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Effects of Cd and Zn Treatments on Leaf Chemıcal Compounds of Japanese barberry (*Berberis thunbergii*), Boxwood (*Buxus sempervirens* var. *rotundifolia*), and Gold tassel (*Euonymus japonica* var. *aurea*) Species

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Highlights:

- Chemicals
- Metals
- Plants

Keywords:

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- Nutrients
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- Stress

ABSTRACT:

In the present study, the effects of cadmium (25 µM and 50 µM) and zinc (200 µM and 400 µM) treatments on some bioactive compounds and mineral levels in leaves of japanese barberry, boxwood, and gold tassel genotypes were investigated. Given the results, it was determined that photosynthetic pigments were stimulated by 200 µM Zn and 400 µM Zn. Boxwood was found to be tolerant to the treatments in terms of chlorophyll and carotenoid. In contrast, japanese barberry was found to be tolerant in terms of chlorophyll b and total chlorophyll. The amount of anthocyanin was higher in the leaves of gold tassel, and boxwood and the total phenolic was higher level in gold tassel and japanese barberry in all treatments. Proline and nitrate levels were generally high in the treated groups of three plants, as well as RWC in japanese barberry and boxwood. Given the element results, japanese barberry had a higher accumulation capacity for P, S, Mn, Cl, Cd, Fe, Al, Si, Cu, Ba, Zn, Ti, and Cr, boxwood for K, Mn, Cl, Cd, Zn, Fe, Al, Si, and Cr, and Gold tassel for K, P, S, Mn, Cl, and Cd. As a result, boxwood showed a higher tolerance to 400 µM Zn, japanese barberry plant to 200 µM Zn, and 50 µM Cd. It can be said that boxwood and japanese barberry plants can be used in afforestation projects in urban parks, gardens, and roadside, as well as in areas with high soil pollution, to reduce the pollution damage.

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INTRODUCTION

Industrial and technological advancements, industrial wastes, increasing urbanization and consequent growth in population, excessive use of fossil fuels, traffic-related exhaust gases, nuclear wastes (Atmaca and Sevimoğlu, 2020; Bingöl, 2020), and improper use of fertilizers and pesticides in agricultural lands create a degradation in the physical, chemical, and biological structure of the soil and heavy metal pollution that suppress the plant development. Excessive metal accumulation in the soil prevents the intake of nutrients from the soil (Ashraf et al., 2016; Küçük and Karaoğlu, 2021), while influencing the other physiological processes affecting the growth and development such as photosynthesis, nitrogen, and respiration metabolisms, etc. (Asad et al., 2019). Many studies showed that heavy metals inhibited the synthesis of pigments, secondary metabolites, nitrogenous compounds, and other biomolecules due to mineral deficiency (Bernardini et at., 2016; Kapoor et al., 2019; Boudalı et al., 2022). Furthermore, these reactions also affect the results of metal toxicity (Dadea et al., 2016). Besides, in addition to causing physiological drought by leading to water retention in the soil, it also creates a proportional decrease in water content in leaves by suppressing the transfer of water from the soil to the leaf. However, some species called "hyperaccumulators" can accumulate heavy metals without any cellular damage and maintain their lives (Kılıç and İpek, 2019; Isinkaralar, 2022). In such plants, metals can be accumulated in membranes and vacuoles or bound to ligands, like organic acids, amino acids, and metallothioneins, and their damage can be prevented. Hence, they are used as biomonitors in phytoremediation studies (Chadzinikolau et al., 2017; Isinkaralar, 2022). For this reason, selecting species having highly resistan species to heavy metals and using them in highly polluted areas are critical for ensuring the sustainability of the ecosystem and Japaneseucing environmental pollution (Turkyilmaz et al., 2018; Kapoor et al., 2019).

Cadmium (Cd) and zinc (Zn) are two heavy metals that pollute the environment. Although Zn is toxic at low concentrations, Cd is toxic at high concentrations. Cadmium emerging as a result of zinc production and ranking 4th in global annual use of metals are released to the environment via industrial wastes, industries such as the paper industry, metal-steel industry, and phosphatic fertilizer industry, as well as batteries, chimney gases, and traffic gases (Jain et al., 2020; Muradolu et al., 2020). The main objectives of this study are to compare the effects of Cd and Zn on 1) some bioactive compounds 2) relative water content (RWC), 3) macronutrients, and 4) trace elements of japanese barberry, boxwood, and gold tassel. The species used in the study are resistant to drought and low-temperature conditions and are widely used in park-garden and roadside greening initiatives, and hedge plant and landscape studies due to their lack of soil selectivity (Yucedag et al., 2019).

MATERIALS AND METHODS

Experimental Procedure

In the present study, 2-year-old individuals of Japanese barberry (*Berberis thunbergii*), Boxwood (*Buxus sempervirens* var. *rotundifolia*), and Gold tassel (*Euonymus japonica* var. *aurea*) species were used. The plants were assigned to the groups of control (0), cadmium (Cd:25 µM and 50 µM- $CdSO_4H_2O$), and zinc (Zn:200 µM and 400 µM- $ZnCl_2$). Cd and Zn elements were dissolved in Hoagland's nutrient solution at determined concentrations. The treatments were performed in accordance with the water retention capacity of the soil (300 ml) and from the soil. The plants in the control group were irrigated using only the nutrient solution. Metal treatments were performed using the using nutrient solution and metals. The treatments were conducted for eight weeks (2 treatments every week). The procedure was stopped when the areas containing chlorophyll decreased approximately by 50% in leaves of gold tassel, and the leaf samples were harvested from these plants. Some were powdered using liquid nitrogen and then used in chemical analyses. Some samples were used in fresh form in RWC measurements, whereas others were used in elemental analyses.

Biochemical Measurements

Photosynthetic pigment level was determined by following the method of Lichtenthaler and Buschmann (2001). For this purpose, 0.5 g of fresh leaf tissue was extracted with 10 ml of 80% chilled acetone and centrifuged at 3500 *g* for 10 min. The absorbance values of the samples were recorded at 450, 645, and 663 nm, and the amount of pigment was given as mg/g. The amounts of total phenolics were estimated according to the Folin-Ciocalteu method (Singleton et al., 1999). Anthocyanin contents were determined by using the method of Mancinelli (1990). Plant material (0.5g) was homogenized with 0.5 N HCl and was centrifuged at 18000 g for 30 min. The absorbance values of samples were noted at 530 nm (UV-VIS Spectrophotometer). Anthocyanin contents were determined using cyanine chloride and expressed in µg/g of fresh weight. Nitrate concentrations in the leaf samples were determined by following the method by Cataldo et al. (1975). The concentrations of proline were determined by using the method by Bates et al. (1973), and total soluble protein amounts were determined using the method by Bradford (1976). To measure PAL (phenylalanine-lyase) activities of the samples, 1g of fresh leaves were extracted in 5 ml of 0.1 M sodium borate buffer (pH 7.0) containing 0.1 g insoluble polyvinylpolypyrrolidone. The extract was filteJapanese through cheesecloth and the filtrate was centrifuged at 15000 g for 25 min. The supernatant was used in the enzyme analysis. PAL activity was estimated as the conversion rate of L-phenylalanine to trans-cinnamic acid at 290 nm, as defined by Dickerson et al. (1984). Samples containing 0.4 ml of leaf extract were incubated with 0.5 ml of 0.1 M borate buffer, pH 8.8, and 0.5 ml of 12 mM L-phenylalanine in the same buffer for 30 min at 30 °C. The amount of trans-cinnamic acid synthesized was calculated using its extinction coefficient of 9630 1/M·cm. Enzyme activity was expressed proprtionately to the fresh weight basis (nmol 1/min.g).

Determining the RWC (Relative Water Content) (%)

The relative water content of leaves was determined in the fully grown healthy leaf of plants. The fresh weight (FW) of the samples was recorded and the leaves were immersed in distilled water for 4 hours. After 4 hours, the turgid weights of the samples were recorded (TW). Samples were then dried in an oven at 65°C to constant weight, and their dry weight was recorded. The relative water content of leaves was calculated using the formula below (Chen et al., 2009).

$$RWC=(FW-DW)/(TW-DW)\ x100 \quad (1)$$

Preparation of the leaf samples to measure nutrients

Fresh, healthy, and fully developed leaf samples (100 g) were collected from four sides of three species. Leaf prefixes were collected and put into labelled paper bags. The leaves were cleaned using distilled water before dehydration on blotter papers. The cleaned and dehumidified samples were air-dried in a shaded area before being ground into powder using a laboratory blender and used in measurements of nutrients at the Central Research Laboratory of Kastamonu University.

Statistical analysis

One-way ANOVA (Analysis of variance) was applied to analyze the differences in the chemicals, and minerals in the leaf samples of three species. The statistical analysis was performed using the SPSS program (Version 11 for Windows). Following the results of the ANOVAs, Tukey's honestly significant difference (HSD) test (α = 0.05) was used to determine significance.

RESULTS AND DISCUSSION

Effects of Cd and Zn Treatments on Photosynthetic Pigments, Anthocyanin, and Phenolic Compound

Photosynthetic pigments, non-photosynthetic pigments, and phenolic compounds provide plants with different colours, odours, and tastes, as well as playing a role in stimulating tolerance to abiotic and biotic stress factors (Kapoor et al., 2019; Park et al., 2019). The changes in chlorophyll a, chlorophyll b, total chlorophyll, chlorophyll a/chlorophyll b ratio, and total carotenoid (cart.) levels of japanese barberry, boxwood, and gold tassel plants after the cadmium (Cd) and zinc (Zn) treatments are presented in Table 1. Table 2 shows the anthocyanin, total phenolic, and nitrate amounts, PAL activity, and RWC concentrations. As seen in Table 1 and Table 2, the effects of treatments on the examined bioactive compounds and RWC values significantly varied by plant species, metal type, and concentration (p<0.05).

Table 1. Changes in Chlorophyll a, Chlorophyll b, total Chlorophyll, Chlorophyll a:Chlorophyll b ratio, and total carotenoid levels in japanese barberry, boxwood, and bold tassel leaves under Cd and Zn stress

Species	Group	Chlorophyll a mg/g	Chlorophyll b mg/g	Total Chlorophyll mg/g	Total carotenoid. mg/g	Chlorophyll a:Chlorophyll b
Japanese barberry	Control	0.0021±0.0001^{e*}	0.0014±0.0001	0.0034±0.0001^{e}	1.15±0.004^{e}	1.56±0.04^{d}
	25 µM Cd	0.0017±0.0001^{f}	0.0007±0.0001	0.0024±0.0001^{g}	0.92±0.003^{e}	2.28±0.11^{c}
	50 µM Cd	0.0019±0.0001^{f}	0.0017±0.0001^{g}	0.0036±0.0001^{e}	1.02±0.004^{e}	1.13±0.04^{e}
	200 µM Zn	0.0028±0.0001^{e}	0.0014±0.0001^{g}	0.0042±0.0001^{d}	1.32±0.003^{d}	2.05±0.04^{c}
	400 µM Zn	0.0015±0.0001^{g}	0.0014±0.0001^{g}	0.0029±0.0001^{f}	1.32±0.002^{d}	1.10±0.02^{e}
Boxwood	Control	0.0942±0.0001^{c}	0.0473±0.0001^{b}	0.1415±0.0002^{b}	2.39±0.008^{c}	1.99±0.01c
	25 µM Cd	0.1035±0.0001^{b}	0.0297±0.0001^{c}	0.1332±0.0002^{c}	7.95±0.005^{b}	3.48±0.01a
	50 µM Cd	0.0730±0.0002^{d}	0.0238±0.0002^{d}	0.0968±0.0004^{c}	6.01±0.008^{b}	3.07±0.02^{b}
	200 µM Zn	0.1001±0.0001^{b}	0.0276±0.0001^{c}	0.1277±0.0002^{c}	7.34±0.006^{b}	3.63±0.01^{a}
	400 µM Zn	0.1550±0.0001^{a}	0.0884±0.0002^{a}	0.2434±0.0002^{a}	16.35±0.005^{a}	1.76±0.01^{d}
Gold tassel	Control	0.0675±0.0001^{d}	0.0894±0.0001^{a}	0.1569±0.0002^{b}	6.52±0.007^{b}	0.76±0.01^{f}
	25 µM Cd	0.0158±0.0001	0.0058±0.0003^{f}	0.0216±0.0003^{h}	1.41±0.004^{d}	2.73±0.15^{b}
	50 µM Cd	0.0179±0.0001^{f}	0.0063±0.0001^{f}	0.0242±0.0002^{g}	2.25±0.004^{c}	2.85±0.04^{b}
	200 µM Zn	0.0140±0.0001^{g}	0.0031±0.0001^{g}	0.0171±0.0002^{h}	1.73±0.005^{d}	4.53±0.10^{a}
	400 µM Zn	0.0228±0.0001^{e}	0.0102±0.0001^{e}	0.0330±0.000^{e}	2.84±0.005^{c}	2.24±0.02^{c}
F		420339.518	65759.889	197518.879	197518.879	729150.681
P		0.000	0.000	0.000	0.000	0.000

*: Data are mean ± standar error (n=3). Values within the column, followed by the same letter(s), are not significantly different according to Tukey's test (P < 0.05)

Chlorophyll b and total chlorophyll in japanese barberry and chlorophyll a, carotenoid, and chlorophyll a: chlorophyll b ratio, however, were generally higher in leaves of boxwood than the control. In gold tassel, however, the total carotenoid and chlorophyll a: chlorophyll b ratio were higher than the control (in all groups), but chlorophyll pigments were lower (Table 1).

Among the treatments, 200 µM Zn dose in japanese barberry and 400 µM Zn dose in boxwood stimulated the accumulation of photosynthetic pigments. The treatments that harmed plant pigment content were 25 µM Cd in japanese barberry, 50 µM Cd in boxwood, and 200 µM Zn in gold tassel (Table 1). Anthocyanin contents of boxwood and gold tassel leaves were higher than the control in all treatment groups, and it was higher than the control in japanese barberry for 50 µM Cd and 400 µM Zn doses (Table 2). Total phenolic content was found to decrease in japanese barberry for 50 Cd dose, boxwood for 50 µM Cd and 200 µM Zn doses, and gold tassel for 400 µM Zn dose than the control (Table 2). Given the data regarding photosynthetic pigments, anthocyanin, and total phenolic compound contents, japanese barberry showed tolerance to 200 µM Zn dose and boxwood to 400 µM Zn and 25 µM Cd doses. In contrast, japanese barberry showed sensitivity to 50 Cd dose and boxwood to 50 µM Cd dose. In terms of photosynthetic pigments, gold tassel was found to be sensitive to concentrations of both metals. However, considering total carotenoid, anthocyanin, and total phenolic compound contents, it was tolerant to all treatment groups (Table 1, Table 2). The results achieved regarding the pigments here are in parallel with the literature. Similar to the present study, it was determined that photosynthetic pigment, anthocyanin, and total phenolic compound contents were affected by the heavy metal stress in *Camellia* (Mukhopadhyay et al., 2013), *Berberis* (Subba et al., 2014; Chadzinikolau et al., 2017), and *Alyssum* and *Daphne* (Song et al., 2019) species. Yang et al. (2020) reported that, low-dose metal treatments stimulated the amount of pigments in *D. involucrata* plants subjected to Pb and Cd stress, whereas high doses had adverse effects. In another study, Dobrikova et al. (2021) determined that high-dose Zn treatment increased the anthocyanin and total phenolic compound, and the chlorophyll a contents in *Salvia sclarea*, and did not significantly affect the total carotenoid content. As authors reported, carotenoids, anthocyanins, and phenolic compounds chelate the metals or prevent peroxidation reactions through their functional groups (Zhang et al., 2014; Chadzinikolau et al., 2017) and, thus, suppress the synthesis of ROS and other toxic compounds that could damage the structure of photosynthetic instruments and pigment molecules (Park et al., 2019). The effects on heavy metal tolerance of anthocyanin and phenolic compounds were reported in detail for species such as *B. thungbergii* (Chadzinikolau et al., 2017), *Hibiscus sabdariffa* (Apáez-Barrios et al., 2018), *Kandelia obovata* (Chen et al., 2019), and *Euphorbia pulcherrima* (Moustaka et al., 2020). In these plants, it was determined that heavy metals such as Cd, Zn, Mn, and Al caused a decrease in pigment content by breaking the chlorophyll molecules down or preventing their synthesis. At the same time, they stimulated the accumulation of anthocyanin and phenolic compound contents, and that these compounds inhibited the damages by forming complexes with the metals. Cd and Zn concentrations negatively affected chlorophyll a molecule in japanese barberry, chlorophyll b and total chlorophyll molecules in boxwood, and all the pigments in gold tassel (Table 1). It was thought that the difference in pigments' response to metals might be because of the sensitivity differences in species and pigment molecules (Morales and Kaiser, 2020). Researchers claimed that chlorophyll b molecules were dominant in species tolerant of shadowing (Morales and Kaiser, 2020; Park et al., 2019). In their study, the authors related the decrease in chlorophyll pigments in all three plant species to the disintegration of pigments due to the structural damage to the chloroplast membrane by Cd and Zn treatments (Yang et al., 2020). Chen et al. (2019) and Per et al. (2016) reported that, by binding to -

SH groups of protein and enzymes, heavy metals impaiJapanese the functions of enzymes that are responsible for the synthesis of chlorophyll molecules, as well as damaging the pigment complexes localized in chloroplast membranes.

Effects of Cd and Zn Treatments on PAL Activity

PAL is an enzyme that play a role in the biosynthesis of phenolic compounds, anthocyanins, and membrane components (Głowacka et al., 2019). In the present study, when compared to the control samples, PAL activities remarkably decreased in japanese barberry at high Cd (50 µM Cd) and Zn (400 µM Cd) doses. Compared to the control, enzyme activity in boxwood decreased only at the dose of 50 µM Cd, whereas it decreased in gold tassel at all treatment doses (Table 2). When compared to the control, the highest PAL activity among the samples was found in boxwood (25 µM- 0.247 EU), followed by japanese barberry (200 µM Zn-0.159 EU) and gold tassel (200 µM Zn-0.126 EU) (Table 2).

Table 2. Changes in anthocyanin, total phenolic, proline, nitrate, PAL activity, and RWC levels in japanese barberry, boxwood, and gold tassel leaves under Cd and Zn stress

Species	Group	Anthocyanin mg/g	Total phenolic mg/g	Proline Mmol/g	Nitrate mg/g	PAL EU mg/ Protein	% RWC
Japanese barberry	Control	1.841±0.0002^{h*}	16.35±0.03^{b}	91.89±0.14^{f}	7.02±0.06^{c}	0.142±0.001^{g}	48.40±0.13^{b}
	25 µM Cd	2.094±0.0002^{i}	17.88±0.04^{c}	90.39±0.18^{f}	6.29±0.21^{b}	0.157±0.001^{h}	45.14±0.39^{a}
	50 µM Cd	1.724±0.0001^{g}	13.96±0.03^{a}	106.67±0.09^{h}	7.04±0.29^{b}	0.115±0.001^{e}	52.42±0.08^{b}
	200 µM Zn	2.020±0.0002^{i}	17.88±0.03^{c}	109.90±0.09^{i}	8.74±0.06^{c}	0.159±0.002^{h}	54.36c±0.29
	400 µM Zn	1.492±0.0002^{f}	16.88±0.02^{c}	100.21±0.14^{g}	7.88±0.03^{c}	0.114±0.001^{e}	67.15±0.16^{h}
Boxwood	Control	0.294±0.0001^{c}	15.20±0.14^{b}	54.50±0.07^{c}	3.71±0.19^{b}	0.146±0.001	60.53±0.10^{d}
	25 µM Cd	0.385±0.007^{e}	18.40±0.02^{c}	60.33±0.04^{d}	4.94±0.03^{b}	0.247±0.001^{i}	66.76±0.56^{g}
	50 µM Cd	0.322±0.004^{d}	14.79±0.03^{b}	54.34±0.04^{c}	4.58±0.26^{b}	0.128±0.001^{f}	56.8±0.30^{c}
	200 µM Zn	0.324±0.0001^{d}	12.40±0.02^{a}	55.08±0.07^{c}	5.78±0.08^{b}	0.210±0.003^{i}	61.66±0.15^{d}
	400 µM Zn	0.312±0.0001^{d}	16.49±0.04^{b}	63.27±0.03^{e}	7.40±0.06^{c}	0.193±0.001	65.42±0.16^{f}
Gold tassel	Control	0.205±0.0001^{a}	15.06±0.02^{b}	35.35±0.03^{b}	2.26±0.03^{a}	0.075±0.001^{a}	65.93±0.51^{f}
	25 µM Cd	0.339±0.0002^{d}	26.44±0.05^{d}	25.84±0.03^{a}	1.94±0.03^{a}	0.108±0.001^{d}	63.51±0.26^{e}
	50 µM Cd	0.269±0.0001^{b}	17.91±0.03^{c}	36.93±0.05^{b}	3.21±0.15^{a}	0.093±0.001^{b}	65.85±0.29^{f}
	200 µM Zn	0.299±0.0001^{c}	18.10±0.04^{c}	36.11±0.04^{b}	3.33±0.16^{a}	0.126±0.001^{f}	62.39±0.22^{d}
	400 µM Zn	0.338±0.0001^{d}	15.93±0.05^{b}	36.40±0.07^{b}	3.28±0.03^{a}	0.096±0.001^{c}	63.86±0.31^{e}
F		6839.1	4408.33	14985.46	240.23	3188.944	444352.26
P		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

*: Data are mean ± standar error (n=3). Values within the column, followed by the same letter(s), are not significantly different according to Tukey's test ($P < 0.05$)

In terms of the PAL activity, the tolerance of species to treatments was found to be gold tassel > boxwood > japanese barberry (Table 2). The changes in PAL activity overlapped with the changes in anthocyanin and total phenolic content. At the doses with high PAL activity, both compound contents are generally high. PAL activities of gold tassel and boxwood were generally high and this was thought to be because these species generally have a greater cambial change and membrane activity than japanese barberry. Japanese barberry morphologically has a shrub-like form and, thus, a leaner structure in comparison to two other plants (DeGasperis and Motzkin, 2007). Hence, the changes in the phenolic compound and anthocyanin contents proved this result (Table 2). The results regarding the change in PAL activity are in parallel with the literature. The increase in PAL activity in plants subjected to heavy metal stress was proven for pepper (Koç and İşlek, 2015), Plantago (Kundu et al., 2018), and pea (Głowacka et al., 2019) plants. Similarly, Jain et al. (2020) for corn seedlings exposed to Zn stress and Chen et al. (2018) for *K. obovata* plants subjected to Cd (2.5 ppm) and Zn (100 ppm) stress determined that the biosynthesis pathway of phenolic compounds increased the PAL enzyme activities.

Effect of Cd and Zn Treatments on Proline and Total Nitrate Contents

As seen in Table 2, Cd and Zn treatments generally caused an increase in proline and total nitrate contents. Besides that, 25 µM Cd dose in japanese barberry and gold tassel and 50 µM Cd dose in boxwood resulted in a decrease in nitrate and proline content in comparison to the control. When compared to the control, the highest nitrate and proline contents were found at 200 µM Zn dose in japanese barberry and at 400 µM Zn dose in boxwood (Table 2). While the nitrate content was at its highest level in gold tassel at 200 µM Zn dose, proline content reached the highest level at 50 µM Cd dose (Table 2). Given the nitrate and proline results, plants' tolerance to Zn concentrations was found to be higher in comparison to the tolerance to Cd concentrations (Table 2). Similar to the present study, Kandziora-Ciupa et al. (2016) examined Cd, Fe, Mn, Pb, and Zn treatments, and Modirroosta et al. (2014) examined Cd toxicity, and they reported that these treatments increased the proline content in needles of scot pine and they played an essential role in the elimination of the harmful effects of metals on -SH groups of proteinic and non-proteinic compounds. Moreover, Boudalı et al. (2022) determined that high Zn (2 mM) doses significantly increased the proline, free amino acid, total polyphenol, and flavonoid contents of *Lepidium sativum*, while Zhao et al. (2021) reported that proline content increased with increasing Cd concentration in the sassafras plant. Pan et al. (2020) found that Zn accumulation stimulated the nitrate accumulation in *Arabidopsis* and that, in these plants, Zn accumulation might be related to the nitrate-dependent pathway. Zemanová et al. (2013) concluded that Cd stress increased the proline accumulation in *Noccaea caerulescens* and *Arabidopsis helleri*, whereas Singh et al. (2016) reported Cd stress decreased the nitrate content in tissues by preventing the enzyme activities but stimulated the amino acid accumulation. In the present study, high nitrate content was related to high phenolic compounds, anthocyanin, and proline contents. In literature, it was stated that proline accumulation in herbal tissues under stressful conditions was a common phenomenon and that, binding the metals, proline, phenols, and anthocyanins would protect the tissues from damage by metals (Modirroosta et al., 2014; Rady et al., 2016). Chen et al. (2019), and Yoo et al. (2020), reported that proline played an esssential role in the glutamate pathway and nitrate metabolism, as well as ATP synthesis. The differences in amounts of nitrate and proline might probably have arisen from species' nitrogen metabolism, differences in the mechanism of nitrate transfer from the xylem to the cytoplasm, and differences in the effects of metals on nitrogen metabolism (Hachiya and Sakakibara, 2017; Pan et al., 2020).

Effect of Cd and Zn Treatments on Relative Water Content

The water content of the leaf is an indicator of critical water level ensuring the optimal development of plants and plays a vital role in osmosis and turgor events, transpiration, and material transfer (Rucinśka-Sobkowiak, 2016). RWC content of plants ranged between 45.14% and 67.15% and it was found to be generally high in leaves of japanese barberry and boxwood and low in leaves of gold tassel in all the treatments (Table 2). Moreover, RWC content decreased by 25 µM Cd dose in Japanese barberry and 50 µM Cd dose in boxwood. RWC results were found to overlap with the literature data. Researchers reported that heavy metals affected the plant-water relationships such as water absorption from the soil, transfer of water to leaves, and turgor and osmosis processes in the leaf (Yee et al., 2005; De Silva et al., 2012) and, in case of a decrease in RWC, chlorophyll pigments disintegrated, and senescence was stimulated (Rucinśka-Sobkowiak, 2016). In the present study, low levels of RWC, and pigments in gold tassel leaves subjected to stress, well as low RWC values but high levels of chlorophyll pigments in japanese barberry and boxwood leaves prove this finding (Danquah et al., 2014). RWC values that were low in japanese barberry and boxwood species, but high in gold tassel were related to the differences between species' metal stress tolerance (Rady et al., 2016). Besides that, the fact that the proline accumulation and carotenoid content in gold tassel leaves were lower than the other two species might have affected this result (Modirroosta et al., 2014; Rady et al., 2016).

Effects of Cd and Zn Treatments on Macro-Elements and Trace Elements

Plants must take nutrient elements from the air, water, and soil in the environment, where they grow, for optimal growth and development. While the level of essential elements in the plant is high, that of trace elements is low (Kopriva et al., 2019; Küçük and Karaoğlu, 2021). The changes in Mg, P, S, K, and Ca contents (mg/kg) found in the present study are presented in Table 3. The mean Mg, P, S, K, and Ca values (mg/kg) of the species were found to be 1485-7840, 1957-7872, 2653-4126, 13530-25370, and 24600-41260, respectively (Table 3).

Table 3. Changes in Mg, P, S, K, and Ca contents of the leaves of japanese barberry, boxwood, and gold tassel under Cd and Zn stress

Species	Group	Mg	P	S	K	Ca
Japanese barberry	Control	2849±45^{f*}	5009±6^{f}	2849±3^{f}	24000±30^{c}	36750±30^{c}
	25 µM Cd	7639±46^{b}	6948±8^{b}	3754±4^{b}	19790±30^{d}	36430±30^{c}
	50 µM Cd	7152±44^{c}	5982±7^{c}	3401±4^{d}	25370±30^{b}	37460±30^{b}
	200 µM Zn	7317±45^{c}	7872±8^{a}	4126±4^{a}	24030±30^{c}	38970±30^{b}
	400 µM Zn	7504±45^{b}	5675±7^{d}	3319±4^{d}	23000±30^{c}	35650±30^{c}
Boxwood	Control	3855±38^{e}	2369±5^{i}	2927±4^{f}	13530±20^{h}	25500±30^{f}
	25 µM Cd	4009±38^{d}	2737±5^{h}	2668±3^{g}	15460±20^{f}	25950±30^{f}
	50 µM Cd	3819±38^{e}	1957±4^{j}	3012±4^{f}	18820±20^{e}	24760±30^{g}
	200 µM Zn	3598±36^{e}	2313±4^{i}	2763±3^{g}	17840±20^{e}	24600±30^{g}
	400 µM Zn	3951±37^{d}	2563±4^{ı}	2653±3^{g}	14160±20^{g}	25500±30^{f}
Gold tassel	Control	1647±21^{h}	2265±4^{i}	3062±3^{f}	16980±20^{e}	41260±30^{b}
	25 µM Cd	1756±23^{g}	2833±4^{g}	3243±3^{e}	23360±30^{c}	34970±30^{d}
	50 µM Cd	1580±22^{h}	2477±4^{ı}	3559±4^{c}	21360±30^{d}	34330±30^{d}
	200 µM Zn	1787±23^{g}	2425±^{4ı}	3493±4^{d}	20090±30^{d}	31970±30^{e}
	400 µM Zn	1485±21^{ı}	2641±4^{h}	3356±4^{d}	19580±20^{d}	37930±30^{b}
Soil		12950±60^{a}	5195±2.8^{e}	3074±3^{f}	27540±30^{a}	111700±100^{a}
F		9235096.537	6001092.63	327564.155	21837747.43	49502626.68
P		0.000	0.000	0.000	0.000	0.000

*: Data are mean ± standar error (n=3). Values within the column, followed by the same letter(s), are not significantly different according to Tukey's test ($P < 0.05$)

Examining the effects of treatments on Mg content, it was determined that Mg content in Japanese barberry increased in all the treatment groups when compaJapanese to the control. Partial increases, however, were observed at the doses of 25 µM Cd and 400 µM Zn in boxwood and 25 µM Cd and 200 µM Zn doses in gold tassel (Table 3). Mean P contents were higher in japanese barberry and gold tassel than in control. Nevertheless, it was found to be lower in boxwood than the control at the doses of 50 µM Cd and 200 µM Zn (Table 3). But 50 µM Cd decreased P content. This can be explained by the fact that Cd suppressed P uptake in plants (Chafei et al., 2004). Examining the mean S contents, it showed an increase in japanese barberry and gold tassel in all the treatment groups when compared to the control but increased in boxwood only at the dose of 50 µM Cd. The treatments increased the K content in boxwood and gold tassel compared to the control but, in japanese barberry, the doses of 25 µM Cd and 400 µM Zn decreased it (Table 3). Ca contents tended to decrease in all three species compared to the control. However, Ca contents of 200 µM Zn in japanese barberry and at the doses of 25 µM Cd and 400 µM Zn in boxwood were higher than the control (Table 3). As seen in Table 2, the sort of macronutrient elements by the amount is Ca> K> P> Mg>S (Table 3). Japanese barberry was found to be rich in P and S elements, while gold tassel was found to be rich in K, P, and S elements. On the other hand, japanese barberry had low values in terms of Mg and Ca, while boxwood yielded low S values, and gold tassel yielded low Ca values (Table 3). Moreover, japanese barberry was found to be tolerant to 50 µM Cd and 200 µM Zn doses, boxwood to 25 µM Cd and 400 µM Zn doses, and gold tassel to 25 µM Cd and 200 µM Zn doses. The differences between the effects of treatments on the macronutrient contents of leaves of species were related to the differences between species' accumulation capacity differences, element transfer speed differences, and also the differences in interaction with Cd and Zn metals (Maillard et al., 2015). It was reported in detail that macronutrient elements were generally found at high levels because of their biological functions in herbaceous and woody species (Kapoor et al., 2019). K plays a role in controlling the osmosis-turgor events and protein synthesis. In contrast, S plays a significant role in physiological processes such as involvement in the structure of enzymes, proteins, and vitamins, as well as stimulating the resistance to metal stress by creating S-containing phyto-gelatins (Kopriva et al., 2019). Ca participates in enzyme activation and membrane structure, whereas Mg plays an influential role in chlorophyll synthesis and enzyme activations (Zhang et al., 2018). P is an element found in many compounds, such as sugars, ATP, and DNA (Kopriva et al., 2019). Researchers reported that K, P, S, and Mg are the dominant elements in plant cells because of their high transfer speed in the phloem, that high K content suppressed Ca and Mg intake, but high P and Ca contents decreased the K intake, and that it was because of these elements competitiveness about binding to metabolites (Maillard et al., 2015).

Trace elements such as Fe, Zn, Mn, Ni, Cu, Co, and Cr are essential for the biosynthesis of chlorophyll and secondary metabolites, carbohydrates, and other growth substances (Modirroosta et al., 2014). However, they are toxic effects at high concentrations, and hence, they are called heavy metals (Asad et al., 2019; Isinkaralar, 2022). Mn concentration showed an increase in japanese barberry compared to the control, whereas it decreased in boxwood and gold tassel at the treatment dose of 400 mm Zn. Copmared to the control, the highest Mn content was found to be in japanese barberry at 400 Zn doses and in boxwood and gold tassel at 50 µM Cd dose (Table 4). Fe contents of japanese barberry and boxwood were higher than the control in all the treatment groups, whereas it was higher than the control in gold tassel only at the dose of 50 µM Cd. Compared to the control, the highest Fe content (japanese barberry and boxwood) was achieved from the 25 µM Cd treatment. Co content of japanese barberry was found to be higher than the control in all the treatment groups, whereas it was higher than the control in boxwood in Cd treatments and in gold tassel at 200 µM Zn dose. Ni content was determined to be higher than the control in all three plants, while the highest Ni contents were observed in Cd treatments (Table 4). Treatments resulted in an increase in Cu content in japanese barberry and gold tassel. However, there was a decrease in boxwood at 200 µM Zn. Cd and Zn doses decreased the Zn content in japanese barberry and boxwood at the dose of 25 µM Cd but increased in other doses. However, Zn content of the gold tassel at 25 µM Cd was higher than the control. Given the species, Cd content was higher than the control in all the treatment groups. Especially the dose of 50 µM Cd yielded four-fold increase in japanese barberry.

Table 4. Changes in Mn, Fe, Co, Ni, Cu, Cd, and Zn (ppm) contents of the leaves of japanese barberry, boxwood, and gold tassel under Cd and Zn stress

Species	Group	Mn	Fe	Co	Ni	Cu	Cd	Zn
Japanese barberry	Control	63.9±0.5^{f*}	1226±3.0^{h}	9.5±1.4^{c}	11.6±0.3^{c}	5.2±0.3^{e}	3.2±0.3e	22.7±0.3^{e}
	25 µM Cd	89.7±0.6^{b}	3650±6.0^{a}	10.9±1.4^{b}	19.0±0.3^{a}	8.4±0.3^{d}	13.2±0.5^{b}	22.2±0.3^{e}
	50 µM Cd	74.4±0.5^{d}	2269±4.0^{d}	7.6±1.3^{d}	14.1±0.24	7.5±0.3^{d}	14.2±0.5^{b}	35.7±0.3^{b}
	200 µM Zn	90.1±0.6^{b}	2818±5.0^{c}	10.5±1.5^{c}	16.4±0.3^{b}	8.2±0.3^{d}	4.3±0.4^{d}	37.6±0.3^{b}
	400 µM Zn	91.5±0.6^{b}	3413±6.0^{b}	13.6±1.6^{b}	16.3±0.3^{b}	7.9±0.3^{d}	5.2±0.4^{c}	35.3±0.3^{b}
Boxwood	Control	79.6±3^{d}	1112±0.5^{ı}	8.8±1.4^{d}	13.8±0.3^{c}	6.7±0.3^{e}	3.0±0.3^{e}	18.9±0.2^{f}
	25 µM Cd	88.8±0.6^{b}	2090±4.0^{e}	12.5±1.7^{b}	15.3±0.3^{b}	6.9±0.3^{e}	11.4±0.5^{b}	17.4±0.2^{f}
	50 µM Cd	93.6±0.6^{b}	1755±4.0^{f}	10.5±1.6^{c}	15.5±0.3^{b}	8.2±0.3^{d}	30.3±0.5^{a}	26.1±0.3^{d}
	200 µM Zn	85.2±0.5^{c}	1415±3.0^{g}	6.3±12^{e}	14.4±0.3^{b}	6.8±0.3^{e}	5.7±0.4^{c}	31.4±0.3^{c}
	400 µM Zn	78.7±0.5^{d}	1385±3.0^{g}	7.1±1.3^{d}	13.9±0.3^{c}	5.6±0.3^{e}	3.8±0.4^{d}	24.7±0.3^{d}
Gold tassel	Control	53.0±0.4.0^{g}	605.8±2.2^{i}	9.6±1.5^{c}	12.3±0.3^{c}	13.8±0.3^{b}	3.0±0.3^{e}	24.6±0.3^{d}
	25 µM Cd	60.5±0.5^{f}	539.8±2.1^{j}	6.9±1.2^{d}	12.3±0.3^{c}	11.0±0.3^{c}	3.4±0.4^{d}	25.7±0.3^{d}
	50 µM Cd	68.4±0.5^{e}	608.4±2.2^{i}	5.9±1.1^{e}	13.1±0.3^{c}	13.2±0.3^{b}	3.9±0.4^{d}	22.9±0.3^{e}
	200 µM Zn	62.6±0.5^{f}	507±2.0^{j}	11.5±1.6^{b}	11.7±0.3^{c}	12.1±0.3^{b}	3.5±0.4^{d}	20.0±0.2^{f}
	400 µM Zn	52.7±0.4^{g}	543.6±2.1^{j}	8.7±1.4^{d}	12.5±0.3^{c}	14.6±0.3^{b}	3.1±0.3^{e}	23.3±0.3^{e}
Soil		460.5±2.0^{a}	34960±30^{b}	33.8±3.8^{a}	19.88±0.3^{a}	36.8±0.7a	3.45±0.3^{d}	71.0±0.7^{a}
F		1996904.146	2532200.378	107899.665	23531.404	230174.122	469285.63	153249.26
P		0.000	0.000	0.000	0.000	0.000	0.000	0.000

*Data are mean ± standar error (n=3). Values within the column, followed by the same letter(s), are not significantly different according to Tukey's test ($P < 0.05$)

Considering the effects of Cd and Zn treatments on the Na and Cl elements, it was determined that Na content decreased in all treatment groups in comparison to the control in all plants, except for 400 µM Zn dose (japanese barberry). On the other hand, Cl content was found to increase in all the treatment groups, except for 25 µM Cd dose (japanese barberry and boxwood) (Table 5). Al, Cr, and Ti contents increased compared to the control in all the treatments in japanese barberry and boxwood (Table 4). In japanese barberry, in comparison to the control, Al and Cr were found to decrease with all treatments, whereas Ti increased by only 50 µM Cd (Table 5). The recorded values of Al, Cr, Mn, Fe, Co, Ni, Cu, Zn, and Ti concentrations in the three species were agreed with the early investigation. Many studies have shown that the ten most important in atmospheric heavy metal pollution arising from heavy traffic, fossil fuels, steel and iron industry, and mining are Fe, Al, Zn, Cd, Cr, Ti, Mn, Cu, V, Ni, Cr (Dobrikova et al., 2021; Turfan et al., 2021). Dadea et al. (2016) determined that Cd, Cu, Mn, and Zn concentrations in some woody plants, growing under polluted areas increased compared to the unpolluted sites. Turkyılmaz et al. (2018) similarly determined that the dominantly accumulated metals in the rings of *Acer platanoides* grown in polluted areas were Al, Fe, Mn, Zn, Ni, Cd, and Co. In another study, it has been shown that Cr, Mn, Fe, Co, Ni, Cu, Zn, and Pb concentrations were at the highest level in the leaves of the six trees grown in the polluted area (Turfan et al., 2021).

Table 5. Changes in Na, Cl, A, Cr, and Ti contents of the leaves of japanese barberry, boxwood, and gold tassel under Cd and Zn stress

Species	Group	Na	Cl	Al	Cr	Ti
Japanese barberry	Control	3480±210^{c*}	3629±3.0^{g}	4518±14^{f}	8.1±0.2^{g}	158.4±1.4^{f}
	25 µM Cd	3120±220^{d}	3579±3.0^{g}	9655±21^{b}	30.0±0.4^{b}	404.0±2.3^{b}
	50 µM Cd	2730±210^{e}	3945±3.0^{f}	6422±17	14.4±0.3^{e}	268.9±1.9^{d}
	200 µM Zn	1900±220^{g}	3934±3.0^{f}	6253±17	24.8±0.3^{c}	300.3±2.1^{c}
	400 µM Zn	3620±220^{b}	4333±3.0^{e}	8550±20^{c}	21.6±0.3^{d}	426.4±2.2^{b}
Boxwood	Control	2230±250^{f}	6894±5.0^{c}	3156±13^{h}	6.5±0.2^{h}	113.0±1.1$^{\imath}$
	25 µM Cd	1570±240$^{\imath}$	6594±5.0^{d}	5288±17^{d}	9.6±0.2^{f}	198.8±1.5^{d}
	50 µM Cd	1860±260^{g}	8977±6.0^{a}	4176±15^{g}	7.8±0.2^{g}	173.0±1.3^{e}
	200 µM Zn	1540±250$^{\imath}$	8072±5.0^{a}	4172±15^{g}	7.6±0.2^{g}	122.9±1.3^{h}
	400 µM Zn	1720±240^{h}	7563±5.0^{b}	4784±15^{e}	7.4±0.2^{g}	149.0±1.2^{g}
Gold tassel	Control	710±160^{i}	703.3±1.0^{i}	2179±9$^{\imath}$	5.9±0.2^{h}	100.3±1.1^{i}
	25 µM Cd	410±170^{k}	823.9±1.1$^{\imath}$	1892±9^{j}	5.2±0.1$^{\imath}$	88.9±1.0^{j}
	50 µM Cd	620±170^{j}	1068±1.0^{h}	2039±9^{i}	5.0±0.1$^{\imath}$	100.5±1.1^{i}
	200 µM Zn	420±170^{k}	859.9±1.2$^{\imath}$	2064±9^{i}	5.4±0.1^{h}	81.9±1.0^{k}
	400 µM Zn	390±170^{k}	783.1±1.1^{i}	1572±8^{k}	5.2±40.1$^{\imath}$	90.9±1.0^{j}
Soil		9600±200^{a}	6977±60^{c}	65180±70^{a}	127.8±1.3^{a}	3746±10^{a}
F		1955420.668	15770304.06	5229942.238	114476.203	558096.166
P		0.000	0.000	0.000	0.000	0.000

*Data are mean ± standar error (n=3). Values within the column, followed by the same letter(s), are not significantly different according to Tukey's test ($P < 0.05$)

It has been reported that although Fe, Zn and Cu are low mobility and Mn is an immobile element, they are essential minerals in metabolic, and physiological processes and can be found in high amounts in leaf tissue (Maillard et al., 2015; Muradolu et al., 2020). It is well documented that tolerant genotypes can accumulate more heavy metals in their tissues without any damage, and the concentrations of As, I, Sn, and Cr in trees are generally lower than that of Fe, Mn, Al, Zn, Ni, and Cu (Bernardini et a., 2016; Zhang et al., 2017).

CONCLUSION

In the present study, the effects of Cd (25-50 µM) and Zn (200-400 µM) treatments on some bioactive compounds and minerals in japanese barberry, boxwood, and gold tassel plants were examined. The sorting of species in terms of chlorophyll molecules was Japanese barberry, Boxwood, and Gold tassel. Considering the raito of chlorophyll a: chlorophyll b ratio, the same sorting was Gold tassel, Boxwood, and Japanese barberry. The sorting of species by total phenolic, anthocyanin, proline, and nitrate contents was gold tassel, boxwood, and japanese barberry. Considering all the bioactive components examined here, japanese barberry tolerated 200 µM Zn, boxwood tolerated 25 µM Cd and 400 µM Zn, and gold tassel tolerated 50 µM Cd. Given the effects of treatments on the nutrient elements in species, the macronutrient elements found in leaves at the most were Ca, K, Mg, P, and S. Mg, P, S, Al, Cr, Mn, Fe, Ni, Cu, Cd, and Ti contents showed an increase in japanese barberry leaves in all the treatments. In contrast, K, Al, Mn, Fe, Ni, Cd, and Ti increased in boxwood leaves and P, S, K, Cl. However, Cd contents increased in gold tassel. Among the metals used, Cd increased together with increasing concentration in all three species, but Cd accumulation in gold tassel leaves was found to be lower than in the other two species. Zn accumulation increased depending on the concentration. Besides that, compared to the control, Zn content in japanese barberry and boxwood leaves decreased at 25 µM Cd dose, whereas it increased in gold tassel leaves at this dose but decreased at other doses. Given all the results, it can be stated that japanese barberry was tolerant to 200 µM Zn and 50 µM Cd doses and Boxwood was tolerant to 400 µM Zn dose, and that both of these plants can be effectively used in urban parks, gardens, and roadside planting projects. Additionally, using these species in highly polluted areas might be effective in air pollution studies.

Conflict of Interest

The article authors declare that there is no conflict of interest between them.

Author's Contributions

The authors declare that they have contributed equally to the article.

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