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THE EFFECT OF HEAVY METAL-CONTAINING WASTEWATER SEDIMENT ON THE MICROANATOMICAL CHARACTERISTICS OF THE LEAVES AND STEM OF *SALIX VIMINALIS* L.

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Abstract: A comparative microanatomical study of *Salix viminalis* L. shoots was performed in order to get an idea of the effect of toxic elements stress on the microanatomical parameters of the shoot (leaves, stem). The examined *Salix viminalis* L. shoots originated from the Lovász-zug suburban area of Debrecen city, where a sewage settling pond was formerly operated as a secondary biological purification unit. The control *Salix triandra* x *viminalis* L. 'Inger' samples originated from the Nyíregyháza experiment with uncontaminated soil. As a result of our research, we can state the following in the case of the leaf samples grown on contaminated soil: the leaf lamina thickness decreased; the extent of the palisade parenchyma decreased; the extent of the intercellular spaced increased inside the spongy parenchyma; the width and the height of the main veins increased; the extent of the collenchyma bordering the main vein increased; the stomatal density increased both in the case of the adaxial and abaxial epidermis; the size of the stomas decreased. In the case of the stem samples, we observed the following: in the case of the samples grown on contaminated soil the extent of the primer cortex increased; the cell wall of the cells building the sclerenchimatic fibers thickened; the number of Ca-oxalate crystal rosettes and sclereids increased; the extent of secondary phloem increased; the lumen of the tracheas in the secondary xylem increased; the average width of the annual rings decreased; the extent of the central stele of the stem increased.

Keywords: heavy metals, toxic elements, *Salix viminalis* L., leaf and stem microanatomical parameters

1. Introduction

As a result of today's highly intensified human activity, a large amount of sewage sludge, and wastewater sediment is generated, the disposal of which causes waste management and environmental protection problems. Due to their influence, certain parameters of the soil change: the pH value of the soil decreases, the total N-, P-, Na-, K- and Ca content increases, the Pb-, Cr-, Cd-, Cu-, Zn- and Ni concentration in the soil also increases. The main problem is that the metals they contain are generally non-degradable pollutants and can accumulate in the food chain in the process of biomagnification (Simon et al. 2022, Asati et al. 2016, Singh & Agrawal 2007). Some plants have a natural ability to uptake inorganic chemicals (including metals) from soil and sediment, and accumulate them in their tissues. *Phytoremediation* is the use of plants and their associated microbes for environmental clean-up. For example, the willow species growing in polluted areas can accumulate significantly higher concentrations of Cd, Pb, and Zn in all their organs than species found in unpolluted areas (Simon et al., 2022).

The toxic elements accumulated by plants from the soil cause characteristic histological changes, supported by many authors (André et al. 2006, Vollenweider et al. 2006, Hermle et al. 2007, Tóth, 2021, Tóth et al. 2022): the abaxial epidermis cells collapse as a result of heavy metal accumulation, the stomata shrink, the cells of the spongy parenchyma age, their size decreases, the chemical composition of the cell walls changes, their lignin and pectin content increases, while their thickness decreases. The cell wall thickness of palisade parenchyma's cells decreases. As a result of the pollution, a decrease in the number of cambium cells, the appearance of pseudo-annual rings, a reduction in the number of sieve elements that build up the phloem, and a decrease in the number of ray parenchyma can be observed. The structure of early and late wood changes, the thickness of the tangential wall of the tracheids of the late wood, the extent of the lumens of the tracheids, and the width of the annual rings.

The aim of our study was to make a comparative microanatomical study of *Salix viminalis* L. and *Salix triandra* x *viminalis* L. 'Inger' shoots were performed in order to get an idea of the effect of toxic elements (heavy metals) stress on the microanatomical parameters of the shoot (leaves, stem). For examinations, the plant samples were collected from two different experimental sites, contaminated and uncontaminated areas. In order to compare the soil properties of the two sample areas, soil sampling, and soil analysis were also carried out.

2. Materials and methods

For our studies, *Salix viminalis* and *Salix triandra* x *viminalis* L. 'Inger' shoot fragments and soil samples were collected from two different experimental sites.

The examined *Salix viminalis* L. shoots 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. The examined *Salix viminalis* L. plants were planted in 2013. For the examination of shoots and leaves, the samples were taken on September 19, 2017. For the microanatomical characterization of the leaves, the 5th position leaf of the best-developed shoot was collected in 5-5 cases of several plant individuals scattered in the area. The samples required for the microanatomical examination of the stem were collected in the middle thirds of the 3-4-year-old shoots (height 70-110 cm).

During October of 2017, soil samples were collected from this recultivated sewage settling pond (geographical point EOV X: 240876 m; EOV Y: 842073 m), where municipal sewage sediment (MSS) was located under soil cover (BS) in a 70-110 cm depth. Soil (BS) was collected from 0-30 cm depth. 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). According to the WRB Soil Classification, the soil belongs to ANTHROSOLS Reference Soil Group.

The control *Salix triandra* x *viminalis* L. 'Inger' samples originated from the open-field small plot long-term experiment with uncontaminated soil. The research area is located in parallel to Westsik Street in Nyíregyháza (Hungary; geographical coordinates: 47°58'41.8"N 21°42'00.7" E) in the experimental field of the University of Debrecen, IAREF, Research Institute of Nyíregyháza. Willows were planted in April 2011, cuttings originated from Holland Alma Ltd., Piricse, Hungary. For the examination of stems and leaves samples of control *Salix triandra* x *viminalis* L. 'Inger' were taken on April 17, 2018 (for the leaf examination the 5th position leaf of the best-developed shoot was collected in 5-5 cases, the stem samples were collected in the middle thirds of the 3-4-year-old shoots (height 70-110 cm)).

The basic characteristics of the uncontaminated Cambisol (brown forest soil with clay stripes) were the following at 0–25 cm depth: loamy sand texture; pH-H_2O: 8.10; pH-KCl:7.52; total salt (mm^{-1} %): <0.02; $CaCO_3$ (mm^{-1} %): 4.80; humus (mm^{-1} %): 1.51%; CEC ($cmol_c$ kg^{-1}): 10.4 P-621, K-2,918, Ca-16,307, Mg-4,603; As-9.60, Ba-57.5, Cd-0.21, Cr-13.7, Cu-9.18, Mn-372, Ni-14.0, Pb-9.89, and Zn-35.5 mg kg^{-1}; as determined from cc. HNO_3-cc. H_2O_2 extract followed the instructions of the Hungarian Standard MSZ 21470-50 (2006).

Stem and leaves samples were stored in Strasburger-Flemming's preservative mixture. After softening in the mixture, the stem samples were sectioned with a slide microtome. On the stem cross sections we examined the following parameters: cork thickness (μm), collenchyma thickness (μm), parenchyma of cortex thickness (μm), cortex thickness (μm), cell wall thickness of sclerenchymatous cells (μm), phloem thickness (μm), hard bast thickness (μm), soft bast thickness (μm), secondary xylem thickness (μm), the diameter of the largest trachea (μm), trachea wall thickness (μm), pith (μm), stem diameter (μm). We conducted the measurements using 10x20 and 10x40 zoom.

We made epidermis imprints and cross-sections out of the leaf samples. The epidermis imprints were made by following the classic method of Gardner et al. (1995). We conducted the following micromorphometric measurements: stomatal density, length, and width of the stoma. The measurements and the counting of the stomas were 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 examined the following parameters: the thickness of the leaf lamina, the thickness of the adaxial and abaxial epidermis, the thickness of mesophyll, palisade, and spongy parenchyma layer, and the width and length of the midrib. We conducted the measurements using 10x20 and 10x40 zoom. We measured each examined quantitative characteristic 10 times with each treatment, and we averaged the measurement values.

The statistical analyses were done by IBM SPSS Statistics 25 (Armonk, NY, USA) software.

3. Results

In the samples from Lovász-zug (contaminated sample area), the decrease of the thickness of the leafblade was observable, the extent of the columnar parenchyma decreased, but there was no significant difference (*Table 1*). Compared to the control samples, in the case of the samples from the contaminated area, the width and the height of the main veins increased, in the case of the height of the main veins, the difference was significant. In the width of the walls of the tracheas, visible growth was observable, which can be explained by the increasing lignification caused by toxic elements, but the difference was not remarkable. A significant amount of collenchyma borders the main vein, and the cell walls of the collenchyma cells are rich in pectin, pollutants harmful to plant metabolism are bound in this pectin layer. The facts mentioned above explain, why the extent of the collenchyma tissues is remarkably, roughly three times significantly bigger in the case of the contaminated samples, compared to the collenchyma mass of the willow leaves used as control samples. A remarkable, but not significant difference can also be observed in the extent of the sclerenchymatous tissues.

Typical of the *Salix* species, the stomatal density is more prominent on the abaxial epidermis, but the size of the stomas is slightly smaller on the abaxial epidermis, compared to the ones on the adaxial epidermis (*Figure 1*). In the case of the samples from Lovász-zug, it can be established, that the value of the stomatal density was significantly bigger (*Table 1*), both on the adaxial and the abaxial epidermis, compared to the control samples. The size of the stoma decreased, but there was no significant difference between the two examined samples. These results are similar to previous observations, which reported the same tendencies of changes in stomatal density and stomatal size in the case of heavy metal pollution.

By examining the microanatomical parameters of the stem samples, it can be stated, that in the case of the willow samples grown on the contaminated soil of Lovász-zug, the cortex thickness was bigger than in the case of the control samples, but the difference was not significant (*Table 1*). The major increase in the cortex thickness was caused by the collenchymatous tissue inside it and the increase of the parenchymatic tissues of the cortex. The difference between the control samples and the samples from Lovász-zug was significant, concerning the collenchymatous tissue. In the case of the samples from Lovász-zug, the presence of Ca-oxalate crystal rosettes and sclereids was observed (*Figure 1*). Their increased presence can be explained by the role they fulfill in the detoxification of the plant. A typical response to heavy metal contamination is the accumulation of crystal inclusions. These extract and isolate toxic elements/heavy metals from physiological processes, preventing their damage.

Table 1: The characteristic micromorphometric parameters of the leaf and the stem of examined willows (mean ± standard error)

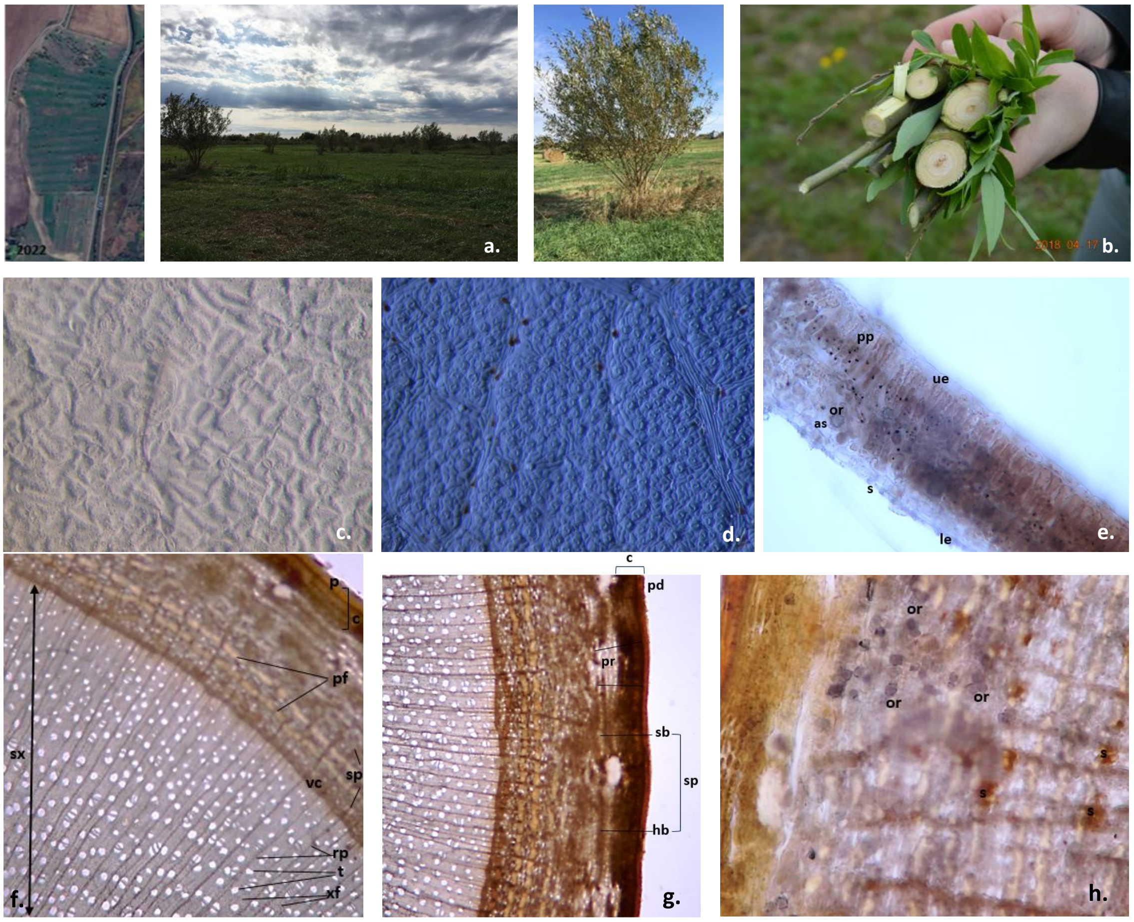
Leaf	Control	Lovász-zug
Lamina thickness (µm)	123,1±3[a]	119±2[a]
Adaxial epidermis (µm)	7,6±0,6[a]	8,1±0,3[a]
Stomatal density (numb./mm^2)	79±10,8[a]	88±4,5[b]
Stomata lenght (µm)	19,6±1,2[a]	22,9±0,3[a]
Stomata widht (µm)	5,7±0,2[a]	5,1±0,3[a]
Abaxial epidermis (µm)	7,3±0,2[a]	7,6±0,1[a]
Stomatal density (numb./mm^2)	118±13,6[a]	129±11[b]
Stomata lenght (µm)	17,1±0,3[a]	16,7±0,2[a]
Stomata width (µm)	6,6±0,7[a]	6,2±0,3[a]
Mesophyllum thickness (µm)	106,1±3,3[a]	101,6±3,7[a]
Palisade thickness (µm)	42,8±2,1[a]	32,2±2,4[a]
Spongy thickness (µm)	63,3±1,2[a]	42,2±3,1[a]
Main vein widht (µm)	708±18[a]	1192,4±11[a]
Main vein lenght (µm)	419±42[a]	678±29[b]
Collenchyma thickness (µm)	30,2±0,8[a]	87±0,3[b]
Scelernchyma thickness (µm)	21,8±1,7[a]	42±0,1[a]
Stem	**Control**	**Lovász-zug**
Cork thickness (µm)	16,13±0,01[a]	16,15±0,02[a]
Collenchyma thickness (µm)	49,17±4,57[a]	79,03±2,89[b]
Parenchyma of cortex thickness (µm)	54,04±5,65[a]	91,95±4,84[a]
Cortex thickness (µm)	119,36±9,69[a]	187,11±4,93[a]
Cell wall thickness of sclerenchymatous cells (µm)	3,53±0,53[a]	4,51±0,80[a]
Phloem thickness (µm)	185,42±12,00[a]	227,43±15,11[a]
Hard bast thickness (µm)	85,49±0,9[a]	124,20±0,7[a]
Soft bast thickness (µm)	100,01±5,27[a]	104,84±8,42[a]
Secondary xylem thickness (µm)	493,58±17,88[a]	500,03±9,03[a]
Diameter of largest trachea (µm)	43,20±4,41[a]	54,00±2,32[a]
Trachea wall thickness (µm)	1,12±0,40[a]	1,69±0,80[b]
Pith (µm)	462,93±23,09[a]	435,51±18,16[a]
Stem diameter (µm)	2056,49±30,71[a]	2146,90±32,32[a]

Source: Own data (n=10±S.D., significant difference compared to the control: $p<0.05$

In the willow samples that grew in areas heavily contaminated with heavy metals, the phloem thickness exceeded (by 22,7 %) the value measured in the case of the control samples, but the difference was not significant. Compared to the control samples, the extent of the hard bast thickness increased. The extent of the soft bast thickness did not show a significant difference between the two samples examined. It can be established, that the heavy metals/toxic elements found in the soil of Lovász-zug, did not have a significant effect on the development of the extent of secondary xylem thickness (*Table 1*). Out of the xylem elements there was a significant difference in the development of the diameter of the tracheas between the samples examined as controls and those from Lovász-zug. In the case of the control samples, the lumen of the tracheas did not reach the value of the samples from contaminated area. The thickness of the walls of the tracheas was much bigger in the case of the samples from Lovász-zug (the heavy metals are neutralized by accumulating in the cell walls to avoid the damages of the vital physiological processes, such as photosynthesis).

An interesting observation was that the average width of the growth rings was reduced in the case of the samples from contaminated soils compared to the control samples. It is also worth mentioning, that although the lumen of the tracheas increases on the contaminated sample areas, their density showed a slight decrease.

Figure 1: The sample area (Lovász-zug) and the microanatomical characteristics of the leaf and stem of examined *Salix viminalis* L.



a. Sampe area of Lovász-zug, b. Collecting of *Salix viminalis* L. samples, c. Upper epidermis, d. Lower epidermis, e. Leaf dorsiventral cross section - ue: upper epidermis, le: lower epidermis, pp: palisade parenchyma, sp: spongy parenchyma, s: stoma, as: air space, or: Ca-oxalate rosettes, f. *Salix viminalis* L. stem cross-section - p: periderm, c: cork, pf: phloem fibres, sp: secondary phloem, vc: vascular cambium, rp: ray parenchyma, t: trachea, xf: xylem fiber, sx: secondary xylem, g. *Salix viminalis* L. stem cross-section II. - c: cork, pd: periderm, pr: phloem ray, sb: soft bast, hb: hard bast, sp: secondary phloem, h. Ca-oxalate crystal rosettes (or) and sclereids (s) in the stem of Salix viminalis L.

In the development of the extent of the pith there was no significant difference in the case of the control samples and the samples from Lovász-zug, but overall the increase of the extent of the stem diameter was observed, which was caused by the increase of the extent of the secondary wood (*Table 1*).

4. Discussion

The effects of heavy metal-containing wastewater on microanatomical parameters of white willow shoots (stem, leaf) were investigated in an open-field experiment. As a consequence of examination the following leaf microanatomical parameters changes could be recorded: reduction of the leaf lamina thickness, decrease in the extent of the palisade parenchyma (Souza et al. 2011), an increase in the extent of the intercellular spaces within the spongy parenchyma, an increase in the width and height of the main vessels, an increase in the thickness of the walls of the xylem's tracheas (Kovačevič et al.,1999), reduction of the lumen of the tracheas, a tripling of the collenchyma tissue's expansion (Bowes, 1997), increase in the extent of the sclerenchyma tissue's expansion, increase in stomatal density in both adaxial and abaxial epidermis (Chardonnens et al. 1998, Baryla et al. 2001, Shi & Cai, 2009), the decrease in the size of the stomas (Cosio et al. 2006), increase in the presence of Ca-oxalate crystals. In the case of the stem, changes in the following leaf microanatomical parameters can be observed: an increase in the thickness of the primer cortex of the stem, the expansion of the collenchymatous tissue, an increase in the cell wall thickness of the cells that build the sclerenchymatous bundles, an increase in the number of the Ca-oxalate crystal rosettes and sclereids (Franceschi & Nakata 2005), a large increase in the thickness of the secondary phloem, an increase in the extent of the hard bast, decrease in the lumen of the trachea, increase in trachea's wall thickness, a decrease in the average width of the annual rings, a decrease in the density of the tracheas. The changes caused by the treatment in the micromorphometrical parameters show an increase in the heavy metal intake, and the changes in the histological structure show the adaptation of the plants to the changed environment.

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References

André, O., Vollenweider, P., Günthardt-Goerg, M. S. (2006): Foliage response to heavy metal contamination in Sycamore Maple (*Acer pseudoplatanus* L.). For. Snow Landsc. Res. 80, 3: 275–288.

Asati A., Mohnish P., Kumar N. (2016): Effect of Heavy Metals on Plants: An Overview, IJAIEM. Volume 5, Issue 3, 55-66.

Baryla, A., Carrier, P., Franck, F., Coulomb, C., Sahut, C., Havaux, M. (2001): Leaf cloroses in oilseed rape plants (*Brassica napus*) grown on cadmium polluted soil: causes and consequences for photosynthesis and growth. Planta, 212: 696-709.

Bowes, B. G. (1997): A color atlas of plant structure. London: Manson.

Chardonnens, A.N., Bookum, W. M. T, Kuijper, D. J., Verkleij, J. A. C., Ernst, W. H. O. (1998): Distribution of cadmium in leaves of cadmium tolerant and sensitive ecotypes of *Silene vulgaris*. Physiol. Plant., 104: 75-80.

Cosio, C., Vollenweider, P., Keller, C. (2006): Localization and effects of cadmium in leaves of cadmium-tolerant willow (*Salix viminalis* L.) Part I. Microlocalization and phytotoxic effect of cadmium. Environmental and Experimental Botany, 58, 64 –74. doi:10.1016

Franceschi, V. R., Nakata, P. A. (2005): Calcium oxalate in plants: Formation and function. Annual Review of Plant Biology 5: 641–671.

Gardner, S.D.L., Taylor, G., Bosac, C. (1995): Leaf growth of hybrid poplar following exposure to elevated CO_2. New Phytologist. 131: 81–90.

Hermle, S., Vollenweider, P., Günthardt-Goerg, M. S., McQuattie, C. J., Matyssek, R. (2007): Leaf responsiveness of *Populus tremula* and *Salix viminalis* to soil contaminated with heavy metals and acidic rainwater. Tree Physiology 27, 1517–1531.

Hungarian Standard MSZ 21470-50. 2006. Environmental testing of soils. Determination of total and soluble toxic element, heavy metal and chromium(VI) content. Hungarian Standards Board, Budapest (in Hungarian).

Kovačevič, G., Kastori, R., Merkulov, L. J. (1999): Dry matter and leaf structure in young wheat plants as affected by cadmium, lead and nickel. Biologia Plantarum, 42(1), 119–123.

Liu, F., Xiong, X., Wu, L., Fu, D., Hayward, A., Zeng, X. (2014): BraLTP1, a lipid transfer protein gene involved in epicuticular wax deposition, cell proliferation and flower development in *Brassica napus*. PLoS One 9(10):10272. doi:10.1371/journal. pone.0110272

Shi, G.R., Cai, Q.S. (2009): Cadmium tolerance and accumulation in eight potential energy crops. Biotechnol. Adv., 27: 555-561.

Simon, L., Makádi, M., Uri, Zs., Vigh, Sz., Irinyiné Oláh, K., Vincze, Gy., Tóth, Cs. (2022): Phytoextraction of toxic elements and chlorophyll fluorescence in the leaves of energy willow (*Salix* sp.), treated with wastewater solids and wood ash. Agrokémia és Talajtan (Agrochemistry and Soil Science) 71(1):77-99.

Singh R.P., Agrawal M. (2007): Effects of sewage sludge amendment on heavy metal accumulation and consequent responses of *Beta vulgaris plants*. Chemosphere, 67: 2229–2240.

Souza, V. L., De Almeida, A. A. F., Lima, G. C. S., Cascardo, J. C. M., Silva, C. D., Mangabeira, A. O. P., Gomes, P. F. (2011): Morphophysiological responses and programmed cell death induced by cadmium in *Genipa americana* L. (Rubiaceae). Biometals, 24, 59 –71.

Tóth, Cs. (2021): Effects of wood ash on the histological characteristics of the stem of *Sinapis alba* and *Vicia villosa*. In: Tóth, C. (ed.). Indigenous and Landscape Varieties - Ecological Products - Healthy Nutrition - Rural Development Conference. University of Nyíregyháza. Proceedings of the Conference. Publisher: University of Nyíregyháza. pp. 167–177. (ISBN 978-615-5545-69-6).

Tóth, Cs., Irinyiné-Oláh, K., Simon, L. (2022): The effect of sewage sediment containing toxic elements on the microanatomy of the leaf of *Sorghum* species. In: Páy, Gábor László (ed.) International Multidisciplinary Conference, 14th Edition. Nyíregyháza, Hungary: University of Nyíregyháza. 124-133.

Vollenweider, P., Cosio, C., Günthardt-Goerg, M. S., Keller, C. (2006): Localization and effects of cadmium in leaves of cadmium-tolerant willow (*Salix viminalis* L.) Microcolonization and cellular effects of cadmium. Environmental and Experimental Botany. 58: 25–40