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# ВЛИЯНИЕ ТЯЖЕЛЫХ МЕТАЛЛОВ НА МОРФОЛОГИЧЕСКИЕ И ФИЗИОЛОГИЧЕСКИЕ ХАРАКТЕРИСТИКИ КЛОНОВ *SALIX*

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Объектом исследований послужили три клона *Salix alba* и один клон *Salix viminalis*. Предмет исследования изучение влияния тяжелых металлов (Ni, Cu, Cr, Cd, Pb, As) на морфологические и физиологические процессы клонов ивы. Цель исследования – обоснование и выбор клона наиболее перспективного для получения биомассы в целях производства энергии при выращивании на загрязненной тяжелыми металлами почве. Перспективные клоны ивы оценивались по морфологическим параметрам (высота растений, димаметр стеблей, биомасса, площадь листев) и физиологическим индикаторам (интенсивность фотосинтеза и транспирации, устьичная проводимость, эффективность использования воды, межклеточная концентрация $CO_2$). Загрязнение почвы тяжелыми металлами отрицательно сказалось на морфологических и физиологических характеристиках клонов ивы. По приведенным данным были выделены клоны, физиологические характеристики которых были менее зависимы от загрязнения почвы тяжелыми металлами. Физиологические показатели клонов положительно коррелировали с морфологическимим параметрами. Теплота сгорания биомассы ивы, выращенной на загрязненной и незагрязненной почве, существенно не различалась. В результате исследований с учетом физиологических и морфологических показателей выделены два клона *Salix alba*, перспективные для проведения дальнейших исследований и испытаний на загрязненных тяжелыми металлами почвах.

***Ключевые слова:*** клоны *Salix*; тяжелые металлы; морфологические параметры; физиологические процессы.

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# THE INFLUENCE OF HEAVY METALS ON MORPHOLOGICAL AND PHYSIOLOGICAL PARAMETERS OF *SALIX* CLONES

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Three clones of *Salix alba* and one clone of *Salix viminalis* were used for research purposes. The research aims to study the influence of heavy metals Ni, Cu, Cr Cd, Pb and As, on morphological (primary and secondary growth) and physiological (rate of photosynthesis, transpiration, water use efficiency) processes and determine the most favourable clone that would have its application in phytoremediation of contaminated soil and productivity of different fast-growing clones, with the aim of energy production. Prospective clones of willow were evaluated based on morphological (plant height, stem diameter, biomass, leaf area) and physiological (photosynthesis intensity and transpiration, stomatal conductance, efficient use of water, intercellular concentration of $CO_2$) parameters. Contamination of soil with heavy metals negatively affected the morphological and physiological characteristics of willow clones. Clones with physiological characteristics less dependent on soil contamination with heavy metals were selected on the results of evaluation. Physiological parameters of clones positively correlated with morphological parameters. Heat of willow biomass combustion planted on contaminated and uncontaminated soils didn't differ significantly. Among the studied genotypes, two *Salix alba* clones, namely clones 3 and 4, stand out. The contaminated habitat substantially reduces willow biomass and physiological parameters of willow. Nonetheless, the thermal energy derived from biomass showed no significant variance between contaminated and uncontaminated plants.

***Keywords:*** clones of *Salix*; heavy metals; morphological parameters; physiological processes.

***Acknowledgement.*** This research was funded by the Science Fund of Republic of Serbia through research project «Landfill Remediation with the Use of Short Rotation Biomass Woody Crops (SRWC) Energy Plantations and Provisioning Multiple Ecosystem Services (TreeRemEnergy) 5357».

## Introduction

The development of society, with a view to improve the comfort of life through development, industrial upgrading and urbanization results in environmental pollution, and thus the deterioration of living conditions. The environmental pollution itself is caused by an increase in harmful substances in the soil, air and water, and therefore we can also say in the plants and food that people consume. The biggest pollution is caused by metals and metalloids, regardless of where they come from. One of the compromise solutions is the application of plants in the remediation of habitats polluted by heavy metals, that is, the establishment of plantations in such habitats that would carry out phytoremediation and be used in co-combustion processes with coal for energy purposes [1].

Bearing in mind that woody species are increasingly used for phytoremediation, in addition to other plants, including poplars and willows, which in addition to phytoremediation also show energy values, we paid special attention to willows both in the process of phytoremediation and energy production. From taxonomy aspect the genus *Salix* represents a large complex and according to certain data it encompasses 350–370 species [2], while other literature data point out that there are even over 400 species of willow with more than 200 hybrids [3].

Willow used for bioenergy production has been studied as a possible phytoremediation crop since the 1990s [4–12]. Research has shown the highly prominent ability of these species to take up and accumulate large amounts of zinc (Zn) and cadmium (Cd) [13], while at the same time they produce high quantities of biomass [14]. Based on the results of a large number of studies, it can be concluded that each individual incident of pollution with a certain heavy metal implies the selection of specific willow genotypes. Thus, willow plants grown in different ecological conditions showed that clones of *Salix alba* and *Salix dasyclados* species as well as *Salix aurita* and *Salix dasyclados* hybrids are fair candidates for biomass production on degraded peat soils [15]. The production of willow biomass and its burning as raw material for energy production ensures both ecological and rural development [16].

For the research purposes of this work, three clones of white willow – *Salix alba* and one clone of basket willow – *Salix viminalis* were used, and what needs to be pointed out is that there are currently no published results of similar analyses in the available professional literature. The purpose of this research was to examine the potential of four autochthonous willow genotypes in the phytoextraction of heavy metals Ni, Cu, Cd, Pb, Cr and As, their accumulation, translocation and adaptation to the presence of the mentioned heavy metals. This paper provides morphological-physiological indicators for the purpose of selecting the most favourable clones for phytoremediation and energy use of plantations. It researches the variability of the morphological and physiological characteristics of willows, different clones, growing on contaminated and uncontaminated (control) soil, with a view to select the most efficient clones for growing in plantations, that is, for obtaining bioenergy.

## Materials and methods

The material and method of work include testing the reaction of willow genotypes to two habitats: control – uncontaminated soil and contaminated to which heavy metals were added. Trials were set up at the University of Belgrade, Faculty of Forestry, with 4 autochthonous willow clones: I. *Salix viminalis* – the basket willow; II. *Salix alba* – clone B-44, III. *Salix alba* – clone 347 and IV *Salix alba* – clone NS 73/6.

For additional contamination, aqueous solutions of heavy metal salts were used, namely: Cd $(NO_3)_2$, $CuSO_4 \cdot 5H_2O$, $K_2 Cr_2 O_7$, $Na_2 HAsO_4 \cdot 7H_2 O$, $NiCl_2$ $6H_2 O$ and $PbNO_3$ in concentrations of $10^{-3}$ mol/dm$^3$ i. e. Cd 112.4, Cu 63, Cr 104, As 74.9, Ni 58.7 and Pb 207.2 mg/kg.

The plants were grown in field experiments (control) located in the Arboretum of the Faculty of Forestry and in containers with contaminated soil with a volume of 10 liters, in the period from March 2019 to September 2021. On the contaminated land, three cuttings were planted for each variant of heavy metals in 20 repetitions per pollution element, that is, 120 repetitions for all elements.

The variability of morphological plant characteristics of different willow clones was carried out on samples of 50 plants from each clone that were grown in uncontaminated (control) soil and the contaminated soil. Plant parameters were analyzed twice, at the beginning and at the end of the vegetation period, namely: plant height (cm), measured with a metric folding ruler; plant diameter – the diameter in the root crown(mm), measured with a micrometer, leaf area (cm$^2$) measured with LI-1800 Portable Spectroradiometer.

Photosynthetic indicators of gas exchange were measured using the LCpro+ system, manufactured by ADC Bioscientific-UK. The measurement was performed on four plants of each clone and treatment, in four technical repetitions, which means that there were 16 repetitions in total. The following parameters were analyzed: rate of photosynthesis (A) (μmol $CO_2$ $m^{-2}$ $s^{-1}$); stomatal conductance (gs) (mol $m^{-2}$ $s^{-1}$); intercellular concentration of $CO_2$ (ci) (μmol $mol^{-1}$); transpiration rate (E) (mmol $H_2O$ $m^{-2}$ $s^{-1}$); water use efficiency (WUE) (μmol $CO_2$ $mmol^{-1}$ $H_2O$);

The first four parameters were read directly from the device. Water use efficiency (WUE) was calculated subsequently as the ratio of the rate of photosynthesis and transpiration (A/E) [μmol mmol$^{-1}$], and endogenous water use efficiency (iWUE) as the ratio of the intensity of photosynthesis and stomatal conductance (A/gs) [μmol mol$^{-1}$] (Zhang, et al., 2003) [17].

Water use efficiency WUE, intrinsic water-use efficiency (iWUE), The STATGRAPHICS CENTURION XVI.I software was used to determine the variability of the morphological properties of the analyzed clones in different treatments [18]. In order to determine the variability between clones, descriptive statistics and One-way Analysis of Variance-ANOVA with Fisher's test of least significant differences (LSD) were applied.

## Results and discussion

*Morphological indicators.* Heavy metals also have adverse effects on morphological indicators. Their adverse effects are mostly reflected in the disruption of the chloroplast structure, the synthesis of chlorophyll, carotenoids, plastoquinone, by destructive action on pigment-protein complexes, enzyme conformation and activity, electron transfer in the transport chains of respiration and photosynthesis [19–22]. Vasilev, et al. [23] found a decrease in the concentration of chlorophyll, and consequently the decreset of photosynthesis rate in two willow clones treated with elevated concentrations of Cd in water cultures. More intense accumulation of cadmium and nickel in young willow leaves results in impaired photosynthetic and respiratory metabolism. This level is maintained for some time, and with the aging of the leaf it begins to decrease, as a result of which old leaves have a smaller contribution to the production of organic matter than young ones.

*Primary growth – hight increase in plants.* Based on the mean values of plant heights of all clones that were measured on plants grown in uncontaminated (control) soil, the values ranged from 104.2 cm to 206.9 cm for clone 1, from 106.8 cm to 216.9 cm for clone 2, from 140.4 cm to 293.5 cm for clone 3 and from 129.1 cm to 177.4 cm for clone 4 (Figure 1).

The obtained results indicate that after the third year, clone 3 exhibited the most significant height growth among all clones, while clone 4 showed the least height growth.

All clones reached almost the same height between the second and third year, when cutting – rotation could be carried out. The data indicate that clone 3 has the fastest growth rate, which reached a height of 272 cm in the second year and 293 cm in the third year. Our data are in line with the data of Greene [24] who obtained remarkable growth rates of willow Salix babylonica obtained from cuttings. The willows showed good results in terms of tree height, diameter, trunk, leaf area and root growth rate already after two years. Based on the mean values of plant heights of all clones that were measured on plants grown in contaminated soil, the values ranged from 56.5 cm to 122.1 cm for clone 1, from 43.8 cm to 110.4 cm for clone 2, from 61.4 cm to 125.5 cm for clone 3 and from 66.1 cm to 119.8 cm for clone 4 (Figure 2).

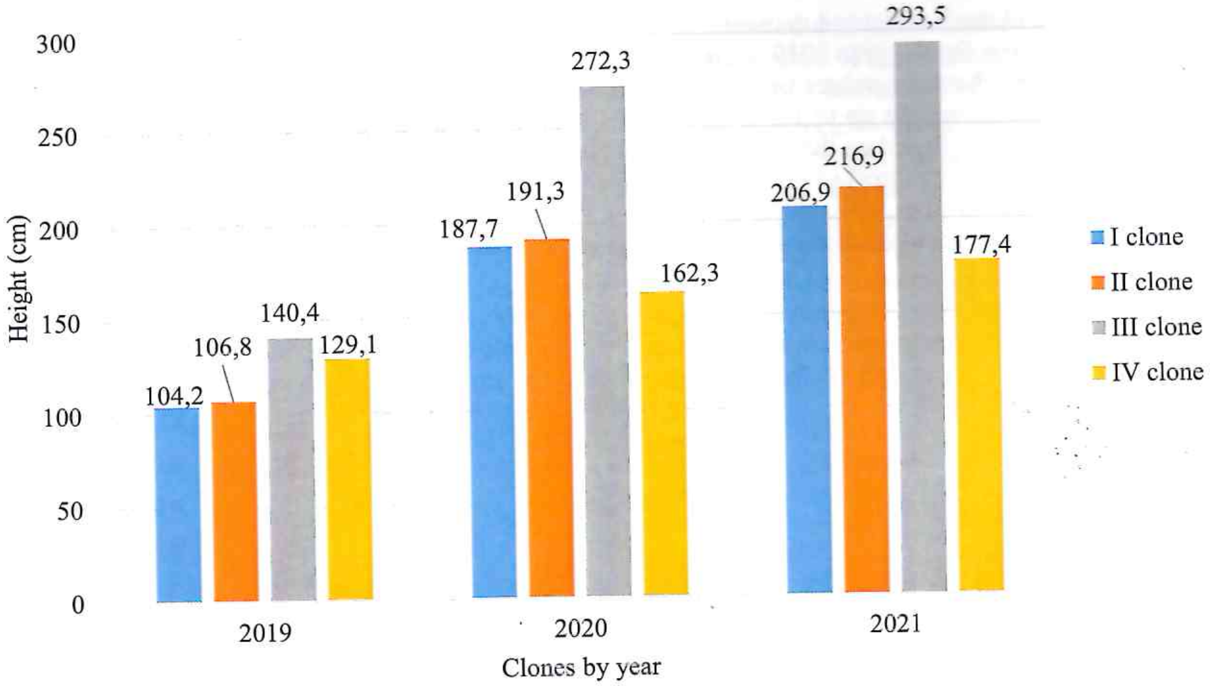

*Fig. 1.* The height of willow clones in uncontaminated soil (2019–2021)

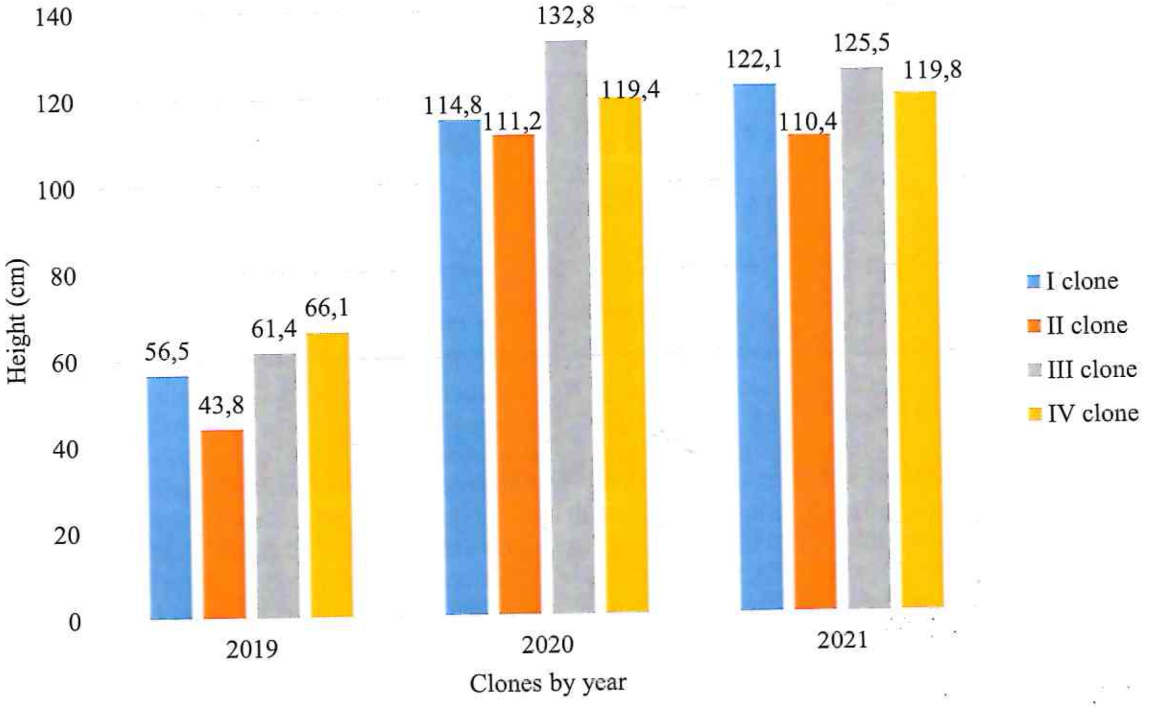

*Fig. 2.* Height of clones of willow in contaminated soil (2019–2021)

The obtained results indicate that the clone 3 is the clone with the greatest height growth, while clone 4 is the clone with the smallest height growth. The studied data (mean values) show that the plants grown on the contaminated soil are almost twice as low (Hsr = 120 cm) as the control plants (Hsr = 223 cm), but still clones 3 and 4 stood out for their height, although the other colons were also very close in height, so that cutting could be done in the plants after the second year.

*Secondary growth – the diameter of plants.* Based on mean plant diameter values of clone 1 measured on plants grown in uncontaminated (control) soil, we obtained the values ranging up to 7.80 mm for the year 2019, from 9.65 mm to 11.65 mm for 2020 and from 13.09 mm for 2021. Based on mean plant diameter values of clone 2 measured on plants grown in uncontaminated (control) soil, we obtained the values ranging up to 8.57 mm for the year 2019, from 10.02 mm to 12.48 mm for 2020 and from 13.59 mm for 2021. Based on mean plant diameter values of clone 3 measured on plants grown in uncontaminated (control) soil, we obtained the values ranging up to 9.07 mm for the year 2019, from 12.56 mm to 16.77 mm for 2020 and from 19.97 mm for 2021. Based on mean plant diameter values of clone 4 measured on plants grown in uncontaminated (control) soil, we obtained the values ranging up to 11.73 mm for the year 2019, from 13.11 mm to 13.82 mm for 2020 and from 14.35 mm for 2021. Based on the mean values of plant diameter of all clones that were measured on plants grown in uncontaminated (control) soil, the values ranged from 7.8 mm to 13.09 mm for clone 1, from 8.57 mm to 13.59 mm for clone 2, from 9.07 mm to 19.97 mm for clone 3 and from 11.73 mm to 14.35 mm for clone 4 (Figure 3). The obtained results indicate that the clone 3 is the clone with the largest diameter.

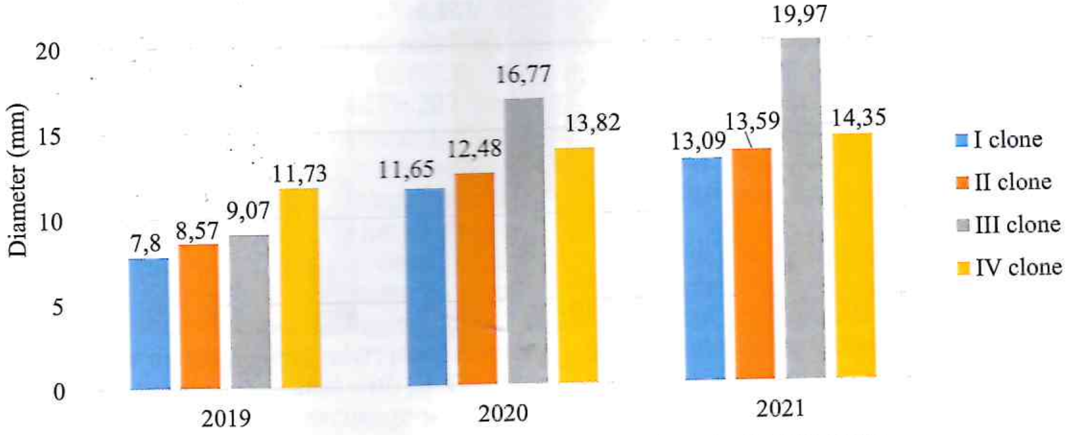

*Fig. 3.* Diameter of clones of willow in uncontaminated soil (2019–2021)

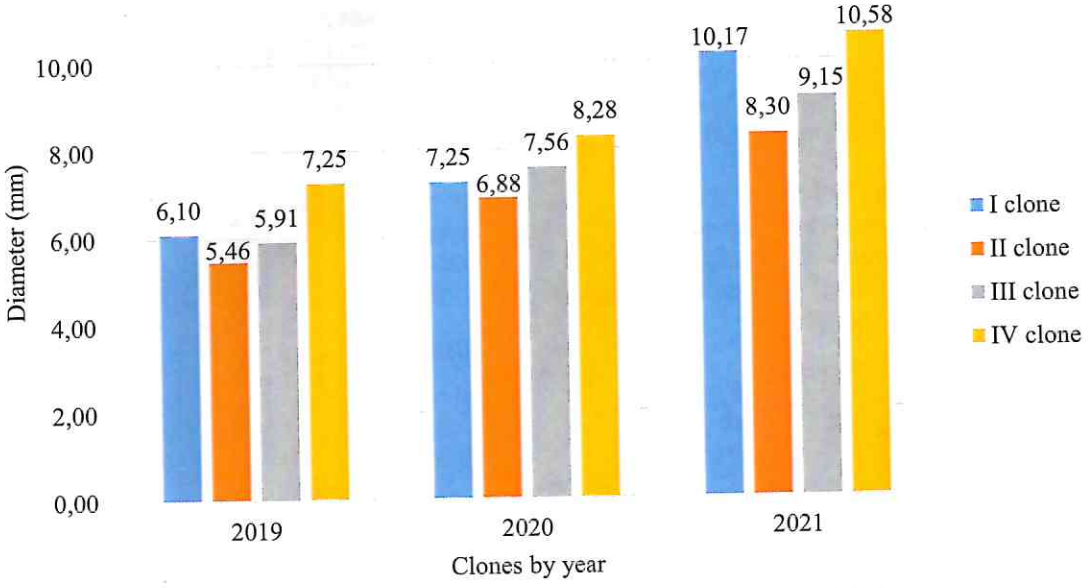

*Fig. 4.* Diameter i of clones of willow in contaminated soil (2019–2021)

*Leaf area.* The leaf areas of plants grown in uncontaminated soil ranged from 34.73 cm$^2$ (clone 2) to 42.89 cm$^2$ (clone 4), and in plants grown in contaminated soil, they ranged from 22.94 cm$^2$ (clone 2) to 31.63 cm$^2$ (clone 4). Based on the results, it can be concluded that contamination with heavy metals has an adverse effect on the morphology of the plants – the leaf area of the plants for all four clones Table 1.

Table 1

**Differences in leaf area between plants on contaminated and non-contaminated soils (cm$^2$)**

| Factor | | Clone | | | |
|---|---|---|---|---|---|
| | | I | II | III | IV |
| | Count | Average | Average | Average | Average |
| 1 | 100 | 25.36 | 22.94 | 27.89 | 31.63 |
| 2 | 100 | 36.24 | 34.73 | 36.92 | 42,89 |
| Total | 200 | 30.80 | 28.83 | 32.41 | 37.26 |

Note. Factor 1 – contaminated soil; Factor 2 – uncontaminated (control) soil.

In all four clones, the differences in leaf area between contaminated and uncontaminated plants are highly significant, but there is a difference in leaf area size among clones. Regardless of the treatment of plants, ie. whether grown in contaminated or uncontaminated soil, clone 4 always stood out by leaf area, and clone 2 was the lowest.

*Photosynthetic indicators.* The photosynthetic activity of plants is a decisive factor in the yield of plants and depends on a number of factors such as the rate, quality and duration of daylight, the concentration of $CO_2$ in the atmosphere [25; 26], the concentration of $O_2$ , temperature, water regime and the specifics of mineral nutrition [27 ], and especially from the genotype of the plant species and the status of the soil, i. e. its pollution [28; 29]. A comparison of 11 different Salix genotypes by Andralojc, et al. [30], showed that the timber yield is positively correlated with the total leaf area per plant, as well as that the rate of photosynthesis depends on the studied genotypes. Habitat pollution had a statistically significant effect on all examined clones, which reacted differently to the degree of pollution. he clone number 1 showed the highest rate of photosynthesis (16.21 µmol $CO_2$ $m^{-2}$ $s^{-1}$) and the clone number 4 showed the lowest rate, only 10.33 µmol $CO_2$ $m^{-2}$ $s^{-1}$ (Figure 5).

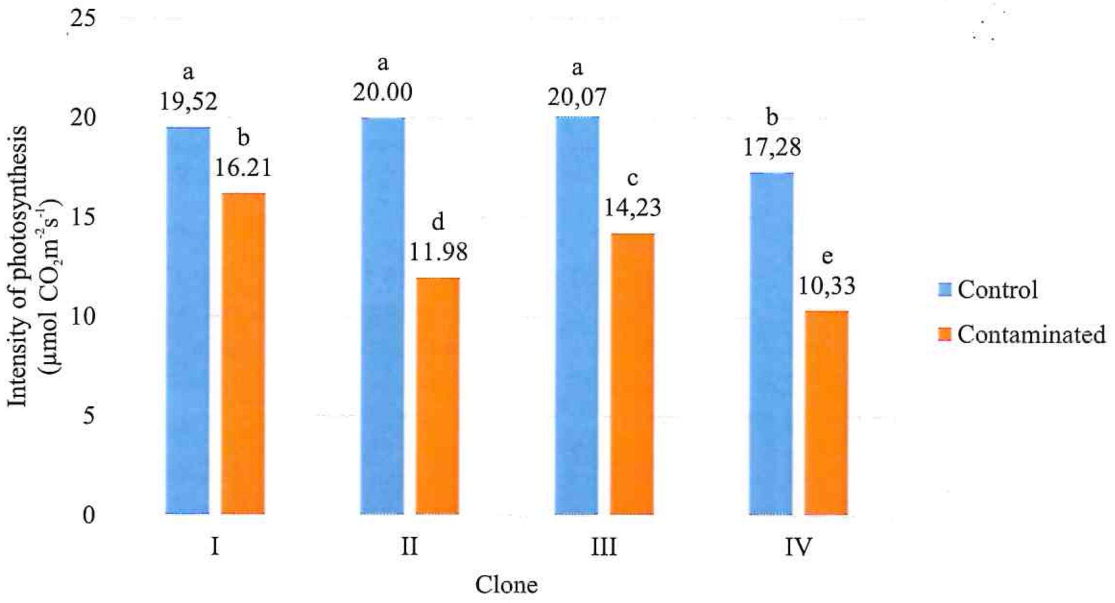

*Fig. 5.* The intensity of photosynthesis of willow clones depending on the soil contamination

Literature data [31] on the impact of soil pollution (Pb, Cr, Ni and diesel fuel) with heavy metals indicate the genotypic specificity of photosynthesis of the studied willow clones grown in glasshouse with different degrees of pollution.

*Stomatal conductance (gs) (mol $m^{-2} s^{-1}$).* In the process of water release and gas exchange, $CO_2$ and $O_2$ stomata occupy a central place because they regulate stomatal conductance by opening and closing, that is, by changing the size of the stoma opening. Changing the size of the stoma opening also changes the stoma's conductance [32; 33]. The stomatal conductance indicator is defined as the flux of carbon dioxide and water vapor inside the stoma and water vapor at the border of stoma-leafand air, and this indicator is the opposite of stomatal resistance [34].

In the control habitat (Figure 6), clone number 4 stands out with the highest stomatal conductance (0.48 mol $m^{-2}$ $s^{-1}$), and clone number 2 is the clone with the lowest stomatal conductance (0.25 mol $m^{-2}$ $s^{-1}$). The situation is different in the contaminated habitat because heavy metals inhibit the process of stomatal conductance. Namely, in the contaminated habitat, clone number 4 is the clone with the lowest stomatal conductance (0.13 mol $m^{-2}$ $s^{-1}$), while clone number 1 is the clone with the highest stomatal conductance (0.30 mol $m^{-2}$ $s^{-1}$).

*Intercellular concentration of $CO_2$ (ci) (µmol mol-1).* Considering that all leaves are in direct contact with the atmosphere, mesophyll cells take up $CO_2$, during photosynthetic assimilation, so there is a concentration gradient in the air outside the leaf and the intercellular space. Having that in mind, the intercellular concentration of $CO_2$ in leaves (Ci) stands as a critical parameter in photosynthesis [35]. This means that during photosynthesis, the concentration of carbon dioxide in the intercellular space of the leaf determines the flow of carbon dioxide into the leaf if the stomatal openings and the external concentration are constant.

Comparison of 4 willow genotypes on control variants clearly shows statistically significant differences between clones. The highest intercellular concentration (Figure 7) is found in clone number 4 (220.7 µmol $mol^{-1}$), while clone number 2 shows the lowest one (144.9 µmol $mol^{-1}$).

Comparison of 4 willow genotypes on contaminated variants clearly shows that there are no statistically significant differences in the intercellular concentration between the 3 clones (clone 1, 2 and 3) and that only clone number 4 is statistically different from them.

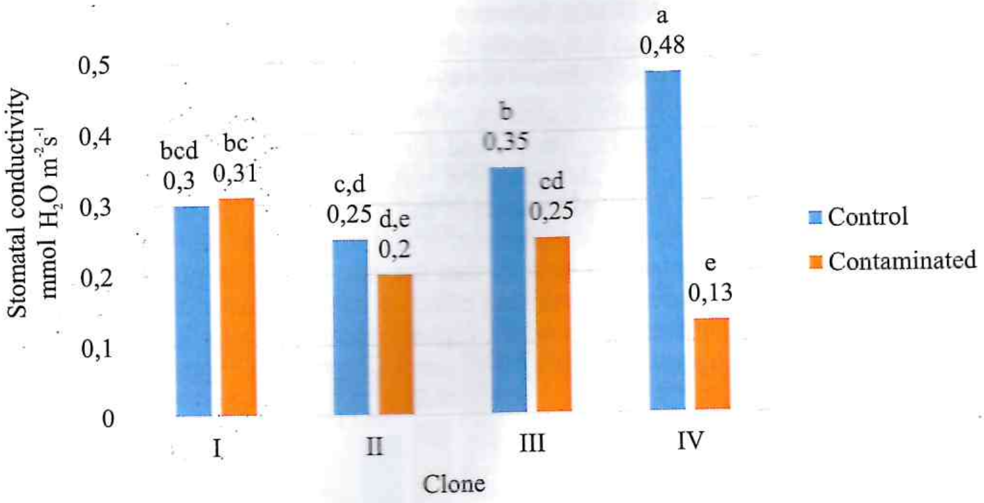

*Fig. 6.* Stomatal conductance for water vapor of willow clones depending on the soil contamination

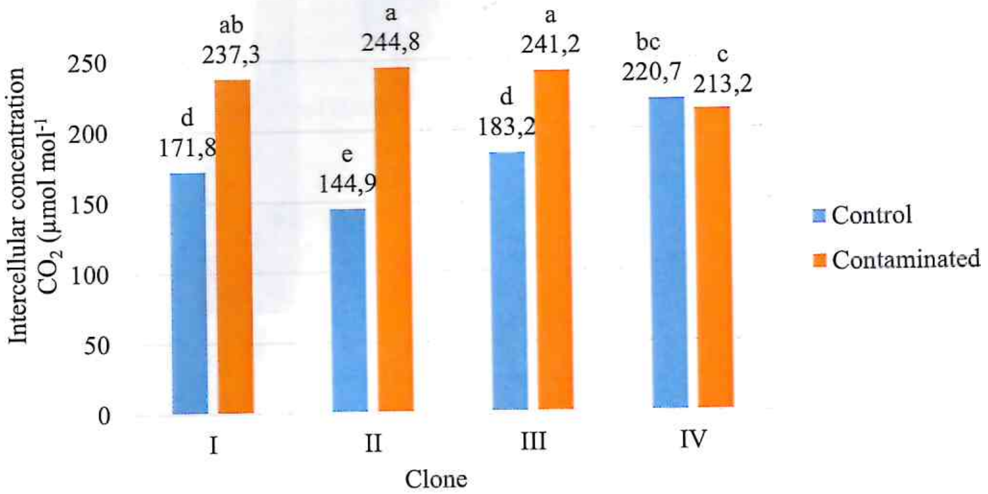

*Fig. 7.* Intercellular concentration of $CO_2$ willow clones depending on soil contamination

*Rate of transpiration (E) (mmol $H_2O$ $m^{-2}$ $s^{-1}$).* The measurement of leaf transpiration and the conductivity of water vapor from the leaf in the atmosphere is a very important indicator for plants in relation to their water regime. Transpiration primarily determines the leaf's energy balance, gas exchange, and determines the efficiency of water use. The exchange of $CO_2$ and water vapor (water) affects the intercellular concentration of $CO_2$ and thus limits other biochemical processes of photosynthesis [36; 37]. The rate of transpiration in different willow genotypes depends on the number, size, arrangement of stomata as well as external and internal factors affecting the degree of stomata opening during the day and night [38].

Comparison of 4 willow genotypes (Figure 8) on control variants clearly shows statistically significant differences between clones. The clone number 3 shows the highest rate of transpiration (2.98 mmol $H_2O$ $m^{-2}$ $s^{-1}$), while the clone number 1 shows the lowest rate (1.93 mmol $H_2O$ $m^{-2}s^{-1}$). Comparison of 4 willow genotypes on contaminated variants clearly shows statistically significant differences between all four genotypes. The clone number 1 shows the highest rate of transpiration (2.31mmol $H_2O$ $m^{-2}$ $s^{-1}$), while the clone number 4 shows the lowest rate (1.68 mmol $H_2O$ $m^{-2}$ $s^{-1}$).

*Water use efficiency.* Weih and Nordh [39] characterized fourteen willow clones (*Salix* sp.) in terms of growth, nitrogen and water use efficiency under different irrigation and fertilization treatments. The results are discussed in relation to the selection of clones for various willow applications such as biomass production and phytoremediation.

Comparison of 4 willow genotypes on the control variants clearly shows that there are no statistically significant differences between the 3 clones (clone 2, 3 and 4) in water use efficiency, and that only clone number 1, which is significantly more efficient compared to the other clones, is statistically different from them (Figure 9). Soil pollution certainly reduces the ability to use water, but not to a great extent, except when it comes to clone number 1. It should be emphasized that there is no statistically significant difference between the tested clones grown on contaminated soil.

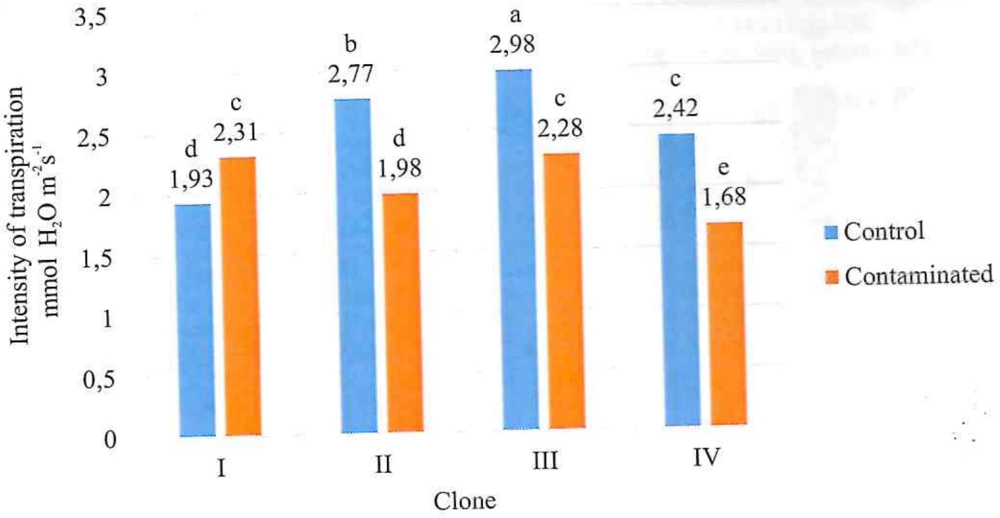

*Fig. 8.* The intensity of transpiration of willow clones depending on soil contamination

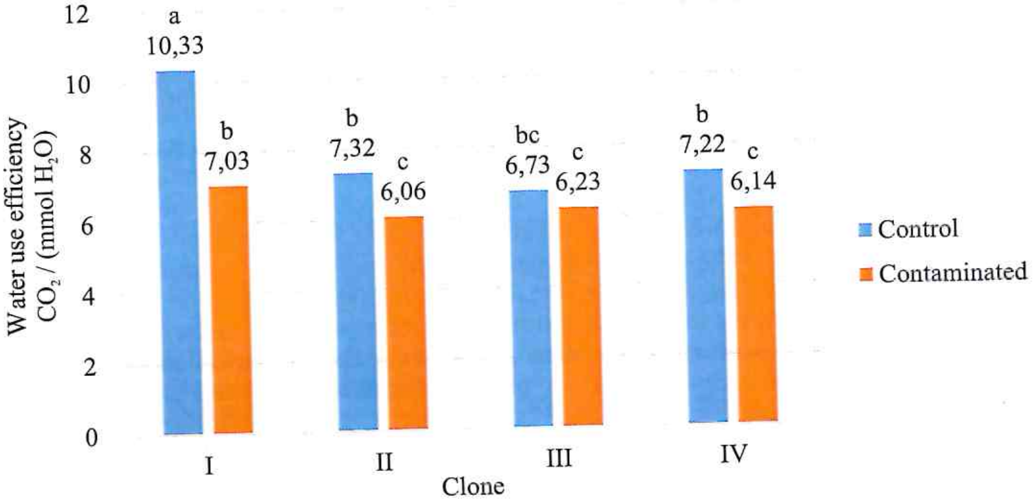

*Fig. 9.* Water use efficiency of willow clones depending on soil contamination

*Intrinsic water use efficiency.* Intrinsic water use efficiency is defined as the ratio between the rate of photosynthesis and the stomatal conductance of water vapor ($H_2O$). The reaction of the stomata to the concentration of $CO_2$ both outside, ie. in the atmosphere, as well as inside the stoma, is extremely important for understanding gas exchange between plants and depends on many factors: plant species, i. e. genotype within the species, leaf area, number of stomata on the face and back of the leaf. During uptake, mesophyll cells consume atmospheric $CO_2$, so the concentration of $CO_2$ n the intercellular spaces is lower compared to air $CO_2$ and iWUE – intrinsic efficiency of water use [40; 41].

Comparison of 4 willow genotypes on the control variants clearly shows that there are statistically significant differences between the clones, with clone number 2 standing out the most (Figure 10). From the obtained results, it can be concluded that the intrinsic water use efficiency depends on the genotype. Contamination in clones 1 and 2 greatly reduces the intrinsic water use efficiency, while in clone number 4 it increases to a great extent. For clone number 3, the results show that the contamination had no significant effect on the intrinsic water use efficiency.

According to the data of Landroth and Cienciala [42] the average long-term efficiency of water use, estimated based on the measurement of the stand level, would amount to 6.3 g of dry biomass per kg of transpired water. This value is high compared to values for other tree species and may be related to the high concentration of nitrogen in the leaves. Water availability is a critical factor in short rotation willow (SRC) forestry despite the relatively high water use efficiency of this species.

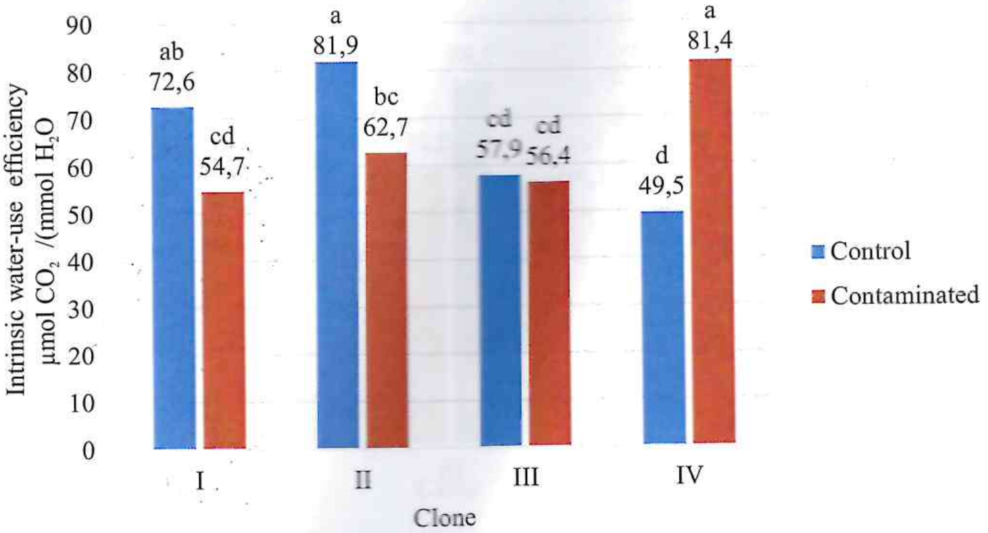

*Fig. 10.* Intrinsic water-use efficiency (iWUE) of willow clones depending on tsoil contamination

## Conclusion

Based on the obtained results, the following conclusions can be drawn:

Significant genotypic differences were observed among willow clones regarding their height, both in plants grown in uncontaminated conditions and those in habitats with heavy metal contamination.

The disparity between willows cultivated in contaminated versus uncontaminated soil is evident in their height and girth growth. Willows grown on uncontaminated soil showed greater height and girth growth compared to willows grown on contaminated soil. The results of leaf area analyses indicated the existence of statistically significant differences between all studied clones that were grown in uncontaminated soil, while statistical analyses of data related to plant material sampled in contaminated soil indicated three homogeneous groups. The first group, with the highest mean value of the leaf area, consists of clone 4, the second group consists of clones 1 and 3, while the clone 2 constituted the third group, with the lowest mean value of the leaf area.

Comparison of the rate of photosynthesis of 4 willow genotypes on the control variants clearly shows that there are no statistically significant differences in the rate of photosynthesis between the 3 clones (clone 1, 2, 3) while clone 4 exhibited distinct differences.

Habitat pollution had a statistically significant effect on all examined clones, which reacted differently to the degree of pollution. The clone number 1 showed the highest rate of photosynthesis (16.21 $\mu$mol $CO_2$ $m^{-2}$ $s^{-1}$) and the clone number 4 showed the lowest rate (only 10.33 $\mu$mol $CO_2$ $m^{-2}$ $s^{-1}$).

Significant differences were also found in other studied parameters, especially in the water use efficiency, which is a significant factor in the cultivation of willow plantations.

As a general conclusion, we can single out clones 3 and 4, as clones with the highest biomass during the experiment. The contaminated habitat substantially reduces willow biomass, nearly halving it and the plants reach the time for cutting in 2–3 years. Nonetheless, the thermal energy derived from biomass showed no significant variance between contaminated and uncontaminated plants, underscoring the disparity in biomass yield.

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