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Effect of essential micronutrients on catalase enzyme activity in *Tilia sp.* leaves growing in urban areas

Dragana Pavlović*, Marija Matić, Veljko Perović, Natalija Radulović, Milica Marković, Miroslava Mitrović, Pavle Pavlović

Department of Ecology, Institute for Biological Research "Siniša Stanković", - National Institute of the Republic of Serbia, University of Belgrade, Bulevar despota Stefana 142, 11000 Belgrade, Serbia

Corresponding_author: dragana.pavlovic@ibiss.bg.ac.rs

Abstract

Changes in the urban environment can have serious effects on plants, including changes in the availability of certain essential micronutrients. Micronutrients are needed in very small amounts and are often required as cofactors for enzyme activity. In this study, the concentrations of selected essential micronutrients (B, Cu, Mn, and Zn) and the activity of the enzyme catalase in leaves of Tilia sp. were measured. The study was conducted in urban parks in Belgrade, Pancevo and Smederevo, exposed to various sources of pollution from traffic and industry. Control site was located in an area without a direct source of pollution. Results of this study revealed toxic B content in leaves of Tilia sp. from Belgrade, while deficiency of this element was measured in Pancevo. Deficit in Zn content was measured in almost all examined individuals, while Mn deficit was measured in Belgrade and at the Control site. The lowest values of catalase activity were measured in Tilia sp. at the Control site, which indicates that the Zn and Mn deficiencies cause slightly lower vitality of Tilia sp. at the Control site compared to the same trees at the other sites. On the other hand, the highest catalase activity measured in Belgrade could be the result of B toxicity. The results of the discriminant analysis (DA) showed that Belgrade site is clearly separated from the other three sites, with B and Mn contributing the most.

Keywords: *urban environment; essential micronutrients; catalase enzyme activity; Tilia sp.*

Introduction

The growth of human population and increasingly pronounced urbanization and industrialization during the last two centuries have led to extensive emission of pollutants that have dramatically changed the quality of the urban environment [1,2]. A particular problem in urban areas represent potentially toxic elements (PTEs), which accumulate in the surface layers of the soil and can easily enter the food chain from there. Significant sources of PTEs in urban areas are emissions from traffic, urban heating plants, and industrial facilities [3-5]. In recent years, increasing attention has been paid to research on the potential impact of accumulation, bioavailability, and monitoring of the content of PTEs in the urban environment [5-9]. Trees, as an integral part of the urban environment, are exposed to the harmful effects of pollutants, including PTEs [2,4]. Plants take up PTEs from the soil through their roots and from the air through their leaves. Their uptake, accumulation, and translocation depend on species characteristics, soil properties, and other ecological factors in the environment [5,6]. Elements such as B, Cu, Fe, Mn, Mo, Ni, and Zn are essential for plants and play an important physiological and biochemical role, being involved in the biosynthesis of chlorophyll and DNA, the process of photosynthesis, redox reactions in chloroplasts and mitochondria, carbohydrate metabolism. In order to develop and grow, plants need to maintain concentrations of essential elements within optimal values [10]. However, when concentrations exceed these values, essential elements can negatively affect the functioning of physiological processes in plants, which is why they are called potentially toxic elements. Through their influence on enzymatic activity, they cause structural and functional damage and also act as antimetabolites, forming stable complexes with essential metabolites, altering membrane permeability, replacing important structural or electrochemically significant elements in cells. Their influence is also reflected in the disruption of the nutritional status of plants, as they enter into active competition with essential nutrients [11,12]. Essential element deficiency can lead to the appearance of visible leaf damage in the form of chlorosis and necrosis, reduced number of leaves, and reduced leaf area, which affects sunlight absorption and biomass production [13,14]. Essential elements are required by plants throughout their life cycle, and each of them has a specific physiological function that cannot be replaced by other elements. Numerous biochemical reactions in plants can be stressed by a deficiency or excess of essential elements. Most of these reactions lead to increased production of reactive oxygen species (ROS) and oxidative stress, which in turn leads to the activation of certain defense mechanisms in the plant. Defense mechanisms include enzymatic (superoxide dismutase, catalase) and non-enzymatic components (ascorbate and glutathione, carotenoids), which have the ability to detoxify ROS [10].

Considering the importance of trees for environmental quality in cities and the role that essential elements play in their functioning, the aim of this work was to determine the content of four essential microelements (B, Cu, Mn and Zn) in the leaves of *Tilia* sp. growing in urban parks in Belgrade, Pancevo, Smederevo and at the Control site. Another objective was to evaluate the adaptation potential of this species by measuring catalase enzyme activity.

Materials and methods

Sampling site description

The study was conducted in urban parks in Belgrade, Pancevo and Smederevo, which are exposed to various sources of pollution from traffic and industrial activities. The sampling site in Belgrade was the park "Hall Pioneer" in the city center, which is exposed to pollution from car exhaust. In Pancevo, sampling was conducted in the National Garden, the main city park in the southeastern part of the city. The main sources of pollution are the oil refinery, nitrogen fertilizer factory and petrochemical industry in Pancevo. The sampling site in Smederevo was the city park in the central zone of the city. The main source of pollution in Smederevo is a steel conglomerate located in the industrial area 7 km southeast of the city center. As a Control site we chose the Arboretum of the Faculty of Forestry, a protected natural area and a valuable archive of native and foreign tree species in Belgrade, located in an area without a direct source of pollution. The arboretum is located in the zone of mixed forest of *Quercus frainetto* and *Quercus cerris*.

Sample collection

Sampling was conducted from three to five randomly selected trees of approximately equal age at each site. Leaf samples were taken uniformly from each tree from different quarters of the canopy in all directions around the tree using stainless steel scissors which were then mixed to form a composite leaf sample, resulting in one composite leaf sample per sampling site. The leaf samples were stored in a paper envelope and then placed in a polyethylene bag prior to transport to the laboratory. In the laboratory, the samples were dried to a constant weight of 75°C for elemental analysis (Binder, Tuttlingen, Germany). Leaf samples for enzyme analysis were placed in liquid nitrogen immediately after sampling and stored at a temperature of -80°C until analysis.

Essential micronutrients analysis

Elemental concentrations in leaf samples were determined after wet digestion in a microwave oven (CEM, 39 MDS-2000) using method USEPA 3052. Certified reference materials was analysed to verify the accuracy of the analytical procedure: Plant material (beech leaves BCR-100) provided by the Institute for Reference Materials and Measurements (Geel, Belgium) and certified by the

European Commission - Joint Research Centre. The concentrations of B, Cu, Mn and Zn (mg kg^{-1}) in the studied samples were determined by optical emission spectrometry with inductively coupled plasma (ICP-OES, Spectro Genesis, Spectro-Analytical Instruments GmbH, Kleve, Germany). The detection limits (mg kg^{-1}) for the elements were as follows: B-0.001; Cu-0.006; Mn-0.003; and Zn-0.002. The average recoveries for the elements in the standard reference materials ranged from 95 to 110%.

Leaf extract preparation and enzyme activity analysis

For enzyme extraction, the frozen leaf tissue was homogenized in a mortar with pestle and liquid nitrogen and then extracted in ice-cold 0.1 M potassium phosphate extraction buffer (pH 6.5) containing 3% polyvinylpyrrolidine (PVP) and 5% phenylmethanesulfonyl fluoride (PMSF). After homogenization, the crude leaf extracts were centrifuged at 14,000 x g at 4°C for 20 min, and the supernatants obtained were aliquoted and used to measure protein content and enzyme activity. Protein content was determined according to Bradford [15], using bovine serum albumin (BSA) as a standard. Catalase activities were determined spectrophotometrically in duplicate at 20°C using a Shimadzu UV-160 spectrophotometer. Catalase activity was measured by adding 10 µl of the enzyme extract to 1 ml of a reaction mixture containing 50 mM K-phosphate buffer (pH 7) and 30% H_2O_2 and measuring the changes in absorbance at 240 nm for 3 min [16]. The results of catalase activity were expressed in units per mg of protein (U mg^{-1}).

Data analysis

Results were analyzed using one-way analysis of variance (ANOVA) by calculating the statistical significance of micronutrient (B, Cu, Mn and Zn) concentrations in relation to their locations. Discriminant analysis was performed to evaluate the differentiation among the studied sites based on the variations of B, Cu, Mn, Zn and catalase activity.

Results and discussion

The content of B in *Tilia* sp. leaves ranged from 11.89 mg kg^{-1} in Pancevo to 221.18 mg kg^{-1} in Belgrade (Table 1). Significant differences ($p<0.001$) in B content compared to the Control were found at all sites (Table 1). The Belgrade site is burdened with increased B content, which is more available to plants than at other sites. It is noticeable that the leaves of *Tilia* sp. growing in this park accumulate significantly more B than the leaves at other sites ($p<0.001$). Boron is an essential micronutrient required for plants at very low concentrations for normal growth and development, but its biochemical role is not well understood [17]. The specificity of B is reflected in the narrow range of concentrations between deficient and toxic, whereas normal concentrations in plant tissues are in a wide range of values (10-100 mg kg^{-1}, [11]). In this study, toxic B concentrations (>100 mg kg^{-1}, [11]) were determined in leaves of *Tilia* sp. in a park in Belgrade, while individuals from Pancevo were characterized by deficient B content (5-30 mg kg^{-1}, [11]). Boron deficiency affects a number of metabolic processes such as the ascorbate/glutathione cycle, membrane transport processes, photosynthesis, etc. [18], while B toxicity causes physiological and morphological defects such as reduced shoot and root growth, inhibition of photosynthesis, increased membrane permeability, peroxidation of lipids, and altered activities of antioxidant enzymes [19]. As a result of the physiological disturbances, toxic B levels cause oxidative stress and ROS are overproduced in plant cells [19,20].

Table 1. Comparison of B, Cu, Mn and Zn concentrations and catalase enzyme activities in leaves of *Tilia* sp. sampled from urban parks in Belgrade, Pancevo, Smederevo and Control site

	B [mg kg^{-1}]					Cu [mg kg^{-1}]				
	M±SD	**B**	**P**	**S**	**C**	**M±SD**	**B**	**P**	**S**	**C**
Belgrade	221.18±2.35	/	***	***	***	6.81±0.44	/	ns	***	ns
Pancevo	11.89±0.57	***	/	***	***	7.08±0.15	ns	/	***	ns
Smederevo	32.15±2.36	***	***	/	***	3.32±0.29	***	***	/	***
Control	86.80±2.44	***	***	***	/	6.85±0.12	ns	ns	***	/
	Mn [mg kg^{-1}]					**Zn [mg kg^{-1}]**				
	M±SD	**B**	**P**	**S**	**C**	**M±SD**	**B**	**P**	**S**	**C**
Belgrade	17.79±0.36	/	***	***	***	26.07±1.36	/	***	***	***
Pancevo	32.56±0.51	***	/	ns	***	18.67±1.15	***	/	***	***
Smederevo	32.97±0.40	***	ns	/	***	11.37±0.36	***	***	/	ns
Control	29.56±0.61	***	***	***	/	13.02±1.51	***	***	ns	/
	Catalase [U mg^{-1}]									
	M±SD	**B**	**P**	**S**	**C**					
Belgrade	812.08±65.60	/	ns	ns	***					
Pancevo	749.14±13.30	ns	/	ns	***					
Smederevo	731.47±29.20	ns	ns	/	***					
Control	524.84±5.32	***	***	***	/					

ANOVA, n=5, ***p<0.001, ns-not significant

The Cu content in the leaves of *Tilia* sp. ranged from 3.32 mg kg^{-1} in Smederevo to 7.08 mg kg^{-1} in Pancevo (Table 1). No significant differences (ns) in Cu content in the leaves in the sampled parks compared to the Control site were found. The exception is *Tilia* sp. from Smederevo, where a significant difference ($p<0.001$) was found compared to the Control (Table 1). Similar Cu content in leaves of *Tilia argentea* L. was found in the study of Greksa et al. [21], while Hrotkó et al. [22] found higher Cu content in *Tilia tomentosa*. Copper, like B, belongs to the group of essential micronutrients necessary for normal plant growth and development. As a structural component of regulatory proteins, Cu is involved in a number of processes necessary for the normal functioning of plants, such as photosynthesis, respiration, reduction and fixation of nitrogen, carbohydrate metabolism, response to oxidative stress, etc. [11,18]. For normal functioning of basic physiological processes in plants, 5-30 mg kg^{-1} Cu is required [11], and its average content in plant tissues is about 10 mg kg^{-1} [23], while the deficit occurs at concentrations <5 mg kg^{-1} [11]. The Cu content was below average values, but in a range that could be regarded as sufficient (Table 1), except for *Tilia* sp. from Smederevo, where a deficit of this element was measured. According to literature data, the activity of Cu enzymes decreases rapidly under Cu deficiency, and in most cases this decrease is associated with metabolic changes and inhibition of plant growth [18]. In addition, due to its low mobility in the plant, insufficient supply of Cu leads to morphological changes in the apical parts of leaves, which spread along the leaf margins as the growing season progresses [4,24,25].

The amount of accumulated Mn in *Tilia* sp. leaves ranged from 17.79 mg kg^{-1} in Belgrade to 32.97 mg kg^{-1} in Smederevo (Table 1). Significant differences ($p<0.001$) in Mn content compared to the Control were found at all sites (Table 1). The influence of sampling site on the differences in Mn content was also observed in the research of Tomašević et al. [26], who found that in the leaves of *Tilia cordata* sampled at different locations in Belgrade, the Mn content ranged from 9-103 mg kg^{-1}. Manganese is another essential micronutrient, which is primarily involved in the process of oxygen production by photosynthesis and plays a key role in the transport of electrons in this process [10]. In addition, Mn is an important component of several enzymes, and its most important function is to participate in various oxidation-reduction processes and build resistance to abiotic and biotic stress factors [10]. The optimal Mn concentration in plants is in the range of 30-300 mg kg^{-1}, and the deficit occurs at concentrations of 10-30 mg kg^{-1} [11]. From the obtained results, it appears that the *Tilia* sp. trees from Belgrade and the Control site have a deficit of this element (Table 1). Oliveira et al. [27] found that Mn deficit induces oxidative stress and decreases the activity of antioxidant enzymes and total phenols, affecting the quantum efficiency of the photosystem II and pigment content.

Zinc content in *Tilia* sp. leaves ranged from 11.37 mg kg^{-1} in Smederevo to 26.07 mg kg^{-1} in Belgrade (Table 1). Significant differences ($p<0.001$) in Zn content compared to the Control site were found at all sites. The exception was *Tilia* sp. from Smederevo, where no significant differences (ns) were found compared to the Control (Table 1). Slightly higher Zn content was found by Greksa et al. [21] (20 mg kg^{-1}) in leaves of *Tilia argentea* L. in Novi Sad and Tomašević et al. [26] (19-32 mg kg^{-1}) in *Tilia cordata* leaves in Belgrade. After Fe, zinc is the second most important essential element, participating as an integral part of numerous enzymes in the metabolism of proteins, carbohydrates and phosphates, in the formation of ribosomes and in the regulation of auxin synthesis. It also affects membrane permeability and stabilization of cellular components [17,28], and it has a particular importance in chloroplasts, i.e., enzymes involved in the process of photosynthesis [29]. From the obtained results, it appears that there is a deficiency of this element at all sites (10-20 mg kg^{-1}, [11]), except for Belgrade, where its content is below the range considered sufficient for the normal functioning of plants (27-150 mg kg^{-1}, [11]). Since Zn ions are involved in the enzymatic defense of cells against free radical damage [30], Zn deficiency can lead to high ROS production and cell damage [29].

Catalase enzyme activity ranged from 524.84 U mg^{-1} at the Control site to 812.08 U mg^{-1} in Belgrade. Significant differences ($p<0.001$) in catalase activity compared to the Control site were found at all sites (Table 1). Lower catalase activity in the Control could be induced by deficiency of Mn and Zn. Zinc deficit was also measured at other sites, but only at Control site it was accompanied by Mn deficit. Manganese is known to play an important role in the antioxidant defense system of plants due to its functions as an enzyme activator, enzyme cofactor, and electron transporter [31]. Manganese deficiency causes changes in oxidative metabolism, leading to an imbalance between the production and elimination of reactive oxygen species (ROS) by modulating the activities of enzymes responsible for the reduction of these compounds in plant cells. In addition to the enzyme system, Mn is also required for the synthesis of the components of the non-enzymatic antioxidant defense system [27]. On the other hand, in Belgrade, despite the Mn deficit, the highest activity of catalase was measured, which may be related to the toxic B content in the leaves. Studies by Carville et al. [32] showed that excess B affects abiotic stress indicators such as proline, carotenoids, etc. Esim et al. [19] investigated the changes in antioxidant catalase enzyme activities in maize roots exposed to two different B concentrations and found that catalase activity was unaffected by both B treatments in the roots of 11-day plants compared to the Control. However, in 15-day plants, root catalase activity was significantly increased in both B treatments. The mechanisms of B tolerance and toxicity are not yet well understood. It has been suggested that antioxidants and antioxidant enzymes may reduce B toxicity in some plants. This antioxidant response is considered to be a crucial process in protecting plants from oxidative damage caused by a wide range of environmental factors [33].

Differentiation among examined sites

Discriminant analysis (DA) was applied to determine differences between sites based on total B, Cu, Mn, and Zn content and catalase enzyme activity in *Tilia* sp. leaves. All sites are clearly separated, with Belgrade standing out on relation to the other sites based on the first discriminant function (DC1), which explains 99.00% of the differences (Figure 1). The parameters that most influenced the separation were B and Mn (Figure 1). Belgrade and Pancevo sites are also separated from Smederevo and the Control site, and based on DC 2, which explains 1.00% of the differences, their separation is mainly influenced by Zn and catalase activity (Figure 1).

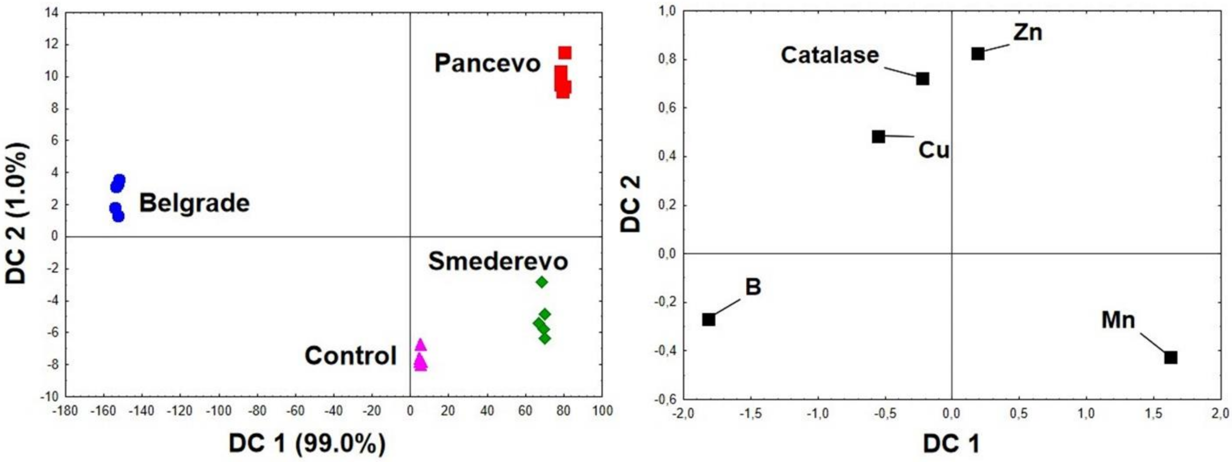

Figure 1. *Discriminant analysis (DA) for four sampling sites based on B, Cu, Mn, and Zn concentrations and catalase activity in leaves of Tilia sp.*

Conclusion

Based on the results presented in this research, it can be concluded that *Tilia* sp. potential for micronutrient accumulation is site specific. Toxic content of B was measured only in *Tilia* sp. leaves from Belgrade, while deficiency of this element was measured in Pancevo. Alongside B deficiency in Pancevo, the Zn deficit stood out in almost all studied individuals, while the Mn deficit was measured in Belgrade and the Control site, and the Cu deficit in Smederevo. In general, the lowest values of catalase activity were measured in the Tilia sp. trees at the Control site, indicating that the deficiency of Zn and Mn causes a slightly lower vitality of the Tilia sp. trees at the Control site compared to the individuals from other sites. The *Tilia* sp. trees in Belgrade showed the highest enzyme activity despite the Mn deficit and toxic B concentrations in the leaves, which could represent some kind of adaptation mechanism of the studied plants for existence under unfavorable site conditions.

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