickness of the spongy parenchyma. The width and the length of the main vein decreased, so did the extent of the xylem cavities. The extent of the collenchymal stock of the leaf venation increased. The number of stomas increased, but the size of the stomas decreased. As a result of toxic element contamination, the number of Ca-oxalate crystals increased within the leaf mesophyll.

Keywords: heavy metals, toxic elements, *Salix alba*, leaf microanatomical parameters

INTRODUCTION

During the last century as a consequence of mining, metal processing, industrialization, transport, burning of fossil fuels, disposal of wastes, etc. soil and water resources were contaminated with metals and certain metalloids all over the world. This becomes of environmental concern when metals (i.e. Pb, Cd, Zn, Cu, Cr, Ni, Hg) in soils and waters enter to the food chain, and begins to affect human health (SIMON, 2014). *Phytoremediation* is the use of plants and their associated microbes for environmental clean-up. Plants have a natural ability to uptake inorganic chemicals (including metals) from soil and sediment, and accumulate them in their tissues. Bioenergy plants (including *Salix* spp.) having the potential to adapt well to the polluted lands, and have the capacity to produce high biomass along with high energy potential. The heavy metals accumulated in the shoot biomass are usually below the standard toxicity levels in the case of majority of the bioenergy plants (SIMON ET AL., 2022a, 2022b).
The toxic elements in soil have strong histological effects on the plant, which can be seen in the alternation of the microanatomical parameters of the leaf, stem and the roots. The toxic element content of the different organs correlates to the alternations of the tissue structure. ANDRÉ ET AL. (2006), VOLLENWEIDER ET AL. (2006) and HERMLE ET AL. (2007) mention the following histological parameters in the case of the leaves, which indicate the presence of toxic elements: thickness of the cuticle; stoma density and size; trichomes; the evolution of the typical characteristics of the epidermis; the evolution of the shape of the cells of the collenchyma; the shape and size of the calcium oxalate crystals and the presence of druses in the cells of the collenchyma bordering the leaf veins; the ratio of palisade and spongy parenchyma in the mesophyll; the thickness of the cell wall of the mesophyll cells. Their point out that parallel to soil pollution, the thickness of the leaf lamina significantly decreases, and as the stoma size decreases, the stomatal density increases.

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The aim of our study was to grow white willow cultivars, tolerant to environmental stress factors, in a growth chamber pot experiment to examine the microanatomical interactions of the test plants with a soil contaminated with toxic elements. The leaves of two test plants (*Salix alba* cv. Pörbölyi, *Salix alba* „82" Naperti clone) were microanatomically examined to determine, that the contaminated soil (amended with wastewater sediment from Debrecen-Lovász-zug) rich in toxic elements how and to what extent effects the micromorphometric characteristics of the leaves of the plants grown in it.

MATERIALS AND METHODS

Growth chamber pot experiment was set up with white willow (*Salix alba* L.) from April to July 2018. Municipal sewage sediment (MSS) originated from Lovász-zug suburban area of Debrecen city, Hungary (47°29′07″ N, 21°35′46″ E), where formerly a sewage settling pond was operated as a secondary biological purification unit. For the experiment, the soil used was the same soil (infill material) used for the reclamation of the pond. According to the WRB Soil Classification it belongs to ANTHROSOLS Reference Soil Group. During October of 2017 MSS samples were collected from this recultivated sewage settling pond (geographical point EOV X: 240876 m; EOV Y: 842073 m), where MSS was located under soil cover (BS) in a 70-110 cm depth. In the same time larger amounts from soil (BS) were also collected at the same location, from 0-30 cm depth. MSS samples were air dried, shredded, thoroughly mixed, and passed through a 5-mm diameter sieve. Soil samples were also air dried, mixed, and passed through a 2-mm diameter sieve. Samples were taken from the MSS and BS for chemical analysis. MSS basic characteristics were the followings: pH-H_2O 7.11; total salt content (m/m%):1.80; dry matter (m/m%):91.98; organic matter (m/m%):26.9; P–5125, K–2963, Ca–29206, Mg–7331; Mg As–12.3, Cd–1.27, Cr–1027, Cu–198, Mn–514, Ni–49.5, Pb–287, and Zn–888 mg/kg; as determined from cc. HNO_3–cc. H_2O_2 extract, followed the instructions of a Hungarian Standard MSZ 21470-50 (2006). BS basic characteristics were the followings: loamy texture; pH-H_2O 7.72; pH-KCl 7.30, total salt content (m/m%):0.057; $CaCO_3$ (m/m%):2.25; humus (m/m %):2.27; NH_4-N (mg/kg):37.0; NO_3-N (mg/kg):10.9; P–1122, K–1859, Ca–17921, Mg–5055; As–7.16, Cd–0.303, Cr–120, Cu–44.4, Mn–306, Ni–31.8, Pb–35.8, and Zn–176 mg/kg; as determined from cc. HNO_3–cc. H_2O_2 extract, followed the instructions of a Hungarian Standard MSZ 21470-50 (2006).

In April 2018 half of the soil was treated with 10% (m/m) MSS. Controls remained untreated. Plastic pots (with 20.5 cm diameter and 23 cm height) were filled with 5000 grams of untreated or treated soil in 4 replications. The growth media were fortified with 33 mg/kg P and 42 mg/kg of K (KH_2PO_4) and 40 mg/kg of N (NH_4NO_3). Soils were weekly saturated with distilled water, to promote the MSS–soil–mineral nutrient interaction. After 30 days of soil incubation 3 willow cuttings per pot were planted to growth media. In half of the pots 'Pörbölyi' willow cultivar, in another half 'Naperti '82' clone was cultivated for 52 or 49 days, respectively. The plants were watered every 3-4 days with distilled water to reach constant weight (75% of the 21% field water holding capacity) of the soil. Illumination intensity (5000 lux for the first 8 days, then 10500-13200 lux for the remaining growth time), illumination time (12 hours daily with 100% intensity and 2 extra hours with 50% intensity), temperature (24–26 °C during the day, 18–19 °C during the night) were controlled. Relative humidity was between 40 and 50%.

Leaf samples for microanatomical investigations were taken after 49 days of plant growth. From the best developed shoots 15^{th} intact leaves from the top were collected in 8 replications. Leaf samples were stored in Strasburger-Flemming's preservative mixture (mixture of 96% ethanol, 99.5% glycerol and distilled water in 1:1:1 ratio).

We made epidermis imprints and cross sections out of the leaf samples. The epidermis imprints were made by following the method of GARDNER ET AL. (1995): we made imprints of the upper and lower of the leaf lamina using clear nail polish and after the nail polish dried, we examined these under microscope. We conducted the following micromorphometric measurements: stomatal density, length and width of the stoma. The measurements and the counting of the stomas was done using an OLYMPUS (type BX51) light microscope, in 10x20 zoom.

We made the leaf cross sections using razor blades following the LIU ET AL. (2012) method, the examination of the cross sections was done by using an OLYMPUS light microscope. We coloured the preparatums with a 0.2% aqueous solution of toluidine blue. We examined the following parameters: thickness of the leaf lamina, thickness of the adaxial and abaxial epidermis, thickness of mesophyll, palisade and spongy parenchyma layer, width and length of midrib. We conducted the measurements using 10x20 and 10x40 zoom. We digitally archived the photos using an OLYMPUS camera. We measured each examined quantitative characteristic 60 times with each treatment, and we averaged the measurement values.

RESULTS

The leaves of the examined willow species are dorsiventral, typically amphistomatic leaves, both on adaxial and abaxial side of there are stoma appliances in-between the polygonal epidermal cells with wavy walls. The stomas are parcytic, the two bordering guard cell has longitudinal axis parallel to the stoma subsidiary cell. The stomas of the abaxial side are bigger in size compared to the stomas on the adaxial side. The position of stomas is random. The epidermises are covered in a thin cuticle layer. In the case of young leaves, both of the leaf sides are covered in trichomes which are mostly unicellular, long coverhairs with thin walls. The mesophyll consists palisade (2 cell rows) and spongy parenchyma (4 cell rows). The mesophyll cells sit close to each other, the intercellular space between the spongy parenchyma cells are very small, they appear as air space above stomas. The presence of rosette Ca-oxalate crystals is a typical characteristic of the leaf mesophyll. However, in the mesophyll cells surrounding the midrib the Ca-oxalate pyramids and crystal sand are typical as a vacuole-filling content part. The crystal bearing idioblast cells sit mostly in-between the mesophyll cells bordering the midrib, a small amount of them sits further away from the midrib, in-between the cells of the leaf mesophyll (spongy parenchyma) in the layers above the abaxial epidermis. The idioblast cells showed no characteristic difference in shape and size compared to the mesophyll cells (*Figure 1.*).

As an effect of the sewage sludge treatment, in the case of the *Salix alba* cv. Pörbölyi, due to the presence of toxic elements, the thickness of the lamina increased, the increase of the thickness of the spongy parenchyma layer was especially characteristic. In contrast to this, in the case of the *Salix alba* „82” Naperti a small decrease of the thickness of the lamina was noticeable due to the decrease of the thickness of the epidermis and the mesophyll. The treatment had further effects: in the case of cv. Pörbölyi the width and the lenght of the midribs decreased more significantly than in the case of the „82” Naperti clone. This is explicable with the strong lignification of the walls of the cellular elements of the wood part.

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A characteristic of the willow is that there are many collenchymas surrounding the midrib, which is important in this case, because the walls of collenchyma cell's rich in pectin. This pectin is able to build up an effective protective zone around the veins because the elements which are toxic to the plant metabolism can bound in the pectin-layer.

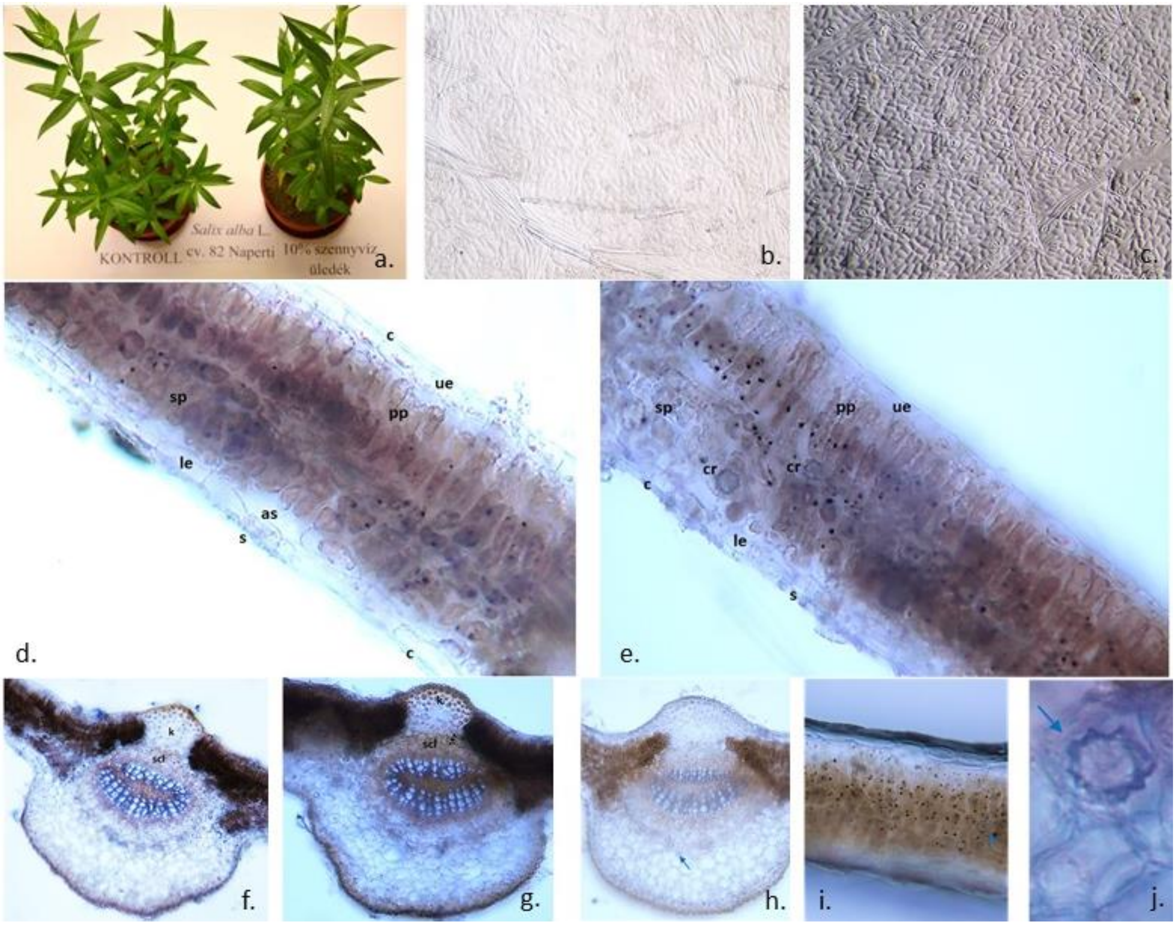

Figure 1. Pot experiment with white willow and the microanatomical characteristics of *Salix alba* cultivars

a. Pot experiment with white willow (University of Nyíregyháza, July 2018) - left: control, right: control + 10% D-L, b. Upper epidermis (cv. „82" Naperti), c. Lower epidermis (clone „82" Naperti), d. Leaf dorsiventral cross section (cv. Pörbölyi), e. Leaf dorsiventral cross section (clone „82" Naperti), f. Cross section of the midrib (cv. Pörbölyi), g. Cross section of the midrib (clone „82" Naperti), h – j. Ca-oxalate crystals (cv. Pörbölyi); (c: cuticule, ue: upper epidermis, le: lower epidermis, s:stoma, as: air space, pp: palisade parenchyma, sp: spongy parenchyma, cr: crystal, k: collenchyma, scl: sclerenchyma, →: Ca-oxalate rosettes)

It can be established, that in the case of Pörbölyi species the adaptation to the heavy metal contamination was more efficient (as a result of the toxic element content of the sewage sludge the thickness of the leaf plate did not decrease, the decrease of the size of the stomas and the increase of the stoma density was more noticeable than in the case of the cv. „82" Naperti species). However, the „82" Naperti clone has a more extensive collenchyma stock bordering the veins, this way it has a higher pectin content and this may lead to a better protection by bounding a bigger amount of toxic elements. This way of cadmium neutralization can be more effective against the toxic elements which have a negative effect on the life process of the plant than in the case of the cv. Pörbölyi.

We can establish that in the case of both willow cultivars examined, the stoma density is more characteristic on the abaxial epidermis, however the size of the stomas was bigger on the adaxial epidermis. It can be said, that the Pörbölyi cultivar has larger stomas than the „82" Naperti clone, and the value of the stoma density on the adaxial epidermis is also bigger compared to the „82" Naperti. However, in the case of cv. „82" Naperti, the stoma density on the abaxial epidermis is bigger than in the case of the cv. Pörbölyi. The data we measured during our experiment corresponds show that the heavy metal stress increases the number of stomas per unit. With the increase of stoma density, the size of the stomas decreases. As a result of the sewage sludge treatment, in the case of both willow cultivars examined, both in the case of the adaxial and abaxial epidermis, the value of the stoma density increased. Parallel to this, in the case of both species examined, both in the case of the adaxial and abaxial epidermis, the size of the stoma complexes decreased as a result of the treatment (*Table1.*).

Table 1. Leaf lamina micromorphological characteristic of *Salix alba* cv. Pörbölyi and Salix alba „82" Naperti clone (mean ± standard error)

	Control cv. Pörbölyi	Control + 10% D-L cv. Pörbölyi	Control clone „82" Naperti	Control + 10% D-L clone „82" Naperti
Lamina thickness (µm)	139±3.2	141±6.57	158.90±5.19	148.80±6.49
Adaxial epidermis (µm)	9.70±0.55	8.70±0.43	7.30±0.27	7.60±0.39
Stomatal density (numb./mm^2)	79.4±12.9	81.5±13.1	70±13.4	71.9±13.7
Stomata lenght (µm)	19.3±0.2	18.5±0.1	18.9±0.7	18.2±0.8
Stomata widht (µm)	5.7±0.1	5.5±0.1	5.5±0.3	5.2±0.2
Abaxial epidermis (µm)	7.50±0.2	7.50±0.21	6.60±0.26	6.30±0.21
Stomatal density (numb./mm^2)	118±15.6	121±16.4	220±21.6	224±22.3
Stomata lenght (µm)	17.0±0.1	16.4±0.1	19.6±0.2	19.0±0.1
Stomata width (µm)	6.8±0.2	6.6±0.1	5.9±0.2	5.8±0.1
Mesophyllum thickness (µm)	80.6±1.29	83.4±0.99	86.10±0.48	85.70±0.56
Palisade thickness (µm)	37.6±0.64	37.90±1.0	38.4±0.83	38.7±1.52
Spongy thickness (µm)	43.6±2.1	45.20±1.26	46.4±1.07	46.5±1.92
Main vein widht (µm)	761±18.5	742±12.4	713±48	701±36
Main vein lenght (µm)	636±18.7	629±13.6	427±47	421±38

n=60; 10% D-L - 10% (m/m) sewage sludge from Debrecen-Lovászzug

The crystal inclusions have an important role in plant detoxification, stress response and the reaction to heavy metal load. The plants do the detoxification and the adaptation to the extremely toxic elements/metal content in the soil by creating Ca-oxalate deposits, crystals in the vacuole. It can be established, that as a result of the treatment, the increase of the number of the Ca-oxalate crystals in the leaf mesophyll in the case of the cv. Pörbölyi was more characteristic than it was in the case of the „82" Naperti clone. This means that the reaction to heavy metal load was more characteristic in the case of the cv. Pörbölyi.

DISCUSSION

The effects of a heavy metal containing wastewater sediment on two cultivars of white willow was investigated in a pot experiment. As a consequence of treatment with wastewater sediment the following leaf microanatomical parameters changes could be recorded: the thickness of the lamina increased, especially the thickness of the spongy parenchyma. The width and the length of the main vein decreased, so did the extent of the xylem cavities. The extent of the collenchymal stock increased. The data we measured during our experiment corresponds with those statements from the relevant literature which state that the heavy metal stress increases the number of stomas per unit (CHARDONNENS ET AL., 1998, SHI and CAI, 2009), and decreases the size of the stomas (COSIO ET AL., 2006). As a result of toxic element contamination, the number of Ca-oxalate crystals increased within the leaf mesophyll (FRANCESCHI and NAKATA, 2005). The changes caused by the treatment in the micromorphometrical parameters show an increase in the heavy metal intake, the changes in the histological structure show the adaptation of the test plants to the changed environment. Based on the examinations, we can establish, that out of all willow species examined, the *Salix alba* cv. Pörbölyi cultivar has the best ability to adapt to the increasing toxic element concentration.

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