



Response to stress and allergen production caused by metal ions (Ni, Cu and Zn) in oregano (*Origanum vulgare* L.) plants

Kamila Kulbat-Warycha^a, Egli C. Georgiadou^b, Dorota Mańkowska^c, Beata Smolińska^c, Vasileios Fotopoulos^b, Joanna Leszczyńska^{c,*}

^a Institute of Technology and Food Analysis, Faculty of Biotechnology and Food Sciences, Lodz University of Technology, Poland

^b Department of Agricultural Sciences, Biotechnology and Food Science, Cyprus University of Technology, Limassol, Cyprus

^c Institute of Natural Products and Cosmetics, Faculty of Biotechnology and Food Sciences, Lodz University of Technology, Poland

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ABSTRACT

Heavy metals are the cause of one of the most significant biosphere contamination problems worldwide, as they can be highly reactive and toxic according to their oxidation levels. Their toxic effects are correlated with the elevated production of reactive oxygen species (ROS) and oxidative cellular damage occurring in plants. The aim of the present study was the investigation of the effects of three heavy metals (Ni, Cu, Zn) applied to the soil in biochemical defense-related responses and allergen production in the aromatic plant oregano (*Origanum vulgare* L.) from the Lamiaceae family. The concentrations of the three heavy metals used, were based on the 2002 Regulation of the Polish Ministry of the Environment on Soil Quality Standards [(i) agricultural land (group B): Ni 100 ppm, Ni 210 ppm, Cu 200 ppm, Cu 500 ppm, Zn 720 ppm and (ii) industrial land (group C): Ni 500 ppm, Cu 1000 ppm, Zn 1500 ppm, Zn 3000 ppm]. The investigated plants accumulated heavy metal ions in aerial parts to a variable extent. For plants grown in soil contaminated with Zn, phenotypic representation of the growth and development were strongly limited and dependent on zinc concentration. Phenotypic representation of plants grown in soil contaminated with Ni and Cu were characterized by normal growth, slightly lower or equal to that of the control plants. All tested metals (Ni, Cu, Zn) caused a concentration-dependent decrease in photosynthetic pigments especially in total chlorophyll content. Highest cellular damage levels were observed in plants treated with Cu and Zn. Increasing concentration of these metals (especially Zn) caused a further increase in cellular damage. 3000 ppm Zn caused highest increase in the concentration of proline compared with control plants, suggesting osmotic stress imposition. Treatment with 1000 ppm Cu led to increased concentration of the allergenic protein profilin in relation to control plants by profilin ELISA analysis, while increasing concentrations of Cu and Zn led to a decrease in the concentration of phenolic compounds and total antioxidant capacity. On the basis of these findings, Ni stress in oregano plants appears to be less damaging (in relation to Cu and Zn) and with lower allergenic potential, compared with 1000 ppm Cu. The present study provides novel biochemical insight in the defense and allergenic response of aromatic plants to metal ions present in the rhizosphere; however, more comprehensive research under realistic field conditions is needed to fully decipher this interaction.

1. Introduction

Aromatic plants of the *Lamiaceae* family are commonly used as culinary herbs, as well as additives for cosmetics and pharmaceutical products (Bauer Petrovska, 2012). A growing number of people reach for food without artificial colors and preservatives, and also draw attention to natural ingredients that increase the taste and aroma of food. Nowadays, there is growing interest in functional foods which

contain biologically active components that provide health benefits. However, in the face of increasing distribution of heavy metal ions, it seems important to learn more about the impact of excessive concentration of heavy metals in aromatic plants which are commonly used as a source of biologically active compounds.

Heavy metal pollution is one of the most important environmental problems all over the world (Vardhana et al. (2019). Transition metal ions such as nickel, copper and zinc at high concentrations can be highly

* Corresponding author.

E-mail address: joanna.leszczyńska@p.lodz.pl (J. Leszczyńska).

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reactive and toxic to living cells. Their harmful effects are mainly associated with the increased production of reactive oxygen species (ROS) leading to plant cell damage (Bhaduri and Fulekar, 2012). Contamination of soil intended for cultivation, located near large industrial areas is particularly dangerous. Due to the possibility of migration in the air, soil and water, as well as the accumulation in living organisms, the presence of heavy metals in the environment is a serious problem for both natural ecosystems as well as for human health.

Heavy metals Ni, Cu and Zn belonging to microelements are necessary for the proper growth and development of plants. However, too high concentration of these metals in the substrate results in increased uptake and accumulation in the tissues of aerial organs leading to toxic effects. According to most researchers, metal accumulation in plant tissues increases with raising concentration in the substrate; however, the degree and rate of accumulation of individual metals varies depending on the plant species and growth stage (Nouri et al., 2009). Abiotic stress related to excessive concentration of transition metal ions, leads to a significant overproduction of ROS, in particular highly reactive hydroxyl radical (mainly produced in the Fenton reaction). High concentration of ROS may result in significant oxidative damage to cell structures (Hasanuzzaman et al., 2020). The best known example of a free radical reaction leading to the destruction of cell components is the peroxidation of membrane lipids. The highly mutagenic malondialdehyde (MDA) is considered a major biochemical marker of peroxidative damage to biological membranes. Moreover, heavy metals can interfere with basic metabolic processes by blocking functional groups of enzymes, as well as substituting the necessary metal ions in protein molecules (Ghori et al., 2019).

Both excess concentration of copper and nickel results in the formation of ROS that affect the activity of enzymes involved in their metabolism (Yruea, 2005; Kumar et al., 2007). According to Sheoran et al. (1990), nickel ions can inactivate many plant enzymes, including those involved in the Calvin cycle. Similarly, excessive uptake and accumulation of copper in plant tissues leads to inactivation of many enzymes involved in basic metabolic processes such as photosynthesis, cellular respiration and cell-wall metabolism (Cuypers et al., 2011; Mostofa and Fujita (2013)). Plants affected by high concentration of zinc or copper may show symptoms of chlorosis. Chlorophyll content decreases most likely due to the inhibitory effect of metals on the activity of enzymes involved in the biosynthesis of chlorophyll (Zengin and Munzuroglu, 2005). Copper as well as cadmium and mercury were also found to significantly reduce proteins building small and large subunits of RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase), both via suppression of biosynthesis and fragmentation (Hajduch et al., 2001). Other research has shown the enzyme RuBisCO to be degraded after exposure of *Elsholtzia splendens* (*Lamiaceae* family member) to excessive copper concentration (Li et al., 2009). Additionally, inhibition of photosynthesis may be caused by substitution of the central Mg^{2+} ion in the chlorophyll molecule. Zn^{2+} may also substitute Mg^{2+} ions in the enzyme RuBisCO inhibiting carbon fixation in photosynthesis (Ghori et al., 2019).

Similarly, soil contamination with heavy metal ions has an impact on the protein profile of plants, mainly proteins involved in defense against oxidative stress, metal ion chelation, gene expression, photosynthesis and, indirectly, other metabolic processes.

Some of the plant proteins show allergenic potential - inducing various allergic reactions in sensitive people. There is no unambiguous description in the literature of the influence of environmental pollution on plant allergenicity. It is known, however, that treatment of plants with defense stimulating salicylic acid or ethylene increases their allergenicity, mostly by inducing biosynthesis of pathogenesis-related (PR) proteins (Midoro-Horiuti et al., 2001). Despite the information in the literature on the increase in pollen allergies in heavily contaminated areas (Aina et al., 2010; Chehregani et al., 2004), there is still not enough information about the impact of environmental pollution on the allergenicity of culinary herbs. The presence of excess copper ions in

plant cells may lead to changes in the cell walls and increase their permeability, which may result in increased release of proteins from cells (Fry et al., 2002). A similar mechanism may potentially be responsible for the increase in plant allergenicity caused by soil contamination with other heavy metals. Pollen grains of a popular grass *Poa annua* L. released a higher amount of allergens when growing in soil contaminated with cadmium ions (Aina et al., 2010). Qualitative changes in protein profile were detected in leaves from rice (*Oryza sativa* L.) after treatment with copper, cadmium, and mercury. Among others the class 5 PR proteins and superoxide dismutase (SOD) have been isolated, indicating that PR proteins play a role not only in pathogen infection but also in abiotic stress (Hajduch et al., 2001). Some PR5 (Thaumatococcus-like proteins) exert allergenic properties (Breiteneder, 2004).

The aim of this study was to investigate the effect of abiotic stress caused by the presence of specific heavy metal ions in the soil (Ni^{2+} , Cu^{2+} and Zn^{2+}) on defense responses in the aerial parts of oregano (*Origanum vulgare* L.). A research hypothesis was therefore postulated, whereby variable concentration of the selected heavy metals may affect the content of biologically active compounds involved in neutralizing the negative effects of abiotic stress, and may also lead to quantitative and qualitative changes in plant proteins, including allergenic proteins.

2. Materials and methods

2.1. Heavy metals

Ni, Cu, and Zn, were applied in the soil, each one in three concentrations. The Regulation of the Polish Minister of the Environment on the Soil Quality Standards regarding soil and ground quality standards from 9th September 2002 was used to choose the metal concentrations. The lowest used concentrations relate to the highest concentrations permitted in group B land or land classified as agricultural (except land under ponds and ditches), forest land and wooded shrubs, wasteland, as well as built-up and urbanized areas (industrial, fossil and communication/trafficked lands were exempted), while the highest are the maximum allowable concentrations for a group C land or the industrial, fossil and communication/trafficked lands. The concentrations used for the three heavy metals were: [(i) group B: Ni 100 ppm, Ni 210 ppm, Cu 200 ppm, Cu 500 ppm, Zn 720 ppm and (ii) group C: Ni 500 ppm, Cu 1000 ppm, Zn 1500 ppm, Zn 3000 ppm].

2.2. Plant material and growth conditions

A suitable dry weight of the soil mixed with perlite (ratio 3:1) (120 g per pot) was combined with a solution of salts of heavy metals ($Ni(CH_3COO)_2 \cdot 4H_2O$, $Cu(CH_3COO)_2 \cdot H_2O$, $Zn(CH_3COO)_2 \cdot 2H_2O$) to obtain their respective ion concentrations (Ni 100, 210 and 500 ppm; Cu 200, 500, 1000 ppm; and Zn 720, 1500, 3000 ppm). Contaminated soil was left for 3 days, in order to bond the heavy metals with the matrix of the soil, and was subsequently properly hydrated. Oregano seeds (40 per pot) were sown in the top layer of the contaminated or control soil medium. Four pots per replication per treatment were incubated for 4 days in the dark at 4 °C to initiate the germination process (Figure 1). After incubation at low temperature, plants were transferred to a growth room, where average temperature reached 23.2 ± 1.9 °C, average humidity was 55 ± 10 % RH, average illuminance was 1800 lx and the photoperiod day/night was 16/8 h. The day of placing the plants in the growth room was considered the first day of cultivation. After 40 days of cultivation (Figure 1) all leaves were collected (except cotyledons) from the aerial parts of oregano plants, homogenized in liquid nitrogen and then stored at -80 °C until use. Analyses were carried out using three independent biological replicates. Before analyzing total protein content, metal content and performing profilin ELISA analysis, plant material was freeze-dried using a Christ Alpha 2-4 LD Freeze Dryer and then stored at room temperature.

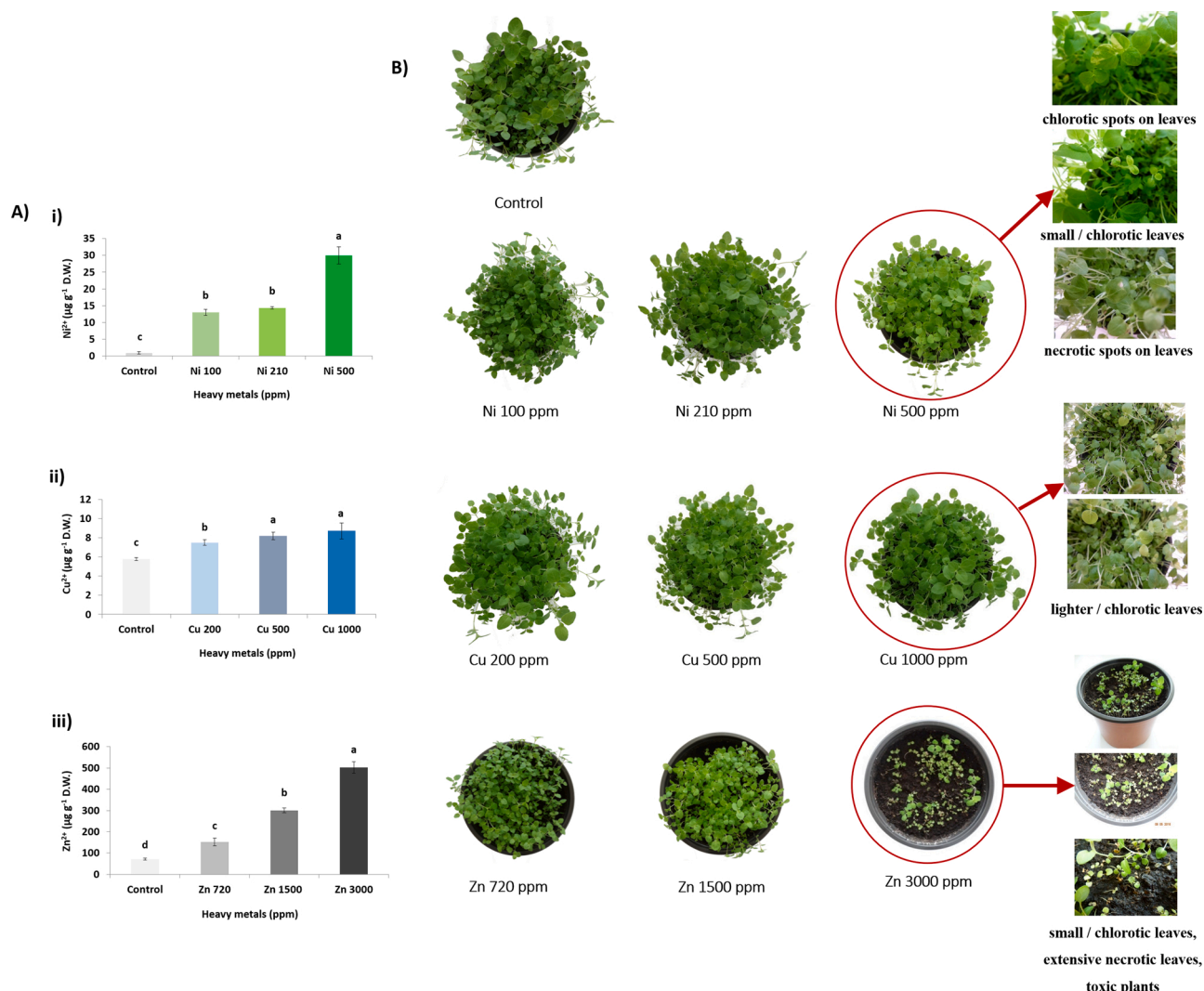


Fig. 1. A) Concentrations of Ni, Cu, Zn in oregano leaves in control plants and plants exposed to three concentrations of heavy metals Ni, Cu, Zn, respectively. Data are means $\pm$ SE of three replications. Bars with different letters are significantly different at $P \leq 0.05$. B) Phenotypic representation of the aromatic plant *Oreganum vulgare* L. treated with heavy metals (Ni, Cu, Zn) at three concentrations (including control plants) at 40 days (harvest day).

2.3. Determination of metal content

Determination of the concentration of selected metals in oregano leaves was carried out through their mineralization in 65 % nitric acid using a microwave mineralizer (Berghof Speedwave2, Eningen Niemcy, # 5305000), followed by the analysis of samples using the ICP-MS (Inductively coupled plasma - mass spectrometry) technique (Berghof Speedwave2, Eningen Germany, # 5305000).

2.4. Photosynthetic pigment analysis

Determination of the level of photosynthetic pigments was performed according to protocol of Richardson et al. (2002). Five leaf disks (approximately 100 mg/mL) per sample were incubated in 5 mL DMSO for 30 min at 65 °C. Absorbance was measured at 661, 643, 470, and 534 nm (TECAN, Infinite 200® PRO). The content of chlorophylls (Chla, Chlb and total) and carotenoids were determined using the equations described by Misra and Dey (2013), while anthocyanin concentration (non-photosynthetic pigment) was determined using the equations described by Nikiforou et al. (2014).

2.5. Lipid peroxidation quantification

The extent of lipid peroxidation was determined by measuring the concentration of the main lipid peroxidation product - malondialdehyde (MDA) formed with thiobarbituric acid (TBA) colored complex (thiobarbituric acid-reactive substances, TBARS). The concentration of the final product was examined spectrophotometrically at 532 and 600 nm (TECAN, Infinite 200® PRO) and estimated using the Lambert-Beer law, with extinction coefficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$ and expressed as nmol MDA g^{-1} fresh weight (F.W.) (Filippou et al., 2011).

2.5.1. Proline content quantification

Accumulation of proline in plant tissues is recognized as a universal marker of osmotic stress. Proline content was determined according to the modified method proposed by Bates et al. (1973). The basis of the assay is the reaction of proline with ninhydrin, which results in a red colored complex at low pH with the maximum absorption at 520 nm (TECAN, Infinite 200® PRO) and was estimated using a standard curve and calculated on a fresh weight (F.W.) basis (μmol proline g^{-1} F.W.) (Filippou et al., 2011).

2.5.2. Determination of total antioxidant capacity

Two analytical techniques (ferric reducing/antioxidant power

(FRAP) and phosphomolybdenum) were used to determine total antioxidant capacity. The two methods used the same methanol extract and are analytically described by Georgiadou et al. (2018). The principle of the FRAP method is based on the ability of antioxidants to reduce Fe^{3+} ions to Fe^{2+} ions, which are complexed by TPTZ (2,4,6-tripyridyl-1,3,5-triazine). The reaction results in a blue product with a maximum absorption at 593 nm (TECAN, Infinite 200® PRO). Standard curve was prepared for L-ascorbic acid and results were expressed as μmol ascorbic acid g^{-1} F.W. (Georgiadou et al., 2018). The phosphomolybdenum method is based on the reduction of Mo (VI) to Mo (V). As a result of molybdenum reduction, at low pH, green phosphate complexes are formed, absorbance was measured at 695 nm (TECAN, Infinite 200® PRO). Standard curve was prepared for L-ascorbic acid and results were expressed as μmol ascorbic acid g^{-1} F.W. (Georgiadou et al., 2018).

2.6. Determination of total phenolic content

Total phenolic content was determined by using Folin-Ciocalteu method according to Singleton and Rossi (1965). Absorbance was measured at 725 nm (TECAN, Infinite 200® PRO). The content of phenolic compounds was calculated using standard curve prepared for gallic acid and expressed as mg gallic acid g^{-1} F.W.

2.7. Protein analysis

For analyzing total protein content and profilin ELISA analysis, oregano samples were extracted with Tris-glycine buffer (T-G, pH = 8.3) composed of 0.05 M Tris and 0.33 M glycine. For this purpose, 0.05 g of dried plant tissue was homogenized in 3 mL of T-G buffer and incubated on a laboratory shaker (TTS 2, Yellow Line, IKA - Werke GmbH & Co. KG, Staufen, Germany) for 2 h. To prevent proteolytic degradation of proteins, 2 μL of protease inhibitor was added to the extracts. Samples were then centrifuged for 10 min (4000 rpm, 4 °C) and then the supernatants were transferred into new tubes and stored until analysis at -20 °C.

2.8. Determination of total protein content

Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher, 23225) following the manufacturer's instructions. Absorbance was measured at 545 nm using a Multiscan RC Reader (Thermo Labsystems, FL). Protein concentration was calculated based on a bovine serum albumin (BSA) as a standard and expressed in mg g^{-1} dry weight (D.W.).

2.9. Profilin ELISA analysis

To assess the allergenic potential of oregano samples, profilin content was measured by indirect ELISA method (Enzyme-Linked Immunosorbent Assay), as previously described by Georgiadou et al. (2018). The immunoreactivity was normalized relative to the content of profilin from peach (Słowianek et al., 2016).

2.10. Statistical analysis

Statistical analysis was carried out using the software package SPSS v17.0 (SPSS Inc., Chicago, IL, United States) and the comparison of averages of each treatment was based on the analysis of variance (One-Way ANOVA) according to Duncan's multiple range test at significance level 5% ($P \leq 0.05$).

3. Results and discussion

3.1. Plant growth and toxicity symptoms

Oregano plants grown on soil contaminated with nickel ions were characterized by normal growth, similar to or slightly decreased

compared with control plants. A noticeable feature of plants grown in soil contaminated with nickel was the formation of finer leaves compared with control plants. The highest concentration of nickel (Ni 500 ppm) resulted in visible, chlorotic white spots on single leaves. For some plants, symptoms like leaves curling, small leaves and necrosis were observed (Figure 1). Growth of plants grown in soil contaminated with copper ions was comparable to the dynamics of growth and development of control plants. Plants developed larger leaf blades compared with plants grown in nickel-contaminated soil. The highest concentration of copper (Cu 1000 ppm) resulted in the appearance of bright green leaves, which could be a result of a lower chlorophyll content (Figure 1). Plants grown in soil contaminated with zinc showed negative phenotypic changes compared with control plants. The lowest zinc concentration (720 ppm) resulted in a slight inhibition of plant growth and development. Medium zinc concentration (1500 ppm) strongly inhibited the growth of oregano plants and resulted in leaf dieback. The highest tested concentration of zinc (3000 ppm) led to a drastic inhibition of plant growth at the stage of developing cotyledons or the first leaves (plants developed toxicity symptoms). Some plants contained very small chlorotic leaves, but most plants showed extensive necrosis in leaves before harvest day (40 days) (Figure 1). Similar results were observed in leaves of basil (*Ocimum basilicum* L.) plants treated with the same heavy metals as the present study (Georgiadou et al., 2018).

3.2. Accumulation of Ni, Cu, Zn

The assessment of metal content in aerial parts of the plants is an extremely important parameter, as heavy metal accumulation reflects the ability to absorb given metals and usually the plants overall ability to absorb metal ions from the soil solution. In regard with concentration of heavy metals in leaves following exogenous application, it appeared to increase with increasing concentrations of Ni, Cu and Zn in contaminated soil compared with controls in leaves of oregano plants, suggesting that successful treatment took place. More specifically, all Ni-treated plants showed significantly higher amounts of Ni in leaves of contaminated plants compared with control plants, with highest amounts of Ni being recorded in plants treated with the highest Ni concentration (500 ppm). Similarly, all Cu-treated plants showed higher amounts of Cu in leaves of contaminated plants compared with control plants, with highest amounts of Cu being recorded in plants treated with the highest Cu concentration (1000 ppm). All Zn-treated plants showed significantly higher amounts of Zn in leaves of contaminated plants compared with control plants, with highest amounts of Zn being recorded in plants treated with the highest Zn concentration (3000 ppm) (Figure 1). For oregano plants, bioaccumulation ability of metals is as follows: $\text{Zn} > \text{Ni} > \text{Cu}$ (Figure 1). Similar results were presented by Szczodrowska et al. (2016) regarding the accumulation of nickel, copper and zinc in aerial parts of plants belonging to *Lamiaceae* and *Brassicaceae* families. Mint (*Mentha piperita* L.) and basil (*Ocimum basilicum* L.) from the *Lamiaceae* family showed a strong tendency for bioaccumulation of zinc ions, while cuckooflower (*Lepidium sativum* L.) from the *Brassicaceae* family accumulated significant amounts of nickel. Similarly, Dghaim et al. (2015) noted a higher accumulation of zinc ions compared with copper in several members (basil, sage, oregano, mint, thyme) of the *Lamiaceae* family.

In addition to determination of nickel, copper and zinc concentration, magnesium and iron were examined (Figure 2). Highest concentrations of copper (500 ppm and 1000 ppm) resulted in a significant decrease in magnesium content in oregano leaves compared with control plants. Similarly, the presence of all Zn treatments caused a decrease in magnesium content. A probable explanation of this phenomenon is the possibility of replacing the central magnesium atom in the chlorophyll molecule with divalent metal cations, especially Cu^{2+} or Zn^{2+} , with the result that the overall magnesium pool may be reduced in aerial parts of plants (Küpper et al., 1998). These metal cations may also

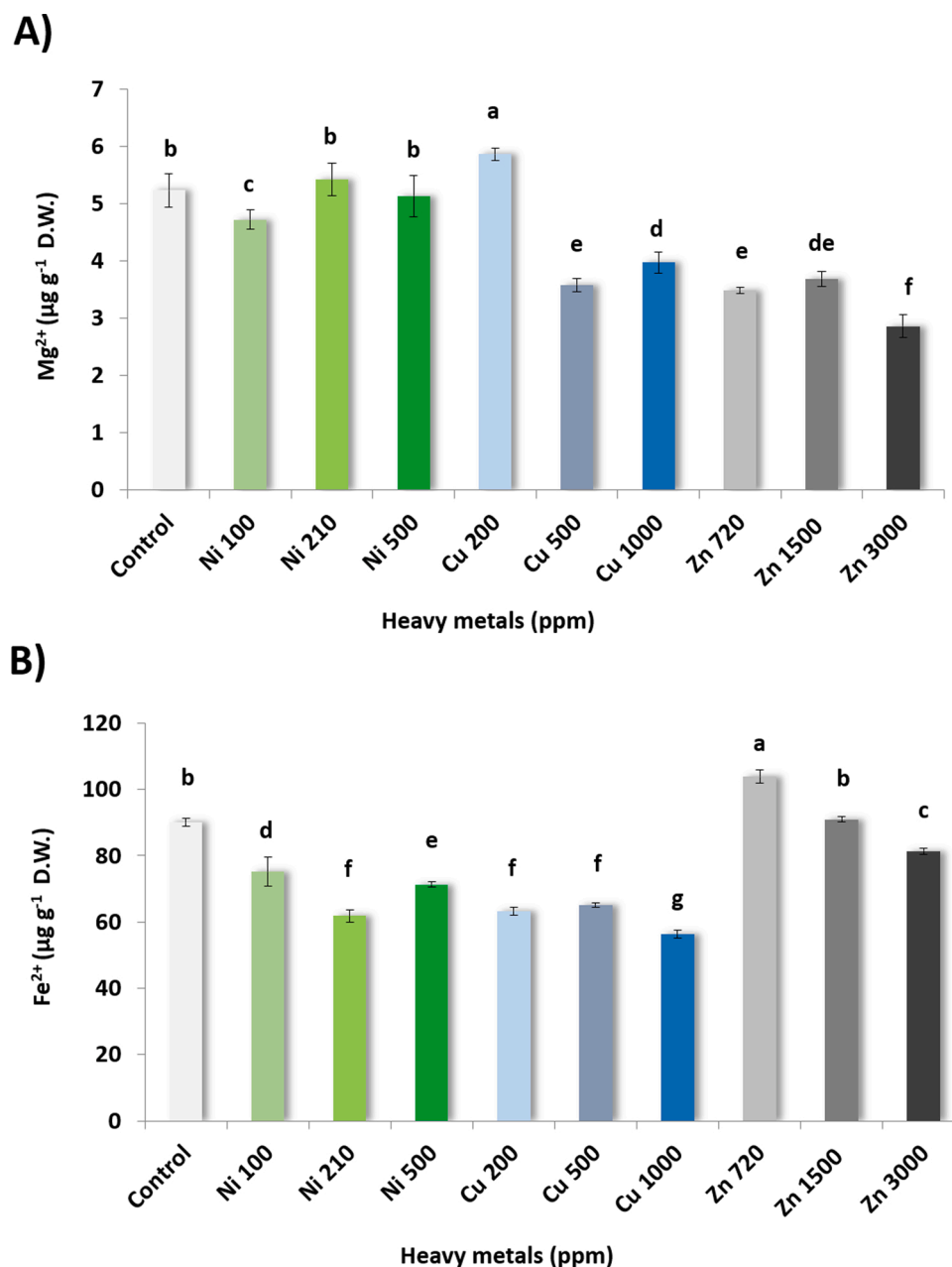


Fig. 2. Concentrations of **A)** Mg and **B)** Fe in aromatic oregano leaves exposed to different concentrations of the heavy metals (Ni, Cu, Zn) and control. Data are means $\pm$ SE of three replications. Bars with different letters are significantly different at $P \leq 0.05$.

competitively displace magnesium during ion uptake from the soil solution (Ouzounidou, 1994).

The presence of nickel, copper and zinc in the substrate affected iron content in the aerial parts of oregano plants. Iron content in plants grown on a substrate contaminated with all Ni and Cu concentrations, as well as Zn (3000 ppm) was significantly lower compared with the iron content in control plants (Figure 2). Similar results were provided by Karimi et al. (2012), who noted a decrease in the content of iron and manganese in the shoots of *Astragalus neo-mobayenii* under the influence of increasing concentrations of copper ions. Iron plays an important role in various physiological processes of plant cells. It is a component of many enzymes, such as cytochromes in an electron transport chain. In plants, iron is involved in the synthesis of chlorophyll and is necessary to maintain the structure and function of chloroplasts. For this reason, iron deficiency may result in reduced chlorophyll content, which is macroscopically visible as a lighter leaf color as well as the appearance of

white chlorotic patches (Rout and Sahoo, 2015).

3.3. Photosynthetic pigments and anthocyanins

The concentrations of total chlorophyll as well as chlorophyll *a* and *b* were significantly lower and strictly dependent on the degree of soil contamination. The most drastic decrease in chlorophyll content was observed in plants growing on zinc-contaminated soils. The downward trend concerned not only chlorophyll, but also anthocyanins and carotenoids, with the largest decrease relative to control in plants grown in soil contaminated with the highest concentration of copper (Cu 1000 ppm), as well as all tested concentrations of zinc. While a clear decrease in the content of photosynthetic pigments in plants grown in zinc-contaminated soil may be the result of general growth inhibition, the downward trend in plants exposed to nickel and copper may be the result of the inhibitory effect on chlorophyll biosynthesis. This theory is

supported by the appearance of white spots on the leaves of plants grown in soil contaminated with the highest concentration of nickel (Ni 500 ppm) and clearly lighter-color leaves of plants grown in soil contaminated with the highest concentration of copper (Cu 1000 ppm) (Figure 3).

Panou-Filotheou et al. (2001), in a study of oregano plants (*Origanum vulgare* subsp. *hirtum*) exposed to increasing concentrations of copper ions in the substrate, noted a decrease in total chlorophyll content in leaves and observed negative morphological changes in the structure of chloroplasts. Similar results were reported by Shakya et al. (2008), who conducted studies on bryophytes treated with heavy metal salts: CuCl_2 , ZnCl_2 and PbNO_3 , separately and in combinations. A significant decrease in the content of chlorophyll *a*, chlorophyll *b* and total chlorophyll was observed under the influence of copper. The presence of zinc and lead slightly reduced chlorophyll content. The largest decrease in the chlorophyll content relative to control plants was noted with the simultaneous influence of three heavy metals. Zengin and Munzuroglu (2005) observed a similar decrease in total chlorophyll content with significant increase in the content of proline, retinol, ascorbic acid and α -tocopherol in bean seedlings (*Phaseolus vulgaris* L.) after 10 days exposure to copper ions.

3.4. Lipid peroxidation levels

Excessive concentration of heavy metals can stimulate the formation

of free radicals and reactive oxygen molecules leading to severe oxidative stress (Demidchik, 2015). The best-known example of a free radical reaction leading to the destruction of cell components is peroxidation of membrane lipids, understood as the process of oxidation of unsaturated fatty acids to lipid peroxides (Puzanowska-Tarasiewicz et al., 2008). The level of oxidative stress is usually determined by measuring the content of malondialdehyde (MDA) - biochemical marker of peroxidative membrane damage (Marnett, 1999). Significantly higher MDA content was noticed in oregano plants grown in soil contaminated with the highest concentration of nickel (Ni 500 ppm), the highest concentration of copper (Cu 1000 ppm) and the two highest concentrations of zinc (Zn 1500 and 3000 ppm) (Figure 4). Existing literature shows that excessive concentrations of nickel and copper ions contribute to the induction of strong oxidative stress in plant cells, which is manifested by changes in biochemical parameters. Level of oxidative stress may be monitored by determining the concentration of reactive oxygen and nitrogen species as well as antioxidants and MDA (Yurekli and Porgali, 2006; Wang et al., 2010; Pena et al., 2011; Yusuf et al., 2012; Chen et al., 2015). According to Wang et al. (2010), excessive concentration of copper ions induces oxidative stress, difficult to neutralize by intracellular antioxidant systems, which was confirmed by a significantly higher concentration of O_2^{*-} , H_2O_2 and MDA in seedlings of *Paulownia fortunei* growing in mine soils contaminated with copper. Similarly, according to Pena et al. (2011), the presence of copper ions induces oxidative stress as a result of excessive formation of ROS and reduced catalase activity caused by

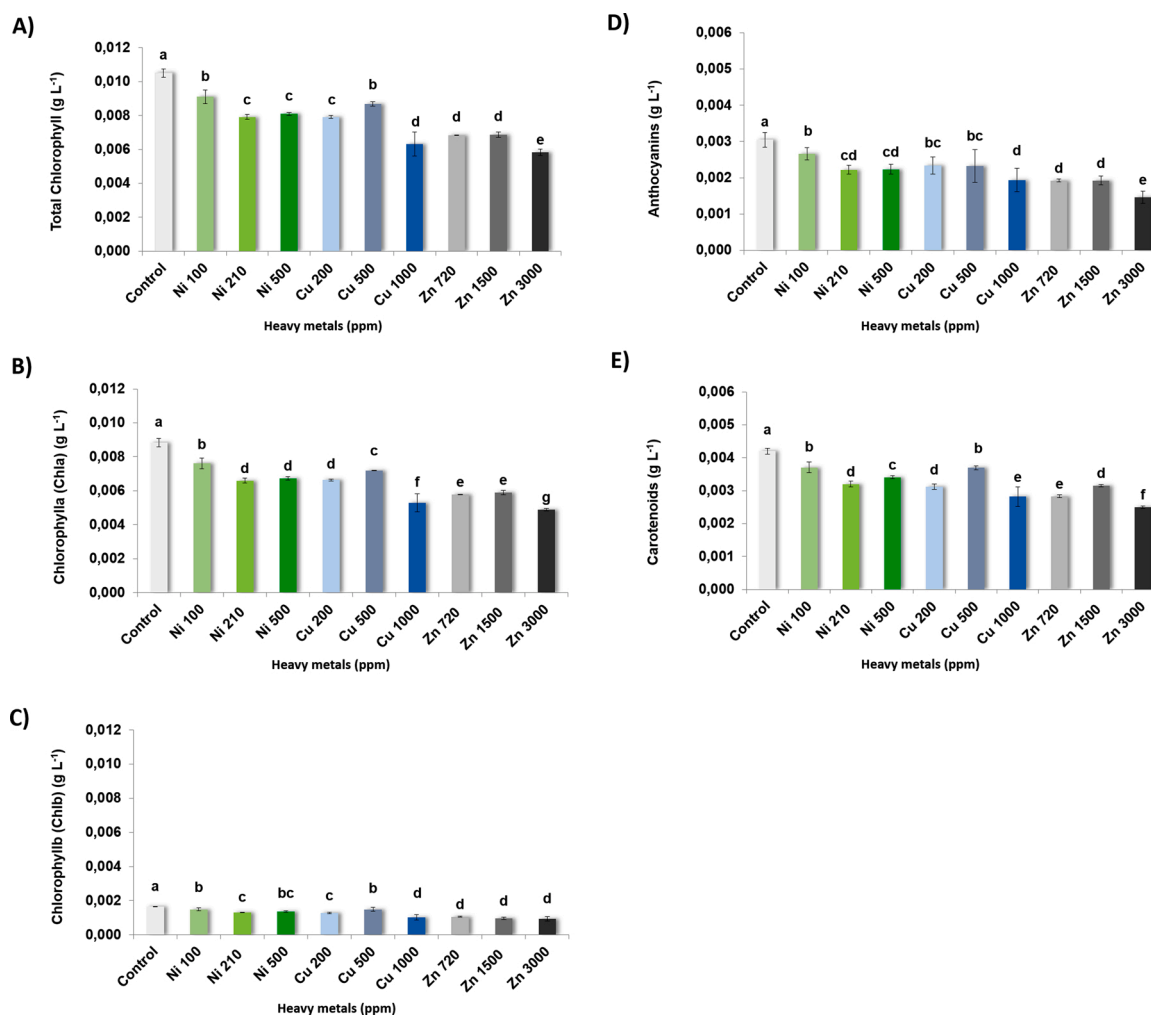


Fig. 3. Effects of heavy metals (Ni, Cu, Zn) at three concentrations (including controls), on pigment content (A - total chlorophyll, B - chlorophyll *a* (Chla), C - chlorophyll *b* (Chlb), D - anthocyanins (non-photosynthetic), E - carotenoids) of aromatic oregano leaves (data are means $\pm$ SE of three replications). Bars with different letters are significantly different at $P \leq 0.05$.

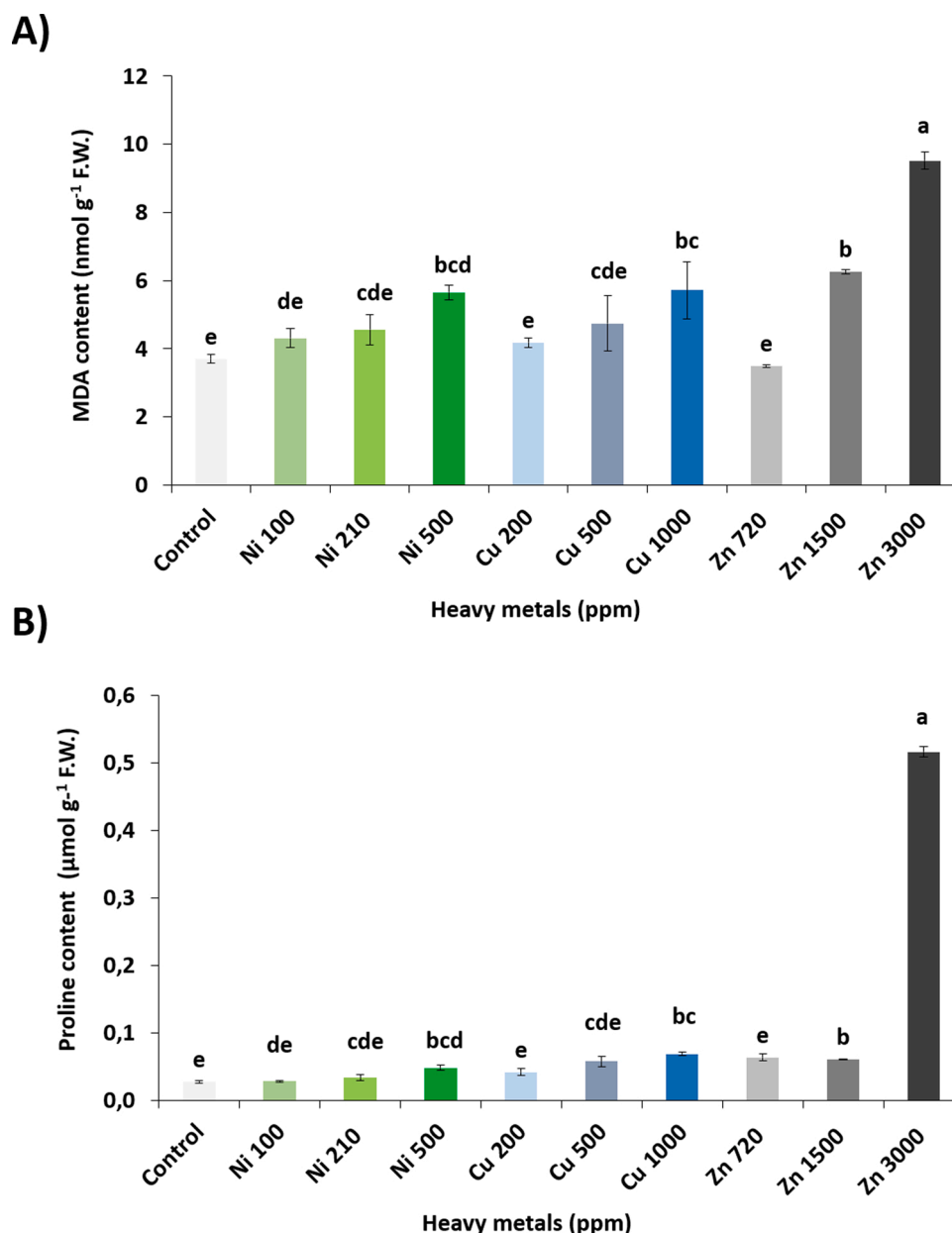


Fig. 4. Effects of heavy metals (Ni, Cu, Zn) at three concentrations (including controls), on (A) malondialdehyde (MDA) content, and (B) proline content of aromatic oregano leaves. Data are means $\pm$ SE of three replications. Bars with different letters are significantly different at $P \leq 0.05$.

oxidation of this protein. Likewise, a marked significant increase of MDA content compared with all treatments was verified in basil plants treated with Cu 1000 ppm, indicative of maximal cell membrane damage (Georgiadou et al., 2018).

3.4.1. Proline content

Under the influence of heavy metal stress, an accumulation of the essential amino acid proline (recognized as a universal marker of osmotic stress) is observed. An increase in proline concentration in plant tissues is noted as a consequence of many harmful factors, including salinity, drought, very high and low temperatures, heavy metals, UV radiation or pathogens. The increasing concentration of free proline as an osmotically active compound helps to protect cellular proteins and membranes against dehydration. Interestingly, proline reduces stress-induced acidification of the cytoplasm (Hare and Cress, 1997; Hare et al., 1998; Verbruggen and Hermans, 2008; Watanabe et al., 2000).

In oregano plants, proline content was significantly higher in plants

stressed by the highest concentration of nickel and copper ions, as well as the two highest concentrations of zinc (Zn 1500 and 3000 ppm) compared with control plants. The highest proline content (almost 19-fold increase relative to control) was observed in plants grown in soil contaminated with zinc under the highest concentration (Zn 3000 ppm) (Figure 4). Higher concentration of zinc was also found in the leaves of basil (*Ocimum basilicum* L.) plants in the concentration Zn 1500 ppm (almost 9-fold increase) as described by Georgiadou et al. (2018). Similarly, Theriappan et al. (2011) noted an increased accumulation of proline as a consequence of exposure of cauliflower seedling (*Brassica oleracea* var. botrytis) to substrate salinity (NaCl). Comparable results were observed under the presence of heavy metal salts: CdCl₂, HgCl₂ and ZnCl₂. Proline content was closely correlated with the concentration of heavy metal ions. Proline as an amphiphilic (hydrophobic-hydrophilic) amino acid can bind to hydrophobic protein moieties, creating more hydrophilic domains on their surface, allowing water molecules to bind. In this way, high proline levels contribute to maintaining the integrity

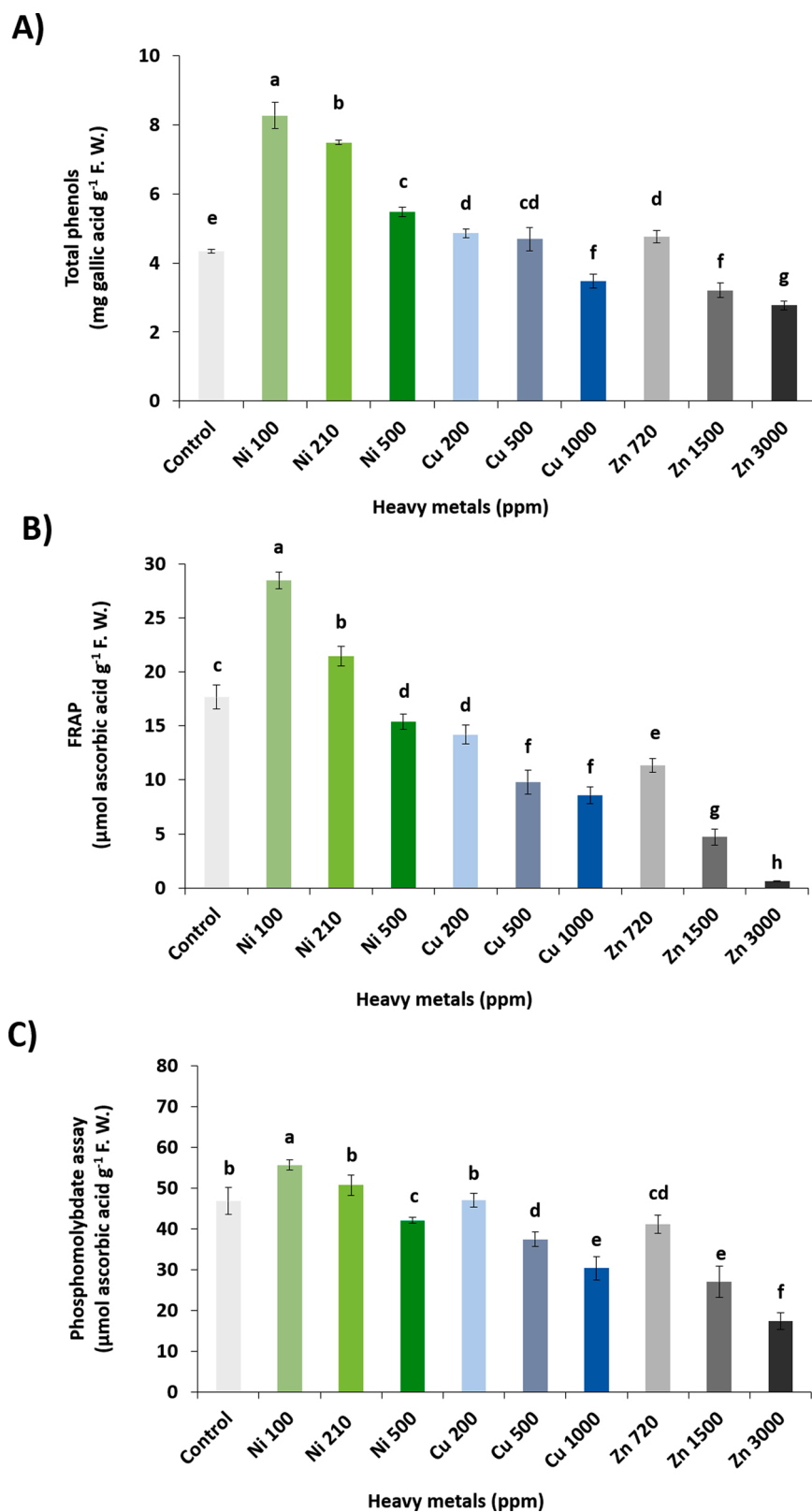


Fig. 5. Effects of heavy metals (Ni, Cu, Zn) at three concentrations (including controls), on A) Total phenolic content with Folin-Ciocalteu, B) FRAP assay and C) Phosphomolybdate assay of aromatic oregano leaves. Data are means $\pm$ SE of three replications. Bars with different letters are significantly different at $P \leq 0.05$.

and functionality of proteins (Sharmila and Saradhi, 2002).

3.5. Antioxidant capacity

The total antioxidant capacity of oregano plants measured for the lowest and medium concentration of nickel (Ni 100 ppm and 210 ppm) was higher than that in control plants. The highest antioxidant potential among all treatments was found when soil was contaminated with nickel ions at the lowest concentration (Ni 100 ppm) (Figure 5). What is worth noting is that the total antioxidant capacity of oregano plants decreases with increasing concentration of heavy metals. A similar trend was observed in the case of phenolic compounds (Figure 5). It is worth mentioning that phenolic compounds are numerous and a very diverse group of strong radical-scavenging antioxidants (Michalak, 2006). The positive correlation between the content of phenolic compounds and the total antioxidant potential suggests that phenolic compounds are a dominant group of antioxidants. Decreasing concentration of phenols in oregano plants when concentration of heavy metals increased may

indicate strong oxidative stress leading to the oxidation of phenolic compounds included in the antioxidant system. Similar results were found in leaves of thyme (*Thymus vulgaris*) treated with nickel ions (Kulbat and Leszczyńska, 2016). These findings are in line with Márquez-García et al. (2012), who noted an increase in the concentration of flavonoids and total phenolic compounds, as well as the antioxidant capacity in the tissue of the endemic species *Erica andevalensis* growing in post-mine soils contaminated with cadmium. Induction of phenolic compound biosynthesis was also described in buckwheat (*Fagopyrum esculentum*) sprayed with an aerosol containing nickel ions. Authors observed the accumulation of nickel in the aerial parts of plants, as well as a significant increase in the concentration of MDA. The total content of phenolic compounds increased depending on the dose and time of plant exposure to nickel (Syta et al., 2013). In addition to performing typical antioxidant functions, phenolic compounds are known for their ability to chelate heavy metal ions, mainly due to the presence of appropriate functional groups - hydroxyl and carboxyl. Binding of metal ions by phenolic compounds was observed in an

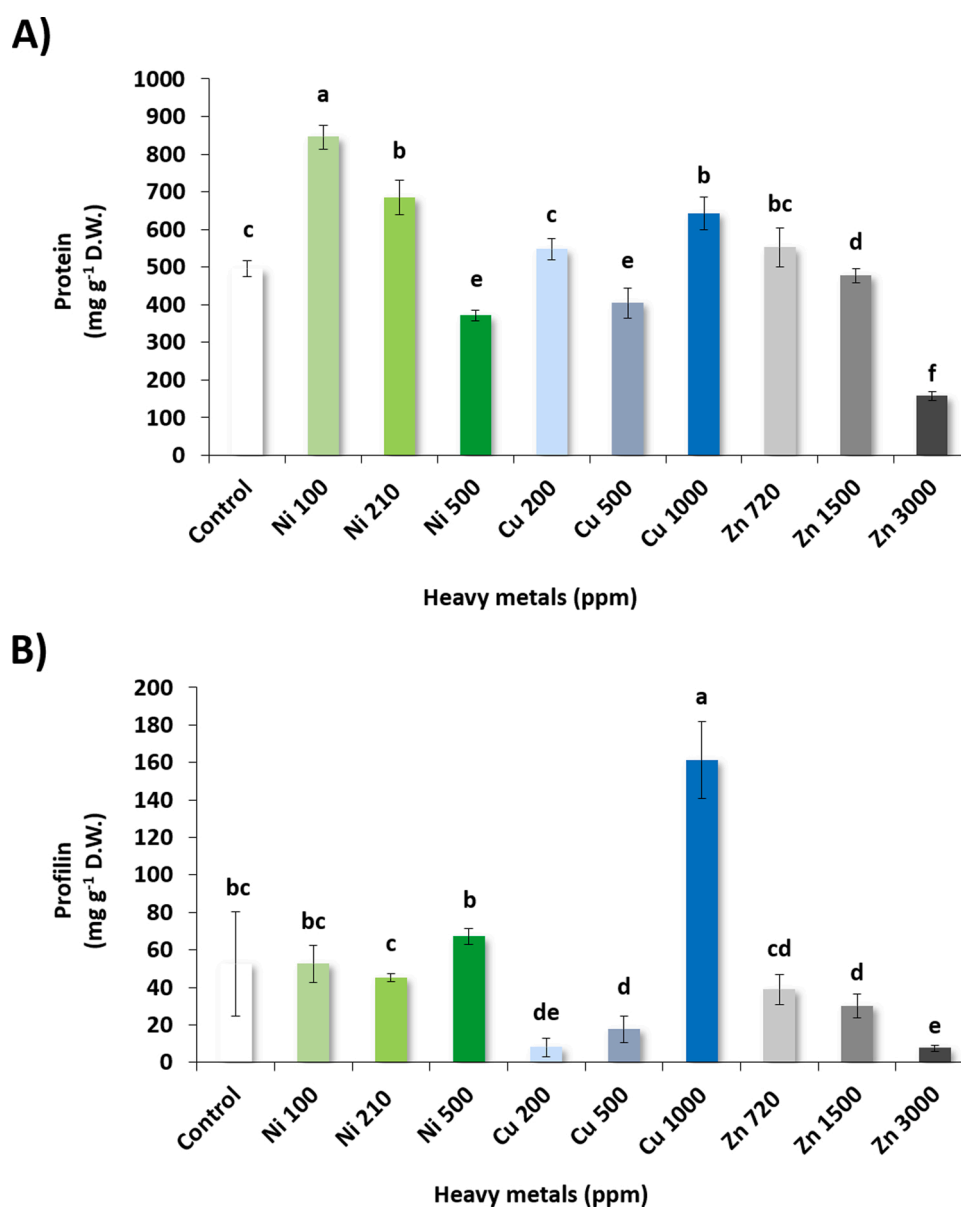


Fig. 6. Effects of heavy metals (Ni, Cu, Zn) at three concentrations (including controls), on (A) total protein quantity assayed by Pierce Protein Assay, and (B) allergenic protein profilin quantity of aromatic oregano leaves assayed by ELISA analysis. Data are means $\pm$ SE of three replications. Bars with different letters are significantly different at $P \leq 0.05$.

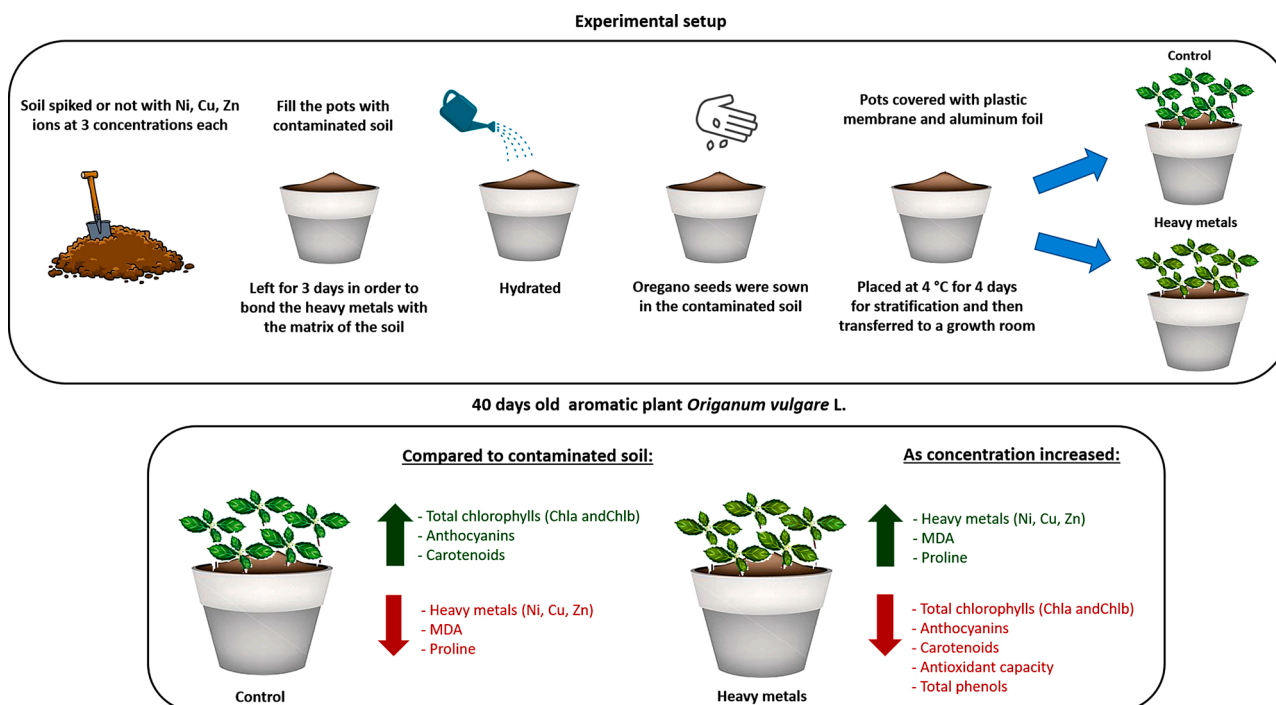


Fig. 7. Schematic overview of the current experimental approach after treatment of the aromatic plant *Origanum vulgare* L. with three heavy metals (Ni, Cu, Zn) at three concentrations, relative to control plants (discussed in detail in the text). Increase is indicated in green arrow and decrease is indicated in red arrow (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

experiment with *Nymphaea*, where heavy metal ions: Hg^{2+} , Pb^{2+} and Cr^{3+} were bound by methanolic extract of polyphenols (Lavid et al., 2001). Similarly, Wojcieszek and Ruzik (2016) demonstrated the ability of polyphenols (flavonoids and phenolic acids) to bind copper ions in the açai berry (*Euterpe oleracea* Mart.) and bilberry (*Vaccinium myrtillus* L).

3.6. Total protein and proline content

The concentration of total protein in aerial parts of oregano plants demonstrated large variation. Plants grown in soil contaminated with Ni 100 ppm, Ni 210 ppm, Cu 200 ppm, Cu 1000 ppm and Zn 720 ppm were characterized by higher content of total protein compared with control plants. In the case of Ni and Zn contamination, total protein content decreased with increasing concentration of metal ions in the soil (likely due to proteolysis). The lowest total protein content was found in oregano plants grown in soil contaminated with zinc ions in the highest concentration (Zn 3000 ppm) (Figure 6).

It is known that accumulation of metals in plant tissues results in protein profile changes. The results of the research suggest that the presence of nickel ions in the medium corresponded to the relatively high content of proteins, which may be the effect of increased synthesis of enzymatic proteins involved in the metabolism of ROS. Accumulation of nickel in the roots of *Nasturtium officinale* caused an increase in plant biomass at low concentrations of nickel, while higher concentrations of nickel ions caused its decrease. Similar relationships were observed in the case of total protein content as well as concentrations of enzymatic proteins involved in the metabolism of ROS - SOD and CAT (Duman and Ozturk (2010)). The change in protein content may also result from the synthesis of phytochelatins - peptides synthesized from glutathione or its derivatives during enzymatic reaction catalyzed by phytochelatin synthase. Phytochelatins are involved in the tolerance of plants to growing metal ions concentration. This is confirmed by studies on *Arabidopsis thaliana* mutants, with a defect in the synthesis of phytochelatins, which are hypersensitive to cadmium (Howden et al., 1995). According to Schat et al. (2002), the tendency of the studied heavy metals to activate the phytochelatins synthesis is as follows: $\text{Cu} > \text{Zn} > \text{Ni}$. Authors

emphasize that in the case of copper, a significant increase in phytochelatins synthesis occurs only at relatively high levels that interfere with the homeostasis of plant cells. Zinc and nickel are considered to be weak inducers of phytochelatin synthase. A much wider group of metal chelators are metallothioneins - low-molecular proteins, rich in cysteine. Metallothioneins, like other proteins, are formed directly in the process of translation (Cobbett and Goldsbrough, 2002). They are currently believed to play an important role in the tolerance of plants to stress induced by the presence of heavy metals, mainly copper and zinc (Cobbett and Goldsbrough, 2002; Castiglione et al., 2007). Expression of the multigene families of metallothioneins is constantly regulated depending on the developmental stage of the plant and environmental stimuli. According to some reports, metallothioneins also participate in antioxidant protection (Viarengo et al., 2000). In order to determine the content of allergenic proteins in oregano plants, ELISA tests were performed using specific anti-profilin antibodies. This study aimed to determine the relationship between the degree of soil contamination by selected heavy metals and the proline content in the aerial parts of oregano plants. Targeted allergen profilin is a small actin-binding protein (12–15 kDa) involved in reconstruction of the cytoskeleton. It is present in the majority of eukaryotic cells (Vidali et al., 2007). In addition to actin, ligands bound by profilins are membrane phospholipids (phosphatidyl-inositol phosphates) and proline-rich polypeptides present in membranes (Mahoney et al., 1999; Moens and Bagatolli, 2007). Profilin is considered as a panallergen, connecting pollen and food allergy (Alvarado et al., 2014). Allergens belonging to profilin family were identified i.a. in birch pollen (Bet v 2), pear (Pyr c 4), cherry (Pru av 4) and celery (Api g 4) and responsible for IgE cross-reactivity (Scheurer et al., 2001). According to international databases (including the official database "The WHO/IUIS Allergen Nomenclature Database" available at www.allergen.org), allergenic proteins from plants belonging to *Lamiaceae* family have not yet been characterized.

The knowledge about the impact of environmental pollution on plant allergenicity is still limited. The presence of excess copper ions in plant cells may lead to changes within the cell walls and increase their permeability. This phenomenon is explained by non-enzymatic

membrane damage triggered by hydroxyl radical formed in the Fenton reaction, which may lead to increased release of proteins from cells (including allergenic proteins) (Fry et al., 2002). It is possible that a similar mechanism is responsible for increasing plant allergenicity resulting from soil contamination by other heavy metals. Aina et al. (2010) noticed the increase of *Poa annua* pollen allergenicity growing in soil contaminated with cadmium ions. The increase in allergenicity resulted not only from the increased concentration of major grass pollen allergens, but also from the presence of additional allergenic proteins such as lipase, nuclease, pectin esterase, peroxidase and chitinase-like protein belonging to the pathogenesis related proteins PR-3. Interestingly, the content of profilin in oregano leaves grown in soil contaminated with the highest concentration of Cu (1000 ppm) was significantly higher than in all other plants (including control plants) (Figure 6). Georgiadou et al. (2018) noticed a similar increase in profilin content in leaves of basil plants contaminated with various concentrations of Cu (200–1000 ppm). The increased profilin content is potentially linked with increased amounts of salicylic acid in an attempt of the plant to defend itself against the stressor (Mishra and Choudhuri, 1999). Application of all Ni treatments and Zn 720 ppm did not affect profilin content significantly, whereas the lowest and medium concentration of Cu (200 ppm and 500 ppm) as well as medium and highest concentration of Zn (1500 ppm and 3000 ppm) resulted in a significant decrease in profilin content compared with control plants (Figure 6). It is possible that lower profilin content could be caused by smaller extractability of this protein due to increased binding to membrane phospholipids and proline, the biosynthesis of which is upregulated in response to abiotic stress condition.

4. Conclusions

The present study shows novel evidence on the effects of three important heavy metals (Ni, Cu, Zn) applied to the soil in three different concentrations in oregano plants compared with control plants (diagrammatic overview of events occurring and key results shown in Figure 7). Current findings show that *Origanum vulgare* L. plants from the *Lamiaceae* family have the tendency to accumulate zinc ions, which considerably inhibit plant growth and development. All tested metals (Ni, Cu, Zn) were the reason of a decrease in photosynthetic pigments, especially total chlorophyll content, while Cu and Zn lead to significant oxidative cellular damage in plants. Increasing concentration of these metals (especially Zn) resulted in a further increase in cellular damage. The concentration of proline increased considerably at Zn 3000 ppm, which may suggest osmotic stress. Copper at the concentration of 1000 ppm caused significantly higher concentration of profilin – a well-known food and pollen panallergen. Moreover, nickel ions applied at concentrations of 100 ppm and 210 ppm resulted in increased total antioxidant capacity, phenolic compounds and total protein content compared with control samples. The presence of nickel ions in the substrate seems to have no effect on the growth and development of oregano plants (when compared with Cu and Zn), and lower allergenic potential compared with Cu 1000 ppm. The present study presents new biochemical evidence related to abiotic stress responses, as well as the induction of allergenic proteins due to the presence of heavy metals in the rhizosphere of aromatic plants, urging the need for more comprehensive research under field conditions in order to fully decipher this important interaction.

Credit author statement

Conceived and designed the experiments: JL, VF and BS. Performed the experiments: KKW, ECG and DM. Analyzed the data: KKW, ECG, DM, BS, VF and JL. Wrote the paper: KKW, ECG, BS, VF and JL. All authors have read and approved the manuscript.

Declaration of Competing Interest

Authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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