

Article

Oxidative Stress Assessment in Poplar and Willow Clones on Landfills and Their Relation to Physiological Response Parameters

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Abstract

Biochemical markers of oxidative stress were used to evaluate the responses of poplar and willow clones grown in phytoremediation buffer plantations on landfill sites in Novi Sad and Vinča (near Belgrade), Serbia. The experiment was conducted using a randomized block design with three replications, each comprising blocks of nine plants arranged in a 3 × 3 layout, with ten leaves collected per block. Key parameters assessed included total phenolic content (TPC), total flavonoid content (TFC), ferric-reducing antioxidant power (FRAP), radical scavenging activity of DPPH (DPPH), and ABTS radical scavenging activity (ABTS). For the parameters FRAP, DPPH, and ABTS, higher values were recorded at the Novi Sad site, likely due to plants' continuous groundwater access. The results indicate significant clone × site interaction for TPC, suggesting environment-specific clone responses for this parameter. There was a significant effect of clone on the variation in TPC, TFC, and FRAP, evidenced by the clear distinction in these parameters between poplar and willow clones, as poplar clones achieved higher values than willow clones. No significant effect of any of the examined sources of variation was found for the TFC/TPC ratio or FPR, and Pannonia was found to achieve significantly higher values compared to willow and clone 135/81. According to principal component analysis, the examined biochemical parameters were divided into three groups. Considering the results from the previous study of physiological and biomass parameters, DPPH and ABTS were included in the first group along with stomatal conductance (g_s), transpiration (E), and total biomass of the above-ground part (m_p), while TPC, TFC, and FRAP comprised the second group and FPR the third group. The first two principal components accounted for most of the variability, with the first PC clearly separating the interaction treatments of poplar and willow clones, and the second PC further distinguishing the treatments according to site. These findings underscore the difference between parameters regarding the effect of site and differences between clones. Further study of the significance of these parameters in selecting appropriate clones for phytoremediation on landfills, as well as on contaminated lands in general, is recommended.

Keywords: landfill; oxidative stress; selection; willows; poplars



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1. Introduction

Landfills represent one of the most persistent environmental challenges due to their potential to release a wide range of contaminants into surrounding ecosystems. Although they enable more controlled waste management, landfills can still be significant sources of pollutants such as nutrients, volatile organic compounds, toxic organic substances, and heavy metals, which may adversely affect both natural and urban environments [1–6]. In order to mitigate this impact, a variety of remediation approaches have been developed, but these are mainly based on mechanical or physicochemical techniques, which are usually costly. In contrast, phytoremediation, as a technology that introduces plants and associated organisms to reduce the toxic effect of contaminants, is a plausible solution for addressing this issue in an environmentally friendly, efficient, and low-cost way [7–9]. Also, phytoremediation is expected to provide numerous other ecosystem services (like improving soil structure, increasing biodiversity, air quality improvement, enhancing carbon sequestration, landscape restoration, and providing renewable energy), mitigating not only the negative effects of landfills but also the impact of climate change [10,11]. Although phytoremediation has been applied for several decades, it remains a developing technology, as future climate conditions will introduce additional challenges that require continuous refinement and enhanced sustainability [12].

The environment of landfills is usually hostile for plant growth and development due to multiple factors such as soil, water, and air contamination, limited nutrient availability, and altered environmental conditions [1]. In this context, plants used in the phytoremediation of landfills should be adapted to the local conditions, easy to cultivate, and tolerant to dominant toxic contaminants. They should possess extensive root systems capable of extracting, transforming, or at least immobilizing substantial quantities of contaminants, along with a high capacity for rapid growth and biomass accumulation, which can subsequently be safely disposed of or utilized as a raw material or renewable energy source [6,13]. In this sense, fast-growing trees, such as willows and poplars, that demonstrate remarkable adaptability could be considered good candidates for the establishment of phytoremediation plantations on landfill sites [1,5,6,14,15]. In this study, we used *Populus deltoides*, *Salix alba*, and the hybrid *P. deltoides* × *P. euramericana*—species known for their pronounced tolerance to both biotic and abiotic stress factors.

Plant growth and development are strongly influenced by the ability of a species or genotype to tolerate abiotic stressors. Considerable variability in abiotic stress tolerance exists both at the interspecific level [16] and within species [6,15,17]. This natural variation provides a critical foundation for identifying stress-resilient genotypes and understanding the underlying physiological and molecular mechanisms of tolerance. The importance of such traits has increased substantially in modern breeding programs, where various contemporary approaches, including advanced phenotyping, molecular breeding, and genomic and biotechnological tools, are increasingly implemented to enhance plant resilience and improve performance under adverse environmental conditions [2,18].

Plant growth and survival in such environments are closely linked to the ability of a species or genotype to tolerate abiotic stressors. Considerable natural variability in abiotic stress tolerance exists both between and within species [11,16,17,19]. This variability forms the basis for identifying stress-resilient genotypes and elucidating physiological and molecular mechanisms of stress tolerance. Given that most abiotic stressors are accompanied by oxidative stress, evaluating biochemical parameters related to oxidative balance provides valuable insight into plant responses under adverse conditions. Maintaining an effective equilibrium between the production and scavenging of reactive oxygen species (ROS) is essential for plant survival [2,20]. For this reason, indicators such as total phenolic content,

total flavonoid content, and antioxidant capacity determined through FRAP, DPPH, or ABTS assays are widely used to assess oxidative stress tolerance and plant adaptation [21].

Although abiotic stress responses in *Populus* and *Salix* species have been extensively studied under controlled conditions involving drought, heavy metals, or single pollutants, such approaches often rely on simplified stress models that inadequately reflect the complexity of contaminated field environments [14]. In contrast, landfill sites are characterized by a heterogeneous mixture of organic and inorganic contaminants that imposes a multicomponent stress regime on plants [22]. The originality of the present study lies in the use of landfill as an integrated and environmentally realistic stress factor, coupled with a genotype-oriented framework that simultaneously evaluates a suite of oxidative stress-related biochemical markers across numerous *Populus* and *Salix* clones. This clone-level analytical approach goes beyond the species-level generalizations dominant in the literature and enables the identification of clonal differences in oxidative stress regulation and adaptive capacity. By integrating antioxidant activities, the study provides a comprehensive assessment of plant physiological performance under realistic contamination scenarios. Embedded within the applied context of phytoremediation at landfill sites, these findings move beyond confirmation of known stress responses and deliver scientifically and operationally relevant insights that directly support the selection of suitable genotypes for effective phytoremediation strategies [23,24].

Numerous studies have highlighted the importance of the antioxidant system in *Populus* species, particularly in the context of responses to abiotic stress. Research on *Populus* species has shown that the regulation of plant oxidoreduction status is associated with changes in the activity of key enzymes, such as superoxide dismutase (SOD), catalase (CAT), and enzymes of the ascorbate–glutathione system, as well as with variations in photosynthetic pigment content and osmoprotectants [25,26]. Investigations involving *Populus nigra* and *Populus × canadensis* have further confirmed the considerable contribution of non-enzymatic antioxidant defenses, especially under the severe abiotic agents that could be monitored by total phenolic and flavonoid contents, as well as in vitro antioxidant capacities assessed by DPPH, FRAP, and ABTS assays [27,28]. In addition, antioxidant activity in these studies was frequently linked to physiological parameters such as relative water content, electrolyte leakage, and lipid peroxidation, indicating the complex role of the antioxidant system in maintaining cellular integrity and facilitating the adaptation of poplar species to adverse environmental conditions.

To characterize oxidative stress, we focused on non-enzymatic markers, including total phenolic content (TPC), total flavonoid content (TFC), and antioxidant capacity assays (FRAP, DPPH, ABTS, and FPR). These markers provide an integrative assessment of the overall oxidative status of plants, reflecting cumulative physiological responses under complex, multicomponent stress conditions such as exposure to landfill leachate [29–31]. Although enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), and enzymes of the ascorbate–glutathione system are widely recognized as central components of the plant redox system, this study is based on non-enzymatic markers, which are widely used, particularly in plant stress physiology, and as such were expected to provide reliable insights into the comparative oxidative stress tolerance of different clones, serving as a reliable basis for the selection of genotypes for phytoremediation applications.

The selected biochemical markers were chosen due to their central role in monitoring plant responses to abiotic stress typical of landfill environments, particularly heavy metal toxicity and water deficit [30,32,33]. Total phenolic and flavonoid contents, together with antioxidant capacity assays (FRAP, DPPH, and ABTS), provide solid insight into a plant's ability to mitigate oxidative damage caused by numerous stressors through reactive oxygen species scavenging. These parameters allow an integrated assessment of

genotypic differences and genotype–environment interactions, which is essential for identifying clones with long-term tolerance and stable growth under complex conditions of contaminated sites. Therefore, the selected markers are considered appropriate indicators of phytoremediation performance.

The present study addresses the limited understanding of how oxidative stress-related biochemical traits can be integrated into the selection of tree clones for landfill phytoremediation under contrasting field conditions. Specifically, we aimed to evaluate the responses of selected poplar and willow clones grown at two landfill sites near Novi Sad and Belgrade, which differ in water availability and soil contamination profiles. We hypothesized that (i) clones would exhibit site-specific biochemical responses reflecting differences in environmental stress intensity, and (ii) clones with higher antioxidant capacity and phenolic content would demonstrate greater tolerance to landfill conditions, making these traits suitable early selection markers. By combining oxidative stress assessment with multivariate analyses of clone performance, this study provides a novel framework for linking biochemical defense strategies with practical phytoremediation selection and offers preliminary guidance for identifying tree species and clones with enhanced adaptability and long-term potential for landfill restoration.

2. Materials and Methods

2.1. Experimental Design and Site Properties

Plantations were established using one-year-old rootless whips planted to a depth of approximately 80 cm, with 1.5 m × 1.5 m spacing and a planting density of 4444 plants per hectare. In the studied landfills, planting was restricted to the upper recultivation layer and did not interfere with engineered protective barriers such as clay liners or geomembranes, which are located at greater depths. The planting depth of 80 cm refers exclusively to the soil cover layer and was selected in accordance with existing recultivation practices at these sites. The two landfill sites differed markedly in soil texture and water availability: the Belgrade (BG) site, characterized by sandy clay loam, lacked direct access to groundwater, whereas the Novi Sad (NS) site, with loamy sand soil, provided continuous groundwater access. The experimental layout followed a block design with three replications. In each replication, the same set of clones was included and arranged into blocks. Each block consisted of nine individual plants, positioned in a 3 × 3 arrangement, ensuring uniform spacing and comparable growth conditions among plants within the block. Both sites contained elevated levels of heavy metals such as As, Ba, Cd, Cr, Cu, Fe, Ni, and Zn [11]. Substrate and shoot samples were first dried at 105 °C, then ground and digested in aqua regia using a microwave system (Milestone D series, Bergamo, Italy). The resulting extracts were filtered, brought to a final volume of 50 mL, and analyzed for heavy metal content using ICP-OES (VISTA-PRO, Varian Australia Pty. Ltd., Melbourne, VIC, Australia) under standard operating conditions [34]. The measured concentrations were then compared to established soil thresholds, including maximum limit (MLV) and remediation (RV) values [35].

The study included two white willow clones (*Salix alba* L. cl. 380, cl. 107/65/9, and cl. 378) and five poplar clones (*Populus deltoides* Bartr. cl. 135/81 and cl. S1-8; and *Populus* × *euramericana* (Dode) Guinier cl. I-214 and cl. Pannonia). The physiological and growth parameters of these plantations—stomatal conductance (g_s), net photosynthesis rate (A), transpiration rate (E), water-use efficiency (WUE), and dry shoot biomass (m_p)—were analyzed in Kesić et al. [11]. In the present study, these previously established physiological and biomass traits are integrated with newly measured oxidative stress indicators to examine their interrelationships and to better understand clone-specific responses to environmental and contaminant pressures.

2.2. Measurement of Biochemical Parameters

Leaf samples for biochemical analyses were collected in July 2024. Immediately after sampling, the material was frozen in liquid nitrogen, freeze-dried for 24 h at $-70\text{ }^{\circ}\text{C}$ using a lyophilizer (Alpha 1-2 LDplus, Martin Christ, Osterode am Harz, Germany), and subsequently stored at $-70\text{ }^{\circ}\text{C}$ until biochemical analyses were performed. The experiment was conducted in three replicates. Clones were arranged according to a block design, with each block consisting of nine plants and being included in all replicates. Leaf sampling was carried out in each replicated block by randomly collecting ten leaves per block for subsequent analyses; biochemical leaf sampling was performed concurrently with physiological measurements, between 9:00 and 11:00 AM, in order to minimize the influence of diurnal variation. In order to measure biochemical parameters of oxidative stress, ethanol extracts of leaf samples were obtained by extracting 0.05 g of dry mass in 2 mL of 96% ethanol.

Total phenolic content (TPC) was determined spectrophotometrically with Folin–Ciocalteu reagent according to the methodology by Kim et al. [36]. Total flavonoid content (TFC) was determined spectrophotometrically using AlCl_3 reagent according to the methodology of Chang et al. [37]. For each sample, 30 μL of ethanol extract was mixed with 90 μL of methanol, 6 μL of NaCH_3COO , 6 μL of AlCl_3 , and 150 μL of distilled water in a microplate, and the mixture was incubated at room temperature for 10 min. Absorbance was then measured at 415 nm, and the results were expressed as mg of quercetin equivalents per g of dry weight ($\text{mg QE g}^{-1}\text{ DW}$).

Ferric-reducing antioxidant power (FRAP) was determined spectrophotometrically according to the method by Benzie and Strain [38]. Briefly, 225 μL of FRAP reagent, obtained by mixing acetate buffer, TPTZ solution, and $\text{FeCl}_3\cdot\text{H}_2\text{O}$ in a ratio of 10:1:1, was added to 20 μL of ethanol extract to measure absorbance at 593 nm. The obtained results are expressed in $\text{mg AAE g}^{-1}\text{ DW}$.

The ability to neutralize ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) radicals was determined spectrophotometrically by the method of Miller and Rice-Evans [39], according to the absorbance at 734, while the ability to neutralize DPPH (2,2-diphenyl-2-picrylhydrazyl) radicals was also determined spectrophotometrically using the method of Perez-Jimenez [40] by measuring the absorbance at 515 nm. The obtained results are expressed in percentages of RSC (radical scavenger capacity), which represents the percentage of radical species that were neutralized by antioxidants present in the leaf extract.

The scavenging activity was calculated using the following formula:

$$\text{Scavenging (\%)} = (A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}} \times 100,$$

where A_{sample} is the absorbance of the test sample and A_{control} is the absorbance of the control.

2.3. Measurement of Heavy Metal Content

For heavy metal determination, leaves were collected and prepared for chemical analysis concurrently with the measurements of oxidative stress markers and physiological parameters. Plant material was dried at constant temperature, finely milled, and subjected to wet digestion using aqua regia composed of concentrated hydrochloric and nitric acids (3:1, *v/v*). After digestion, the samples were filtered and diluted to a final volume of 50 mL with deionized water. Metal concentrations were determined by inductively coupled plasma–optical emission spectrometry (ICP-OES; VISTA-PRO, Varian Australia Pty. Ltd., Melbourne, VIC, Australia). The instrument was operated at a radiofrequency power of

1100 W, with plasma and auxiliary gas flow rates set to 15.0 and 1.50 L min^{−1}, respectively, to ensure stable plasma conditions. All measurements were performed in triplicate [34].

Also, the bioconcentration factor was used as a parameter that describes the ability of a plant to achieve phytoextraction. The bioavailability factor was calculated by the following formula, according to [41]:

$$\text{BCF} = C_{\text{leaf}}/C_{\text{soil}}, \quad (1)$$

where BCF stands for the bioconcentration factor, C_{leaf} for the content of heavy metal in the shoot [mg/kg DW], and C_{soil} for the content of heavy metal in the substrate [mg/kg DW].

2.4. Statistical Analysis

Data was analyzed using two-way factorial analysis of variance for randomized block design. Based on these results, Duncan's post hoc test was applied for multiple comparisons of treatments at the level $\alpha = 0.05$. The relationship between treatments of clone $\times$ site interaction was also examined at the base of the first two principal components. Also, principal component analysis (PCA) was used to examine relationships between parameters based on average values at the level of interaction of clone $\times$ site and the correlation matrix. In the process, the data for physiological and biomass parameters from the study of Kesić et al. [11] were used as well. The parameters were grouped according to their loadings with the first four principal components, rotated by the Varimax method. All statistical analyses were performed using the STATISTICA 14.0.0 software package (TIBCO Software Inc., 2020, Palo Alto, CA, USA).

3. Results

3.1. Analysis of Variance (ANOVA)

According to the two-way ANOVA, the factor clone had a significant effect on most biochemical parameters (TPC, TFC, and FRAP), as well as on several leaf heavy metal contents (Ba, Cd, and Mn) (Table 1). The landfill factor significantly influenced FRAP, DPPH, and ABTS antioxidant activities and also affected the accumulation of Cu, Mn, and Zn in leaves. Significant clone $\times$ landfill interaction was detected only for TPC.

The basis was considerable differences between poplar and willow clones, where willow clones had lower values for all three parameters than poplar ones. According to the Duncan test, the highest TPC and TFC values were recorded in I-214, and the highest FRAP in S1-8. Also, DPPH in 378 was significantly higher than in most of the examined clones, and ABTS in clone 378 was higher than in Pannonia, while Pannonia had a higher FPR than most examined clones (Appendix A, Table A1).

The effect of landfill was significant only in FRAP, DPPH, and ABTS. The values of all of these parameters were significantly higher at the BG site than at the NS site (Table A1).

3.2. Results of Oxidative Stress Parameters

TPC values differed significantly among clones and between landfill sites (Figure 1a). The lowest TPC levels were recorded in clones 378, 380, and 107/65/9 at both locations. In contrast, clones 135/81, I-214, and S1-8 exhibited the highest TPC values, with no significant differences between BG and NS. A significant site-related difference was observed only for clone Pannonia, where BG showed higher TPC compared to NS.

Table 1. Results of the F-test in the two-way analysis of variance block design for biochemical and leaf heavy metal content parameters ⁽¹⁾.

Parameter	Clone (A)		Landfill (B)		Interaction A × B		Block	
	F ⁽²⁾	p	F	p	F	p	F	p
Biochemical parameters ⁽⁴⁾								
TPC	38.48	0.0000 ** ⁽³⁾	1.46	0.2372	3.72	0.0083 **	1.33	0.2810
TFC	18.36	0.0000 **	0.87	0.3604	1.12	0.3791	2.44	0.1072
FRAP	26.54	0.0000 **	24.25	0.0000 **	2.35	0.0603	4.57	0.0199 **
DPPH	2.04	0.0961	5.92	0.0222 *	0.79	0.5878	2.31	0.1196
ABTS	2.00	0.1940	13.00	0.0012 **	2.00	0.1496	2.00	0.1274
FPR	1.64	0.1772	2.05	0.1642	0.53	0.7812	0.63	0.5411
Leaf heavy metal content								
As	1.85	0.1280	0.84	0.3669	1.29	0.2960	0.08	0.9240
Ba	11.37	0.0000 **	0.19	0.6679	0.67	0.6730	2.17	0.1342
Cd	4.60	0.0026 **	0.48	0.4947	0.30	0.9291	2.52	0.1003
Cr	1.31	0.2891	1.42	0.2447	0.87	0.5272	1.10	0.3480
Cu	1.75	0.1503	13.53	0.0011 **	1.36	0.2671	3.25	0.0549
Fe	4.58	0.0027	11.95	0.0019	1.02	0.4332	3.24	0.0556
Mn	3.36	0.0137 *	21.22	0.0001 **	2.37	0.0584	0.55	0.5838
Ni	1.19	0.3408	0.56	0.4593	1.23	0.3227	0.82	0.4527
Zn	1.43	0.2412	14.76	0.0007 **	1.09	0.3934	0.11	0.8965

⁽¹⁾ Degree of freedom: for clone, $df_A = 6$; for landfill, $df_B = 1$; for interaction of clone × landfill, $df_{A \times B} = 6$; for block, $df_{Block} = 2$; for residual, $df_{Residual} = 26$. ⁽²⁾ Labels for parameters of ANOVA: F—F value, p — p value (probability for F test). ⁽³⁾ Labels for the F-test: *— $p < 0.05$, **— $p < 0.01$. ⁽⁴⁾ TPC—total content of phenolics, TFC—total content of flavonoids, FRAP—ferric-reducing antioxidant power, DPPH—DPPH activity [% of RSC], ABTS—ABTS assay [% of RSC], FPR—TFC/TPC ratio

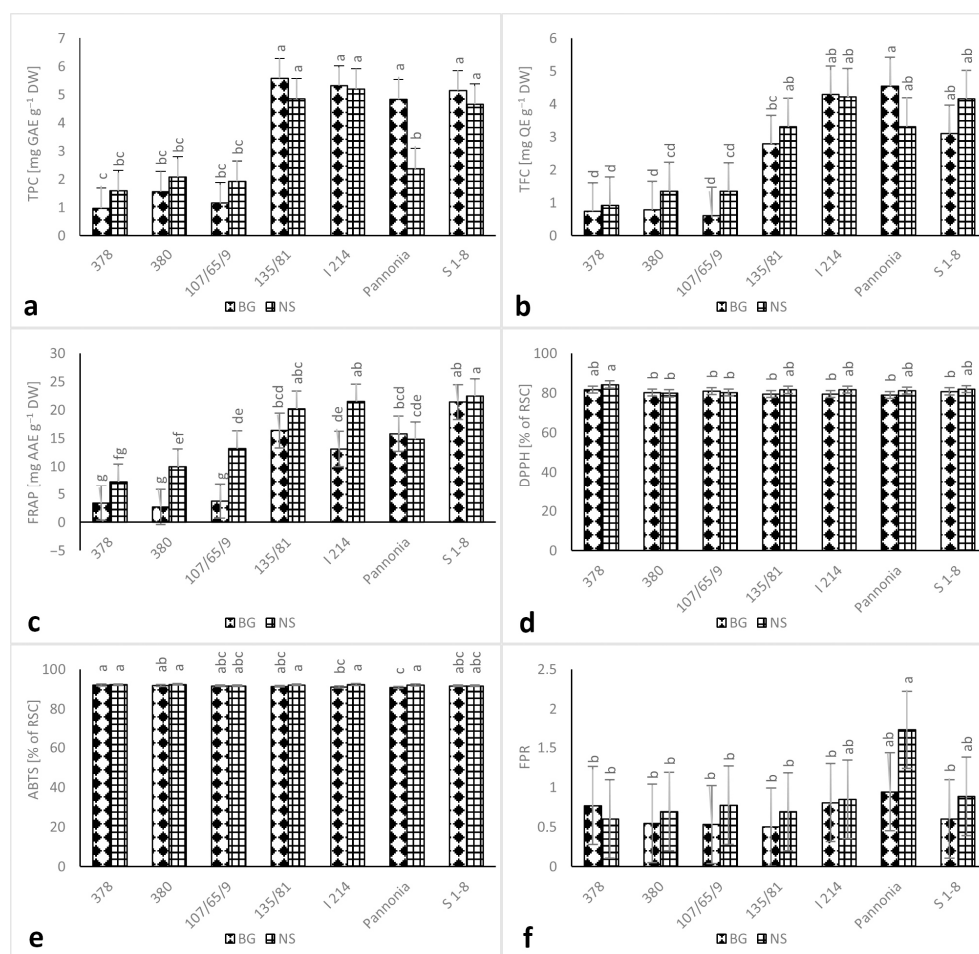


Figure 1. Relationship between clone × landfill interaction treatments according to Duncan test: (a) TPC—total content of phenolics [mg GAE g^{−1} DW]; (b) TFC—total content of flavonoids [mg QE g^{−1} DW]; (c) FRAP—ferric-reducing antioxidant power [mg AAE g^{−1} DW]; (d) DPPH—DPPH activity [% of RSC]; (e) ABTS—ABTS assay [% of RSC]; (f) FPR—TFC/TPC ratio. Clones 107/65-9,

378, and 380 are willow, and the rest are poplar clones. Landfills: BG stands for landfill near Belgrade, and NS for landfill near Novi Sad; values with the same letter are not significantly different according to the Duncan test for $p < 0.05$.

Significant differences in TFC were observed among clones and landfill sites (Figure 1b). The lowest flavonoid content was recorded in clones 378, 380, and 107/65/9 at both locations. Higher TFC values were detected in clones 135/81, I-214, Pannonia, and S1-8. Clone I-214 showed similarly high TFC at both sites, whereas Pannonia and S1-8 exhibited higher values at the BG landfill compared to NS.

The lowest FRAP activity was observed in clones 378, 380, and 107/65/9 at both locations (Figure 1c). Substantially higher FRAP values were recorded in clones 135/81, I214, Pannonia, and S1-8. For most clones, FRAP activity was higher at the NS landfill compared to BG, with the most pronounced differences observed in clones 135/81 and I-214.

Relatively small but not significant differences in DPPH scavenging activity were detected among the examined clones and landfill sites (Figure 1d). Clone 378 exhibited the highest DPPH values at both locations, while slightly lower activities were recorded in clones 380 and 107/65/9. For most clones, DPPH activity was marginally higher at the NS landfill compared to BG.

High ABTS scavenging capacity was recorded across all clones and both landfill sites (Figure 1e). The highest activities were observed in clone 378 at both locations. Slightly lower ABTS values were detected in clones 107/65/9 and Pannonia, particularly at the BG landfill.

The FPR values showed low variability among clones and landfill sites (Figure 1f). Similar FPR were observed across all genotypes, with only slight differences between BG and NS, which were not statistically significant.

3.3. Heavy Metal Content

Leaf heavy metal concentrations varied among clones and between landfill sites, with element-specific response patterns (Figure 2). Arsenic (As) showed generally low concentrations across all genotypes, with minor site-related differences and no pronounced clone-specific trends (Figure 2a). In contrast, barium (Ba) content was highest in clone 378 at both sites, while lower values were recorded in clones I214, Pannonia, and S1-8 (Figure 2b). Cadmium (Cd) accumulation differed among clones, with the highest levels detected in clones 378 and 380, whereas clone S1-8 exhibited consistently lower concentrations at both landfills (Figure 2b).

Copper (Cu) concentrations were generally higher at the BG landfill for most clones, with Pannonia and 380 showing the highest values (Figure 2d). A similar site-dependent pattern was observed for iron (Fe), with elevated concentrations at NS across most genotypes, particularly in clones 378 and 107/65/9 (Figure 2e). Manganese (Mn) displayed pronounced clone-specific variability, with the highest accumulation detected in clone S1-8 at BG, while lower values were observed in clones 378 and 107/65/9 (Figure 2f).

Nickel (Ni) content showed moderate variation among clones, with higher values recorded in clones 380, I214, 107/65/9, and Pannonia (Figure 2g), whereas zinc (Zn) exhibited a clear site effect, with consistently higher concentrations at NS compared to BG for all examined genotypes (Figure 2h).

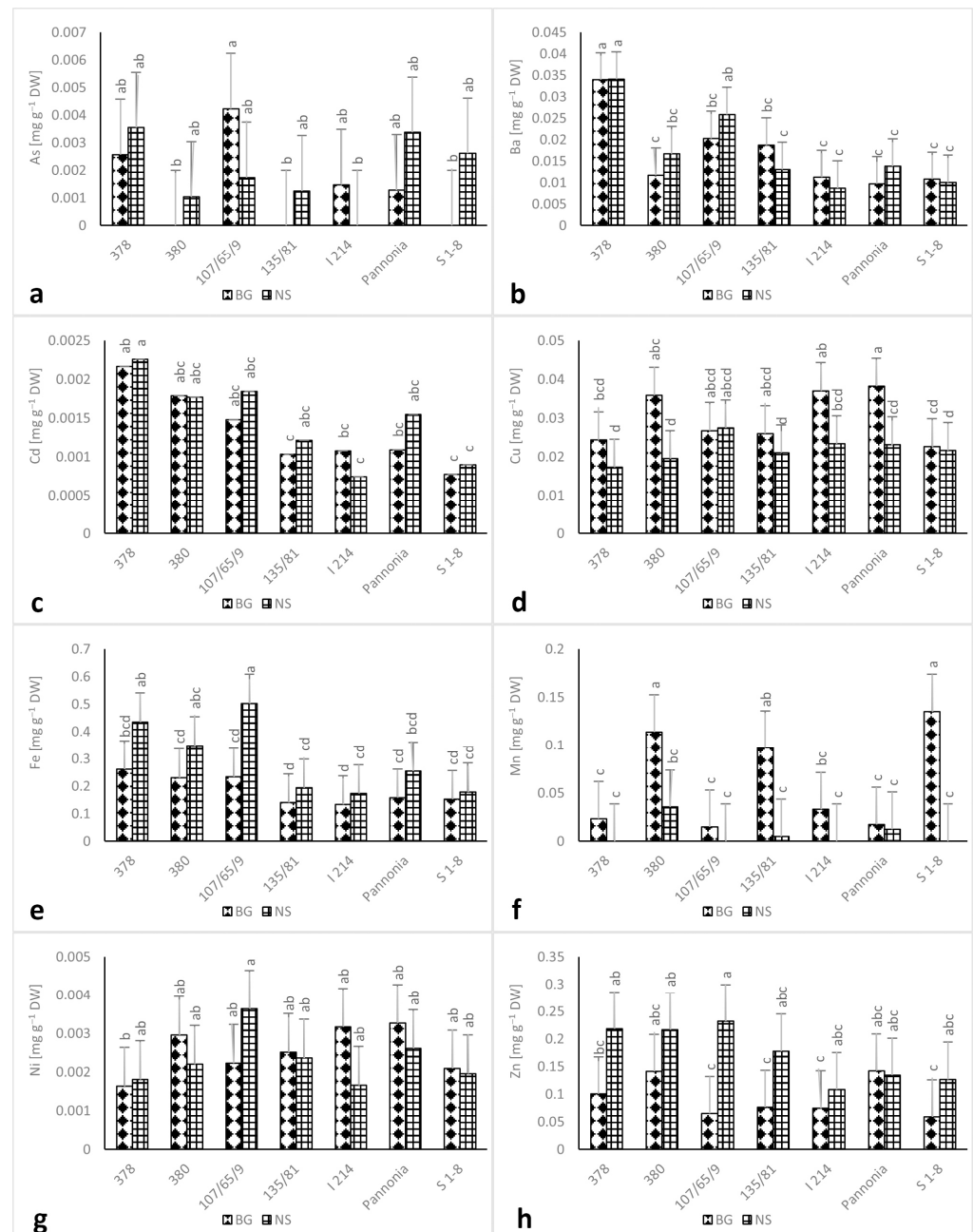


Figure 2. Relationship between clone $\times$ landfill interaction treatments according to Duncan test: (a) As—arsenic [$\text{mg g}^{-1} \text{DW}$]; (b) Ba—barium [$\text{mg g}^{-1} \text{DW}$]; (c) Cd—cadmium [$\text{mg g}^{-1} \text{DW}$]; (d) Cu—copper [$\text{mg g}^{-1} \text{DW}$]; (e) Fe—iron [$\text{mg g}^{-1} \text{DW}$]; (f) Mn—manganese [$\text{mg g}^{-1} \text{DW}$]; (g) Ni—nickel [$\text{mg g}^{-1} \text{DW}$]; (h) Zn—zinc [$\text{mg g}^{-1} \text{DW}$]. Landfills: BG stands for landfill near Belgrade, and NS for landfill near Novi Sad; values with the same letter are not significantly different according to the Duncan test for $p < 0.05$.

According to the measured bioconcentration factor (BCF) for clones at the BG and NS sites, for most of the examined heavy metals, the BCF was higher at the NS than at the BG site, except for Mn, for which it was the opposite, and Cu, for which the BF at the examined sites was similar. In total, some or all willow clones dominated with higher BF for all examined heavy metals except the highest BF for Cu, which was found in the Euramerican poplar clones I-214 and Pannonia. The similarity was at the BG site, where the soil content of examined heavy metals was higher than at the NS site, except willow clone 380 which also had a high BCF for Cu together with I-214 and Pannonia, while Pannonia also had a high BCF for Ni and Zn together with willow clone 380 (Table 2).

Table 2. Bioconcentration factor for leaf in examined clones at landfills near Belgrade and Novi Sad.

	As	Ba	Cd	Cu	Fe	Mn	Ni	Zn
BG site								
107/65-9	0.239	0.109	0.361	0.410	0.004	0.016	0.016	0.475
135/81	0.000	0.101	0.252	0.397	0.002	0.103	0.018	0.558
378	0.145	0.182	0.529	0.373	0.004	0.025	0.012	0.736
380	0.000	0.063	0.436	0.549	0.004	0.121	0.022	1.035
I-214	0.084	0.060	0.262	0.567	0.002	0.035	0.023	0.550
Pannonia	0.073	0.052	0.266	0.585	0.002	0.019	0.024	1.044
S1-8	0.000	0.058	0.188	0.347	0.002	0.144	0.015	0.431
NS site								
107/65-9	0.271	0.295	0.921	0.565	0.021	0.000	0.098	2.995
135/81	0.196	0.149	0.606	0.433	0.008	0.016	0.063	2.296
378	0.554	0.388	1.128	0.357	0.018	0.000	0.049	2.821
380	0.161	0.191	0.885	0.403	0.014	0.111	0.059	2.797
I-214	0.000	0.099	0.371	0.482	0.007	0.000	0.045	1.394
Pannonia	0.528	0.157	0.773	0.476	0.011	0.039	0.070	1.733
S1-8	0.408	0.115	0.449	0.445	0.007	0.000	0.053	1.637

3.4. Principal Component Analysis

Considering only biochemical parameters and heavy metal contents, the first four unrotated principal components had eigenvalues higher than 1 and described 83.0% of the total variance. In this way, they form the base to divide these parameters into four groups regarding the principal component with which they have their highest factor loading, i.e., correlation. The parameters of the first group, those that are closely correlated with the first principal component, are: TPC, TFC, FRAP, Ba, Cd, Fe, and Zn, and the second group consisted of DPPH, ABTS, and Cu. These two principal components together describe more than 57.3% of the total variance (Table 3). The third unrotated principal component is correlated with FPR, As, and Mn, and the fourth to Ni.

Table 3. Principal component analysis table with loadings for different traits and metals across four unrotated principal components.

Original Traits ⁽¹⁾	PC1	PC2	PC3	PC4
TPC	<u>0.907</u> ⁽²⁾	0.184	−0.083	−0.106
TFC	<u>0.865</u>	0.239	−0.422	0.008
FRAP	<u>0.690</u>	0.576	−0.206	−0.049
DPPH	−0.286	<u>0.665</u>	0.389	0.325
ABTS	−0.493	<u>0.549</u>	0.201	0.436
FPR	0.121	0.231	<u>−0.656</u>	0.246
As	−0.517	0.002	<u>−0.553</u>	−0.491
Ba	<u>−0.792</u>	−0.185	−0.029	−0.441
Cd	<u>−0.932</u>	−0.228	−0.060	−0.015
Cu	0.386	<u>−0.797</u>	−0.137	0.318
Fe	<u>−0.864</u>	0.054	−0.219	0.207
Mn	0.307	−0.331	<u>0.730</u>	0.161
Ni	0.131	−0.608	−0.372	<u>0.625</u>
Zn	<u>−0.612</u>	0.262	−0.316	0.475
Explained variation	5.530	2.493	1.997	1.593
Proportion of the total variation	0.395	0.178	0.143	0.114

⁽¹⁾ TPC—total content of phenolics; TFC—total content of flavonoids; FRAP—ferric-reducing antioxidant power; DPPH—DPPH activity; ABTS—ABTS assay; FPR—TFC/TPC ratio. ⁽²⁾ The underlined values represent the highest loadings of examined traits for four selected and rotated principal components.

Principal component analysis (PCA) provided the basis to relate the studied clones regarding their scores for the first two components, which explained 59.53% of the total variability (PC1 = 33.48%, PC2 = 25.05%) (Figure 3). Clones at the Belgrade landfill (BG) were generally positioned on the positive side of PC1, while clones from the Novi Sad site were on the negative side of the same principal component, indicating a strong effect of the site on parameters of the first PC group. PC2 further separated the performance of clones between the same sites, wherein every clone PC2 value was higher at the BG site than at the NS site, except for Pannonia, which at both sites remained near the origin, indicating its moderate and stable behavior (Figure 3).

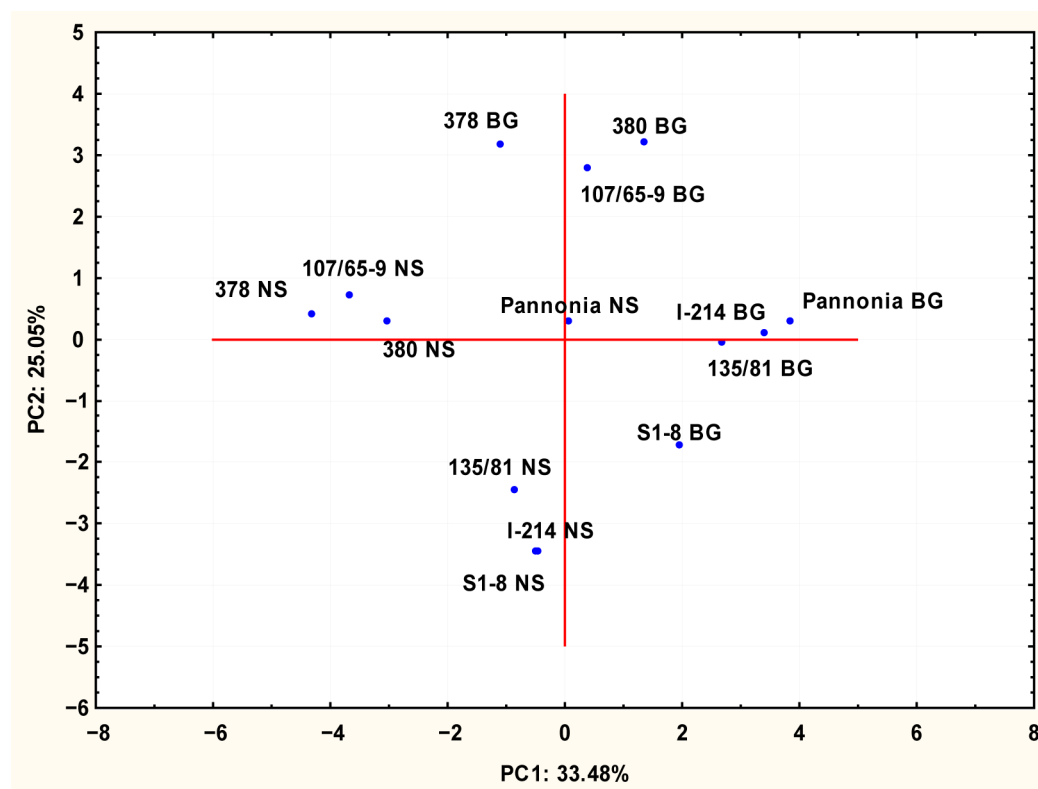


Figure 3. Relationship between clone × landfill interaction treatments according to factor scores of the first two principal components (BG stands for Belgrade landfill substrate and NS for Novi Sad landfill substrate). Clones 107/65-9, 378, and 380 are willow, and the rest are poplar clones.

To enable an integrated assessment of plant responses, the biochemical parameters analyzed in this study were related to the physiological and biomass traits documented by Kesić et al. [11]. These physiological datasets, obtained from the same experimental plots, provided a coherent framework for examining the interplay between oxidative stress markers, plant water relations, photosynthetic performance, and growth dynamics. According to the results of principal component analysis, which included the physiological and biomass parameters together with the parameters measured in this study, the first four principal components met Keiser's rule, having eigenvalues higher than one. These principal components described 89.7% of the total variation between treatments. After their rotation by the Varimax method, original parameters that had their highest loadings with the same principal component were grouped in the same group. The examined parameters were grouped in three groups: the first with Cu and Ni, the second with TPC, TFC, Ba, Cd, and Fe, and the third with FPR, As, and Nb. Only in the fourth group were all examined physiological and biomass parameters in the same group with Zn. The DPPH and ABTS were clustered in a separate, fifth group (Table 4).

Table 4. Factor loadings of original parameters, including physiological parameters provided by Kesić et al. [11], with the first five principal components rotated by the Varimax method.

Trait ⁽¹⁾	RC1	RC2	RC3	RC4	RC5
g _s	0.368	0.194	0.272	<u>0.725</u>	0.364
A	0.129	−0.041	−0.043	<u>0.953</u>	0.039
E	0.356	0.101	0.312	<u>0.742</u>	0.360
WUE	−0.016	−0.131	−0.422	<u>0.740</u>	−0.117
m _p	0.382	0.369	0.324	<u>0.605</u>	0.200
TPC	0.033	<u>0.934</u>	−0.117	0.042	−0.269
TFC	−0.143	<u>0.932</u>	0.224	−0.011	−0.179
FRAP	0.172	<u>0.896</u>	0.090	0.313	−0.025
DPPH	0.299	−0.069	−0.020	0.062	<u>0.901</u>
ABTS	0.067	−0.265	−0.037	0.417	<u>0.733</u>
FPR	−0.344	0.213	<u>0.604</u>	−0.098	0.188
As	0.275	−0.446	<u>0.769</u>	−0.088	−0.211
Ba	0.309	<u>−0.789</u>	0.074	0.305	−0.363
Cd	−0.017	<u>−0.935</u>	0.161	0.162	0.009
Cu	<u>−0.752</u>	0.079	−0.157	−0.433	−0.326
Fe	−0.107	<u>−0.695</u>	0.207	0.646	0.104
Mn	−0.090	0.067	<u>−0.800</u>	−0.357	0.053
Ni	<u>−0.921</u>	−0.003	0.061	−0.016	−0.134
Zn	−0.239	−0.352	0.326	<u>0.654</u>	0.339
Explained variation	2.349	5.179	2.335	4.472	2.236
Proportion of the total variation	0.124	0.273	0.123	0.235	0.118

⁽¹⁾ g_s—stomatal conductance; A—net photosynthesis rate; E—transpiration rate; WUE—water use efficiency; m_p—dry shoot biomass; TPC—total content of phenolics; TFC—total content of flavonoids; FRAP—ferric-reducing antioxidant power; DPPH—DPPH activity; ABTS—ABTS assay; FPR—TFC/TPC ratio. Data for physiological and biomass parameters are used from the study presented by Kesić et al. [11]. The underlined values represent the highest loadings of examined traits for four selected and rotated principal components.

4. Discussion

4.1. Sensitivity of Oxidative Stress Markers to Landfill Effects and Clonal Variation

In this study, biochemical markers of oxidative stress were used to assess the responses of poplar and willow clones grown on landfill sites near Novi Sad and Belgrade. Parameters such as total phenolic content (TPC), total flavonoid content (TFC), ferric reducing antioxidant power (FRAP), and radical scavenging activities (DPPH and ABTS) are widely applied in studies addressing the impact of abiotic stress factors, as these influences are closely linked to oxidative stress in plants. In addition, the accumulation of heavy metals in leaves, including As, Ba, Cd, Cu, Fe, Mn, Ni, and Zn, was analyzed to evaluate clone-specific differences in metal uptake and tolerance and to relate biochemical defense responses to the level of metal exposure under landfill conditions. These indicators provide valuable insight into how plants cope with and adapt to stressful environmental conditions. In addition to differences between species, there are considerable differences between genotypes within species in their tolerance to various abiotic and biotic agencies, which is the basis for plant breeding and selection [42]. In that sense, parameters of oxidative stress can be used in the breeding process used to evaluate the reaction of genotypes to various abiotic stresses [43]. In the case of landfills, there are multiple sources of numerous abiotic stresses, and parameters evaluating oxidative stress give the opportunity to assess the response of plants to that complex influence [44]. The reaction of examined clones by different parameters of oxidative stress in this research suggested that there was a clear distinction between DPPH and ABTS, which showed considerable differences between the examined landfills,

and TPC and TFC for which there were no significant differences between the landfills. This pattern aligns with previous research reporting that DPPH and ABTS radicals are highly responsive to changes in external abiotic factors such as pollution, drought, and temperature stress and consequently often reflect the overall stress load affecting plant material [30,36]. Similar findings have been documented for various poplar and willow genotypes, where environmental conditions more strongly influenced DPPH and ABTS values than genetic background [2,45]. In contrast, the differences between clones were considerable by TPC and TFC but poor by DPPH and ABTS. These results suggest the use of DPPH and ABTS in the evaluation of the effects of landfills and TPC and TFC in the assessment of differences between clones. These results are consistent with earlier studies showing that the biosynthesis of phenolic compounds is largely genetically controlled, leading to pronounced genotype-dependent variation even when individuals grow under comparable environmental conditions [46].

The FRAP parameter emerged as the most informative, distinguishing both landfills and clones. Since FRAP measures total reducing power, reflecting the combined activity of diverse antioxidant metabolites, this assay is frequently described in the literature as highly robust and reliable for assessing complex biological matrices [38]. Its ability to differentiate both genetic and environmental effects has been confirmed in studies examining trees growing on degraded or polluted soils, where FRAP consistently tracks changes in oxidative status induced by stress [47]. On the other hand, FPR proved poorly informative for distinguishing either landfills or clones. However, several findings have been reported by other authors, suggesting differences between genotypes of white poplar and wild cherry under in vitro conditions [2,21,48].

Clone-dependent differences in leaf accumulation of Ba, Cd, and Mn further emphasize the existence of distinct metal uptake and translocation strategies among genotypes. Such variability has been widely reported in forest tree species and reflects differences in root uptake, sequestration, and detoxification mechanisms [49]. From a phytoremediation perspective, clones that combine efficient antioxidant defense with controlled metal accumulation are particularly valuable, as they are able to limit oxidative damage while contributing either to phytoextraction or phytostabilization, depending on management goals.

4.2. Clone $\times$ Landfill Interactions in Antioxidant Capacity

Particularly noteworthy are the findings suggesting variation in how individual clones respond to environmental conditions. TPC showed a significant clone $\times$ landfill interaction, with Duncan's test confirming landfill-related differences only for the clone Pannonia. Likewise, although the interaction effect for FRAP was not statistically significant, post hoc analysis revealed landfill-related differences for clones 107/65/9 and I-214. These results agree with previous studies indicating that antioxidant responses can be modulated by specific clonal adaptations, so that even parameters generally insensitive to landfill effects (e.g., TPC) may still show differential responses in particular genotypes [2].

The results clearly demonstrate that different antioxidant parameters have distinct diagnostic values. DPPH and ABTS are recommended for evaluating environmental effects, and TPC and TFC for discriminating among clones, whereas FRAP appears to be the most robust and comprehensive parameter for assessing variation across both landfills and clones. Additionally, TPC and FRAP may be useful for identifying clones that exhibit specific responses to variable landfill conditions, which is valuable for selecting tolerant genotypes and assessing their adaptive potential on degraded soils.

Generally, willow clones had lower values of TPC, TFC, and FRAP, suggesting a higher antioxidant capacity of poplar clones. Among poplar clones, Pannonia had a lower TPC than the others, I-214 had a lower TFC than clone 135/81, and clone S1-8 had a higher FRAP

than I-214 and Pannonia. These results suggest the high antioxidative potential of S1-8 and the relatively low antioxidative potential of Pannonia. The pronounced variation in total phenolic content (TPC) observed in the Pannonia clone between the two sites, exceeding 100%, indicates a high degree of phenotypic plasticity. This variability likely reflects the clone's sensitivity to site-specific environmental constraints, particularly differences in water availability and soil chemical properties, both of which are known to strongly modulate phenolic metabolism in plants. Enhanced phenolic synthesis under more stressful conditions may represent an adaptive strategy aimed at strengthening antioxidant defense and metal chelation capacity. From a practical perspective, such plasticity can be advantageous in heterogeneous or highly stressful environments by allowing flexible metabolic adjustment; however, it may also pose a limitation for phytoremediation applications that require stable, predictable biochemical performance across contrasting sites [50]. The general opinion is that the high antioxidant potential of the plant suggests a high tolerance to stressors. However, Vuksanović et al. [48] found that the high antioxidative response of white poplar clones *in vitro* suggested a lower tolerance to medium with a low initial pH (pH3), assuming that the need of some clones to increase antioxidant potential in the same conditions might suggest their susceptibility. They also stressed higher tolerance of clones with higher FPR. In that sense, the low TPC, low FRAP, and high FPR of Pannonia might correspond with the well-known tolerance of this clone to many abiotic stressors. Pannonia reacted to differences between two landfills by TPC and ABTS, and I-214 by FRAP and ABTS, suggesting their behavior as specialists and their ability to exploit better conditions. Willow clones 380 and 107/65-9 reacted to differences between the two landfills by FRAP, with higher values at the NS than at the BG landfill. These results also favor FRAP as a potent parameter to be used in the evaluation of the effect of landfills on the antioxidative potential of willow clones.

The observed variation in leaf heavy metal concentrations among clones and between landfill sites reflects both genotype-dependent metal uptake strategies and site-specific environmental conditions. The results for the BG site were of particular interest, as they showed the reaction of examined clones to a higher soil content of heavy metals than at the NS site. For example, there were considerable differences in the reaction of the examined clones at the BG and NS sites according to the bioconcentration factor results. While the poplar clones I-214 and Pannonia were dominated by high BCF for As at the NS site, the willow clones 107/65-9 and 378 dominated at the BG site. However, generally low arsenic (As) concentrations across all genotypes suggest limited As bioavailability in the studied soils or an effective exclusion mechanism at the root or translocation level. Similar low As accumulation in poplar and willow leaves has been reported in previous field studies, where As is often retained in roots or immobilized in the rhizosphere rather than translocated to above-ground tissues [14,51].

In contrast, barium (Ba) and cadmium (Cd) exhibited pronounced clone-specific accumulation patterns, indicating substantial genetic control over their uptake and transport. Willow clone 378, and willow clones in most cases, dominated with a higher BCF for both Ba and Cd at both sites. The consistently higher Ba content in clone 378 and elevated Cd levels in clones 378 and 380 suggest enhanced root uptake or reduced sequestration capacity in below-ground tissues. Cadmium is known to be readily translocated to leaves in fast-growing tree species, particularly in genotypes lacking efficient vacuolar sequestration or chelation mechanisms [52]. Conversely, the low Cd concentrations observed in clone S1-8 point to a more conservative uptake strategy, which may be advantageous for phytostabilization-oriented remediation.

Copper (Cu) and iron (Fe) showed strong site-dependent patterns, reflecting differences in soil chemistry, redox conditions, and organic matter content between landfills.

According to the bioconcentration factor, the examined clones presented a considerably lower Fe phytoextraction ability at the Fe-rich BG site compared to the NS landfill site, but in the case of Cu, the BCF at the BG site remained similar to that at the NS site. Elevated Cu leaf content at the Belgrade site and higher Fe leaf content at Novi Sad are consistent with previous reports indicating that metal availability is strongly influenced by pH, moisture regime, and landfill substrate heterogeneity [34,53]. As both Cu and Fe are essential micronutrients, their accumulation likely represents a balance between physiological demand and detoxification capacity, rather than purely passive uptake.

Manganese (Mn) exhibited marked clone-specific variability, with exceptionally high accumulation in clones S1-8 at the BG site, which was also confirmed by their high BCF. Also, this was the only examined heavy metal for which the examined clones achieved higher values at the BG than at the NS site, suggesting that plants were able to exploit its higher soil content at the BG site, which was not the case with the other heavy metals examined. Manganese uptake is highly sensitive to soil redox conditions and water availability, and its accumulation has been linked to adaptive responses in trees growing on disturbed or water-limited sites [54]. While Mn is essential for photosynthetic function, excessive accumulation may impose oxidative stress, emphasizing the importance of coupling metal data with antioxidant responses when evaluating clone suitability for phytoremediation.

Nickel (Ni) and zinc (Zn) displayed moderate-to-strong site effects, both by leaf content and bioconcentration factor. In particular, there was consistently higher Zn leaf content at the NS landfill. Zinc is relatively mobile in soils and readily absorbed by woody plants, often accumulating in leaves without causing acute toxicity [14]. The ability of all examined clones to tolerate elevated Zn concentrations further supports their potential use in landfill remediation, especially in sites characterized by mixed metal contamination.

4.3. Species- and Site-Level Separation

The results of PCA based on biochemical parameters suggest a clear distinction between poplar and willow clones by the first principal component. This principal component is strongly positively correlated with TPC, TFC, and FRAP, suggesting higher antioxidant potential for poplar compared to willow clones. Similar differences between species of the genera *Populus* and *Salix* have been reported in previous studies, which found that poplars generally accumulate higher levels of phenolic compounds and flavonoids, as well as exhibiting a stronger reducing capacity [55,56]. These results support the similar results gained by Duncan's multiple range test that also showed a clear distinction between clones of the two species.

The second unrotated principal component made a clear distinction between landfills. Almost all of the clone $\times$ landfill interaction treatments that had negative values of PC2 were at the NS site, except 380 at BG site. Also, in all clones, the PC2 values recorded at the BG site were greater than those recorded at the NS site. As DPPH and ABTS were strongly but negatively correlated with PC2, it seems that the examined clones strongly responded in these parameters based on differences between landfills. This observation is in complete agreement with previous studies emphasizing that the free radicals DPPH and ABTS respond strongly to environmental variation and represent reliable indicators of abiotic stress and habitat degradation [2,56]. The significantly higher antioxidant activity observed at the NS site points to stronger stress exposure and more intensive activation of the antioxidant system, which is also supported by the results of Duncan's test. Such a relationship between stress and increased DPPH/ABTS values has been widely documented in woody species exposed to pollution, drought, or heavy metals [57].

In this case, the parameter FPR was related to the third unrotated principal component, which suggests a relatively weak influence of differences between clones and between the examined landfills on its variation. Still, the fact that it is categorized in a separate group suggests that it contains specific information that is not described by the other biochemical parameters, and as such, deserves further attention. Similar observations have been reported in earlier literature, emphasizing that certain antioxidant assays may measure specific aspects of redox status or antioxidant dynamics that are not fully captured by standard phenolic tests [58]. Therefore, despite its relatively weak association with clone and landfill variability, FPR's unique position in the PCA space indicates that it may serve as a useful supplementary indicator, especially in studies aiming to identify specific mechanisms of antioxidant response.

According to the results of principal component analysis, which integrated physiological, biochemical, and biomass parameters, we revealed four groups of traits reflecting different aspects of clone performance and stress response. The first group included the physiological parameters g_s and E related to the plant's water regime, as well as dry biomass yield (m_p). This finding aligns with previous studies showing that poplar clones activate antioxidant systems under drought or water-limiting conditions, which helps to mitigate oxidative stress and maintain growth [2,59,60]. The close relationship of these parameters with the first principal component, which accounts for the largest portion of the total variance, emphasizes their importance and suggests their continued use in similar studies evaluating plant responses to environmental stressors. The biochemical parameters that were included in the second group were DPPH and ABTS. The fact that they were closely related to the water regime of the plant and biomass accumulation, as well as their close relationship with the first principal component which describes the highest partition of the total variance, emphasizes their importance and purposes them for further similar studies (Figure 3, Table 3). Efficient antioxidant defense appears to be closely linked with sustained growth, rather than biomass directly driving detoxification. In trees, exposure to abiotic stresses such as drought or metal contamination triggers the accumulation of antioxidant compounds, including phenolics and flavonoids, which protect cellular structures and maintain metabolic activity. In forest species, higher antioxidant capacity has been linked to improved tolerance to environmental stressors, supporting biomass accumulation by minimizing biochemical damage. Therefore, the PCA pattern likely indicates that effective antioxidant responses enable better growth performance under stressful conditions, rather than biomass itself initiating these protective mechanisms [61]. The second set of parameters, including photosynthetic rate (A) and water-use efficiency (WUE), was grouped along the third principal component, indicating that while these traits are not as dominant as those in the first group, they provide unique information about inter-clonal variability and landfill-specific responses. This observation reflects the complex strategies of woody species, such as *Populus* sp. and *Salix* sp., which often balance water-use traits with antioxidant defenses when exposed to stress, resulting in different physiological and biochemical response patterns among clones [46].

At the same landfill sites, the physiological performance of poplar and willow clones was systematically evaluated with respect to site-specific environmental conditions [11]. The results demonstrated a significant clone $\times$ site interaction for net photosynthesis, indicating that photosynthetic responses are genotype-dependent. While the mean transpiration rates and stomatal conductance did not differ significantly between sites for any clone, the overall photosynthetic activity was higher at the Novi Sad landfill, likely due to continuous groundwater availability which stabilized plant water status and turgor. Clones 378 and I-214 exhibited the highest net photosynthesis and water-use efficiency at Novi Sad, whereas clone S1-8 maintained elevated stomatal conductance and transpiration at

both sites, supporting sustained CO₂ assimilation and photosynthetic performance. These findings underscore the critical importance of stable water availability and site-specific environmental conditions in maintaining photosynthetic efficiency and metabolic activity, which are fundamental for optimal growth and for maximizing the phytoremediation potential of poplar and willow clones. Overall, the combined evaluation of physiological and biochemical traits proves essential for understanding clone adaptability. Physiological parameters provide insights into water management and growth performance, whereas biochemical assays, including DPPH and ABTS, reflect the oxidative stress status and the activation of protective mechanisms. Integrating these complementary datasets allows a more comprehensive assessment of clone performance under variable environmental conditions, supporting the use of multidimensional approaches in future studies on plant stress tolerance and adaptation.

4.4. Study Limitations and Future Research Directions

Despite providing clear evidence of differential antioxidant responses among poplar and willow clones grown on landfill sites, this study has several limitations that should be considered when interpreting the results. First, the research was conducted at only two landfill locations, which limits the broader generalization of the findings to other landfill types with different contamination profiles, climatic conditions, or management histories. In addition, measurements were based on a single sampling period and therefore do not capture seasonal or interannual variability in oxidative stress responses, which may significantly influence antioxidant dynamics in perennial woody species.

Furthermore, the study relied primarily on commonly used spectrophotometric assays (TPC, TFC, DPPH, ABTS, FRAP, and FPR), which provide integrative measures of antioxidant capacity. While these assays are valuable for comparative assessments, they do not allow identification of the individual phenolic compounds or specific antioxidant enzymes responsible for the observed responses. Similarly, soil chemical properties and contaminant concentrations were not directly quantified in parallel with plant responses, limiting the ability to link specific stressors to biochemical changes.

Future research should therefore expand to a larger number of landfill sites and include multi-year monitoring to better capture temporal variability and long-term adaptation of clones. Combining biochemical assays with targeted metabolomic and enzymatic analyses would allow a more mechanistic understanding of antioxidant responses. In addition, integrating detailed soil and groundwater chemistry with plant physiological and biochemical data would strengthen causal interpretations of stress effects. Such approaches would further improve the selection of tolerant poplar and willow genotypes and enhance their application in phytoremediation and biomass production on degraded landfill soils.

5. Conclusions

This study confirms that biochemical markers of oxidative stress are effective tools for assessing both landfill-related environmental stress and clonal variability in poplar and willow. DPPH and ABTS clearly differentiated landfill sites, with consistently higher values at the Novi Sad landfill, indicating stronger oxidative stress, whereas TPC and TFC showed pronounced clonal differences but no significant site effects, supporting their use in genotype discrimination. FRAP proved to be the most informative parameter, successfully distinguishing both landfills and clones and revealing site-specific responses in several genotypes (107/65/9, I-214, and willow clones 380 and 107/65/9). Poplar clones exhibited a higher overall antioxidant capacity than willow, as confirmed by higher TPC, TFC, and FRAP values and clear species separation in PCA. Distinct antioxidant strategies were also identified among clones, indicating genotype-specific adaptation to landfill

conditions. Overall, DPPH and ABTS are reliable indicators of landfill-induced stress, TPC and TFC are suitable for clonal differentiation, and FRAP provides the most comprehensive assessment of antioxidant capacity. The integration of biochemical and physiological traits offers a robust basis for selecting tolerant poplar and willow genotypes for growth and phytoremediation on degraded landfill sites.

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Abbreviations

The following abbreviations are used in this manuscript:

TPC	Total phenolic content
TFC	Analysis of total flavonoid content
FRAP	Ferric reducing antioxidant power
DPPH	Radical scavenging activity of DPPH ⁺
ABTS	ABTS radical scavenging activity
FRP	Flavonoid-to-phenol ratio
g _s	Stomatal conductance
A	Net photosynthesis
E	Transpiration
WUE	Water-use efficiency
m _p	Dry shoot biomass

Appendix A

Table A1. Duncan’s test for parameters of oxidative stress in the examined clones on landfills near Novi Sad and Belgrade.

Clone	Landfill	TPC ⁽¹⁾	TFC	FRAP	DPPH	ABTS	FPR
Means of landfills							
	BG	3.508 ^{a (2)}	2.408 ^a	10.942 ^b	80.210 ^b	91.525 ^b	0.670 ^a
	NS	3.243 ^a	2.658 ^a	15.640 ^a	81.538 ^a	92.082 ^a	0.888 ^a

Table A1. Cont.

Clone	Landfill	TPC ⁽¹⁾	TFC	FRAP	DPPH	ABTS	FPR
Means at the level of the interaction clone × landfill							
378	BG	0.971 ^c	0.741 ^d	3.433 ^g	81.721 ^{ab}	92.107 ^a	0.769 ^b
378	NS	1.594 ^{bc}	0.914 ^d	7.300 ^{fg}	84.250 ^a	92.227 ^a	0.600 ^b
380	BG	1.559 ^{bc}	0.786 ^d	2.695 ^g	80.158 ^b	91.868 ^{ab}	0.544 ^b
380	NS	2.080 ^{bc}	1.354 ^{cd}	10.002 ^{ef}	79.824 ^b	92.390 ^a	0.691 ^b
107/65/9	BG	1.164 ^{bc}	0.607 ^d	3.783 ^g	80.832 ^b	91.661 ^{abc}	0.529 ^b
107/65/9	NS	1.930 ^{bc}	1.352 ^{cd}	13.218 ^{de}	80.263 ^b	91.665 ^{abc}	0.774 ^b
135/81	BG	5.574 ^a	2.790 ^{bc}	16.372 ^{bcd}	79.503 ^b	91.418 ^{abc}	0.499 ^b
135/81	NS	4.851 ^a	3.306 ^{ab}	20.240 ^{abc}	81.551 ^{ab}	92.175 ^a	0.690 ^b
I-214	BG	5.316 ^a	4.291 ^{ab}	13.116 ^{de}	79.464 ^b	91.073 ^{bc}	0.807 ^b
I-214	NS	5.198 ^a	4.213 ^{ab}	21.473 ^{ab}	81.620 ^{ab}	92.349 ^a	0.849 ^{ab}
Pannonia	BG	4.831 ^a	4.545 ^a	15.800 ^{bcd}	79.022 ^b	90.895 ^c	0.942 ^{ab}
Pannonia	NS	2.380 ^b	3.314 ^{ab}	14.813 ^{cde}	81.258 ^{ab}	92.076 ^a	1.729 ^a
S1-8	BG	5.142 ^a	3.099 ^{ab}	21.400 ^{ab}	80.774 ^b	91.649 ^{abc}	0.602 ^b
S1-8	NS	4.666 ^a	4.152 ^{ab}	22.434 ^a	81.998 ^{ab}	91.688 ^{abc}	0.886 ^{ab}

⁽¹⁾ TPC—total content of phenolics [mg GAE g^{−1} DW]; TFC—total content of flavonoids [mg QE g^{−1} DW]; FRAP—ferric-reducing antioxidant power [mg AAE g^{−1} DW]; DPPH—DPPH activity [% of RSC]; ABTS—ABTS assay [% of RSC]; FPR—TFC/TPC ratio. ⁽²⁾ Values with the same letter are not significantly different according to the Duncan test for $p < 0.05$.

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