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Effect of salt stress on prenol lipids in the leaves of *Tilia* 'Euchlora'

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Abstract: Soil contamination caused by the NaCl used to de-ice slippery roads in winter is now recognized as one of the major causes of nutrient disorders and death in urban trees. It is believed that polyisoprenoids may have a specific role in the adaptation of plants to adverse conditions and habitats; it is further believed that in the cell, they may exhibit a protective effect in response to biotic and abiotic stress. The aim of this study was to evaluate the effect of salt stress on the content of prenol lipids in the leaves of Crimean linden (*Tilia* 'Euchlora'). The Cl content in the slightly damaged ("healthy") leaves averaged 0.96%, while that in the heavily damaged ("sick") leaves averaged 2.02%. The leaves of control trees contained on average 0.57% Cl. The Na contents in the healthy and damaged leaves were 208 mg/kg and 1038 mg/kg, respectively, and the Na content in the control areas was 63 mg/kg. A mixture of polyprenols consisting of four compounds, prenol-9, prenol-10, prenol-11 and prenol-12, was identified in the leaves of Crimean linden. This mixture was dominated by prenol-10 (2.16–6.90 mg/g). The polyprenol content was highest in the leaves of "healthy" trees (approximately 13.31 mg/g), was lower in the case of "sick" trees (approximately 9.18 mg/g), and was the lowest in the control trees (mean 4.71 mg/g). No changes were observed in the composition of the mixture of polyprenols under these conditions. The results suggest that polyprenols may affect the accumulation of Cl in leaves. This phenomenon is evidenced by the high content of prenols in the leaves of trees considered "healthy" but growing under conditions of increased soil salinity and the lower content of prenols in the leaves of the "sick" and control trees. It is advisable to further investigate the role of prenol lipids in the leaves of trees subjected to salt stress.

Additional key words: polyprenols, salt stress, de-icing urban trees, linden

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Introduction

The abiotic (salt, osmotic, oxidative, thermal) and biotic stresses to which trees are subjected in urban environments cause major changes in the physiological and biochemical processes of the tree as well as in the tree morphology (Marschner 1995; Munns 2002; Larcher 2005). Soil contamination caused by the NaCl used for de-icing slippery roads in winter is now recognized as one of the major causes of nutrient disorders and death in urban trees (*e.g.*, Czerniawska-Kusza et al. 2004; Franklin and Zwiazek 2004; Green et al. 2008; Dmuchowski et al. 2013, 2014). According to data from the General Directorate for National Roads and Motorways, in Poland in December 2010, more than 200 000 tons of NaCl were used in snow removal from the streets and sidewalks (alone or mixed with sand (Marosz 2011)). For comparison, in Riga, Latvia, 10 000 tons of NaCl are applied each year (4.06 kg NaCl/m^2 of road), while in Germany, the average use is 2 kg NaCl/m^2 (Pedersen et al. 2000; Cekstere et al. 2008).

Damage to trees caused by the pollution of soil with salt is mostly observed in urban areas, usually along transportation routes characterized by heavy traffic (*e.g.*, Sehmer et al. 1995; Marosz and Nowak 2008; Lax and Peterson 2009; Fay and Shi 2012). Depending on the sensitivity of the particular species of tree and the degree of accumulation of Cl, the first visible signs of damage appear during the spring leaf bud burst and leaf growth. This not only contributes to a reduction in the decorative value of the trees but also makes it difficult for them to fulfill their biological functions (Bach and Pawłowska 2006; Dmuchowski et al. 2007).

It was estimated that in 1971, NaCl contributed to the death of more than 700 000 trees in Eastern Europe (Flückiger and Braun 1981). In Liverpool, 39% of all the inner city trees planted in recent years died within five years (Pauleit et al. 2002). In Edmonton, Canada, more than 20 000 trees died due to salinity and drought (Jimenez-Casas and Zwiazek 2014). Research carried out in the center of Warsaw by Dmuchowski *et al.* (2011) showed that over a period of 34 years, more than half (59%) of sidewalk trees died. The greatest losses concerned the following tree species: *Sorbus aucuparia* (94%), *Acer pseudoplatanus* (83%), *Tilia cordata* (65%) and *Tilia* 'Euchlora' (62%). The smallest losses were observed among *Tilia platyphyllos* (44%).

In response to salinity, plants, and trees in particular, have developed a variety of defense mechanisms that allow them to minimize the effects of stress and maintain homeostasis (Munns 2002; Dajic 2006; Goździcka-Józefiak and Woźny 2010). It is believed that polyisoprenoids can play a particular role in the adaptation of plants to adverse climatic and habitat conditions, thus demonstrating protective action in response to biotic and abiotic stresses (Bajda et al. 2009).

Polyisoprenoid alcohols constitute a group of highly hydrophobic polymers (Swiezewska and Danikiewicz 2005; Skorupińska-Tudek et al. 2008; Surmacz and Swiezewska 2011). A characteristic feature of these compounds is their occurrence as a mixture ('family'), with a single predominant species and a Gaussian-like distribution of homologues (Stone et al. 1967). It is postulated that the composition of the polyisoprenoid "family" in plant leaves is a characteristic feature of particular plant species and can be used as a chemotaxonomic marker (Chojnacki and Dallner 1988; Swiezewska and Danikiewicz 2005).

Polyisoprenoid alcohols are localized in membrane structures within the cell (Chojnacki and Dallner 1988; Swiezewska and Danikiewicz 2005). Polyisoprenoids are accumulated mainly as free alcohols and esters of carboxylic acids; in addition, their phosphates can be detected in the cell (Hemming 1985; Surmacz and Swiezewska 2011). The accumulation of polyisoprenoid alcohols varies over the life span of a plant. In some plant species, a 20-fold increased accumulation was noted in older leaves (Swiezewska et al. 1994; Ranjan et al. 2001). There is no information about the influence of temperature on the accumulation of polyisoprenoids. However, seasonal variations in polyprenol content in evergreen plants are observed (Swiezewska and Chojnacki 1988).

Polyisoprenoid alcohols play many important roles within the cell. Low abundant phosphates take part in protein N- and O-glycosylation and in protein prenylation. The latter process has been studied in rat liver (Thelin et al. 1995), spinach (Swiezewska et al. 1993) and *Arabidopsis thaliana* (Gutkowska et al. 2004). Genetic studies on yeast have shown that polyisoprenoids are involved in protein trafficking to the endoplasmic reticulum (ER) and vacuole (Sato et al. 1999; Belgareh-Touze et al. 2003). The role of free polyisoprenoid alcohols and their carboxylic esters remains uncertain. It is generally believed that polyisoprenoid alcohols modulate the biophysical properties of biological membranes (Thelin et al. 1995; Hjertman et al. 1997; Skorupińska-Tudek et al. 2008). This assumption is based on *in vitro* experiments on model membranes, which have shown that isoprenoids and their phosphates promote the formation of hexagonal phase II, increasing the permeability and fluidity of model membranes and also enhancing membrane fusion (Valtersson et al. 1985; Janas and Walinska 2000; Ciepichał et al. 2011). Nevertheless, these observations have not been confirmed *in vivo* (Swiezewska and Danikiewicz 2005). It is also postulated that polyisoprenoids act as scavengers of free radicals. The antioxidant properties of isoprene and other monoterpenes were postulated by Loreto and

Velikowa (2001). Similarly, the antioxidant properties of polyisoprenoids in mammalian cells were described recently (Bergamini 2003).

There is little information describing role of isoprenoids in plant response to stress conditions (Bajda et al. 2005). However, an increase in the biosynthesis of quinones under oxidative stress has been observed (Wanke et al. 2000). The aim of this study was to evaluate the effect of salt stress on the content of prenol lipids in the leaves of Crimean linden (*Tilia* 'Euchlora').

Materials and methods

The subject of the study was Crimean linden (*Tilia* 'Euchlora'). The study area selected was the avenue of Żwirki and Wigury (the median strip between the roadways). This is one of the main streets in Warsaw, characterized by a high intensity of traffic. The control area was a park (8 trees), located approximately 150 meters from the avenue and sheltered from it by a hedgerow of trees and shrubs. The soils in the study area, of anthropogenic character, showed alkaline reactions and a high abundance of exchangeable Ca, K and Na. The second control area (suburban) was a plant nursery in Żbików (8 trees). The trees growing in Żbików were the same age as the trees growing along the Żwirki and Wigury avenues.

The street trees were divided into two categories (with 8 in each): trees with no visible damage to the leaf blade ("healthy") and trees with emerging damage to the leaves ("sick"). Sick leaves were characterized by significant damage to the leaf blade (current number of blemishes in the form of chlorosis and necrosis).

Leaf samples were collected for chemical determination separately from each tree during the last week of July in 2010. The leaves were collected from the external belt of the crown, around its entire circumference, at heights from 2 m to 4 m.

Table 1. Comparisons of measured and certified concentrations of Na (mg/kg) and Cl (%) in plant in certified reference material

Standard	Certified		Measured		Recovery (%)	
	Cl (%)	Na (mg/kg)	Cl (%)	Na (mg/kg)	Cl	Na
Apple leaves – NIST-1515[1]	–	24.4	–	25.5	–	104.5
Beech leaves – BCR 100[2]	0.149	–	0.141	–	94.6	–

[1] National Institute of Standard and Technology, USA

[2] Sigma-Aldrich

Chemical analysis of the leaves

After the mineralization of the dry leaves in a muffle furnace (Allen 1974), Na was determined by atomic spectrophotometry using a Perkin Elmer 1100B (Perkin-Elmer 1990). Chlorine was determined by potentiometric titration using an ion-selective electrode and ion meter, Orion Star Plus (LaCroix et al. 1970).

To provide quality control (QC), the elemental content in the plant samples was determined using certified reference materials, including apple leaves from the NIST – USA and Beech leaves from Sigma-Aldrich (Table 1). The obtained results were in good agreement with the certified values. The recovery range was 94.6% for Cl and 104.5% for Na%.

Qualitative and quantitative analysis of polyisoprenoids

Sample preparation

Dried and milled leaves of linden (50 mg) were placed in a tube, and the internal standard (Prenol-15, 50 μl, C = 1 μg/μl), together with 1 ml of an acetone:methanol mixture (1:1, v/v), were added. Lipids were extracted at 37°C for 30 min. The extract was removed by centrifugation and decantation. The tissue was re-extracted four times with new portions of the solvent mixture. All extracts were pooled and evaporated under a stream of nitrogen. The dry residue was dissolved in 0.5 ml of hydrolysing mixture (7.5% KOH in a mixture of water/toluene/ethanol (1:6.6:5.5 v/v/v) containing 0.2% pyrogallol) and incubated at 95°C for 1 h (Jozwiak et al. 2013).

When the sample reached room temperature, 1 ml of water, 1 ml of hexane and 0.5 ml of saline were added and mixed vigorously. After phase separation, the organic layer was removed, while the water phase was re-extracted with 1 ml of hexane three additional times. The combined organic phases were evaporated under a stream of nitrogen.

The polyisoprenoids were separated and purified on a silica gel column (1 ml of silica gel). The column was washed three times with 2 ml of hexane, and the lipids were then placed on the column. The column was first eluted with 2% diethyl ether in hexane (6–8 ml) to remove carotenoids and was then eluted with 15% diethyl ether in hexane (10 ml) to elute the polyisoprenoids. The fractions were evaporated under a stream of nitrogen, dissolved in 200 μl of IPA and analyzed by HPLC. All analyses were performed in triplicate.

To provide quality control, additional analyses were performed on plant material with a well-characterized polyprenol content and spectrum (photosynthetic tissue of *Sorbus intermedia, Nicotiana tabacum*

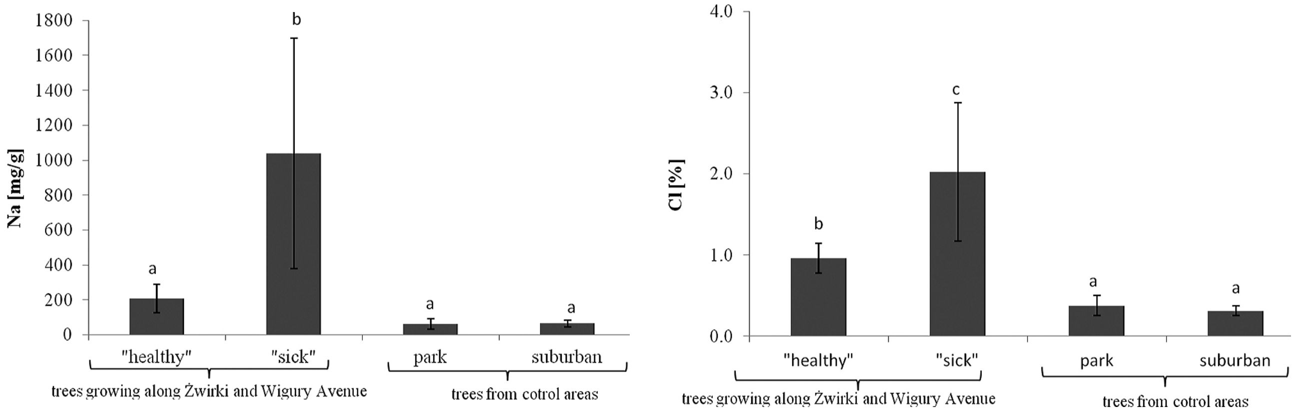

Fig. 1. Average content of Cl and Na in the leaves of Crimean linden

and *Picea abies*). The obtained results were consistent with literature data (Jozwiak et al. 2013).

HPLC/UV analysis

Quantitative and qualitative analyses of the obtained extracts were performed using HPLC/UV. Runs were performed on a 4.6 × 75 mm ZORBAX XDB- C18 (3.5 μm) reversed-phase column (Agilent, Santa Clara, CA) using a Waters dual-pump apparatus, a Waters gradient programmer, and a Waters Photodiode Array Detector (spectrum range: 210–400 nm). The solvents used in this analysis (Table 2) were A (methanol:water; 9:1, v/v) and B (methanol: isopropanol:hexane; 2:1:1, v/v/v). The solvent flow was set at 1.5 ml/min.

Results of the integration parameters

The separated compounds were identified by comparison of their retention times and absorption spectra with those of external standards of a polyprenol mixture (Pren-9, Pren-11 to Pren-23, and Pren-25) from the Collection of Polyprenols, IBB PAS. Extracted chromatograms registered at 2010 nm were integrated using Empower Pro software. The content of polyprenols was estimated based on the signal of the internal standard (Pren-15), and the calculated values were given as mg per 1 gram of dry leaves.

Statistical analysis

The results were subjected to statistical analysis using Statistica software and applying a univariate analysis of variance. Multiple comparisons of the means were performed using Tukey's procedure. Based on the above analysis, a homogeneous group of averages was identified. The analysis assumed a significance level of 0.05 (Sokal and Rohlf 1995).

Table 2. Solvent gradient used in HPLC analyses

Time	% A	% B
0	100.0	0.0
18	28.7	71.3
19	0.0	100.0
24	0.0	100.0
25	100.0	0.0

Results

The study confirmed a statistically significant influence of the Cl and Na content in the leaves on the deterioration of their health status. The Cl content in the leaves of Crimean linden increased with the deterioration of the health status of the leaves and varied depending on their degree of damage from 0.96% (±0.18%) ("healthy" trees) to 2.02% (±0.85%) ("sick" trees) (Fig. 1). The leaves of the control trees contained 0.38% (±0.13%) (park) and 0.31% (±0.06%) (suburban) Cl. "Healthy" trees with no visual damage to the leaves contained significantly more Cl than the trees from the park with no visual damage. There was a positive Pearson correlation (0.37) between the Cl content in the leaves and their state of health.

The differences in the content of Na in the leaves with respect to their state of health were much greater than the differences in Cl with respect to health

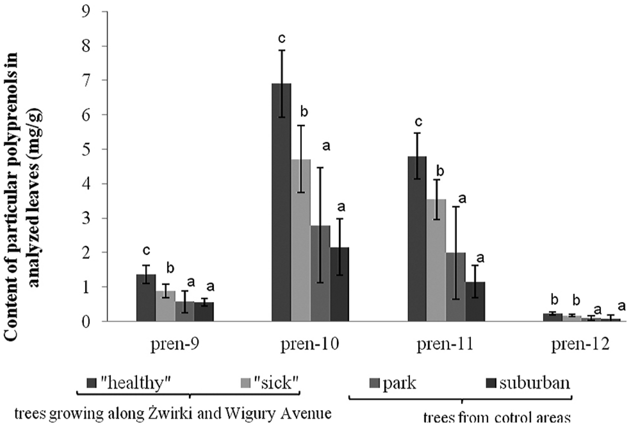

Fig. 2. Content of particular prenol lipids isolated from the leaves of Crimean linden

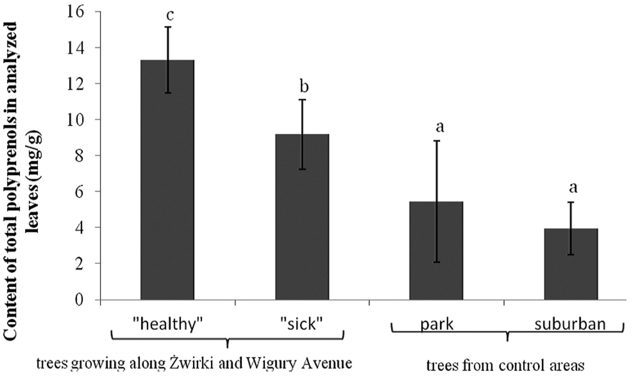

Fig. 3. Comparison of prenol lipid content from the leaves of Crimean linden as a function of location and state of health of the trees

status (Fig. 1). Leaves without damage contained an average of 208±81 mg/kg Na, and the damaged leaves contained 1038 (±660) mg/kg. The undamaged and damaged leaves of the control trees contained an average of 62 (±30) mg/kg and 64 (±18) mg/kg Na, respectively. There was a positive Pearson correlation between the content of Na in the leaves and their state of health.

Based on chromatographic analysis (HPLC), four prenol lipids were identified in the leaves of Crimean linden: prenol-9, prenol-10, prenol-11 and prenol-12. The prenol profile did not change with the conditions governing the growth of the plant; the analyzed leaves were characterized by the highest content of prenol-10 (2.16–6.90±0.82–1.67 mg/g) and the lowest of prenol-12 (0.08–0.23±0.03–0.10 mg/g) (Fig. 2).

There was an interesting tendency toward reduced prenol lipid content in the leaves with an increase in the degree of damage (Fig. 3). The prenol lipid content was highest in the leaves from “healthy” trees (approximately 13.31±1.84 mg/g), lower in the case of “sick” trees (approximately 9.18±1.94 mg/g), and lowest in the control group (mean 4.71±2.41 mg/g). This trend was observed in all identified prenols. Differences in the contents of prenol lipids in the leaves were significant.

Based on the determined functions of polynomial regression (Fig. 4), it appeared that with increasing Cl and Na concentration in the leaves, the prenol content increased but plateaued at a certain Na and Cl content (approximately 2% for Cl and approximately 1000 mg/kg for Na) in the leaves; after exceeding this level of Cl and Na, the content of lipids decreased. For the content of Cl, the correlation was high (r = 0.61) and for Na it is average (r = 0.35).

Discussion

Salt stress and the accompanying osmotic stress are the two main causes of deteriorating health in trees growing in urban areas (Davison 1971; Pedersen et al. 2000; Oleksyn et al. 2007; Cekstere et al. 2008). The presence of excess Cl in leaves causes initially invisible changes inside the cells, and with increasing amounts of compounds containing Cl, morphological changes in the form of chlorosis and necrosis become visible on the leaf blades (Pauleit 1988; Dmuchowski and Badurek 2004; Oleksyn et al. 2007). High concentrations of Cl and Na also contribute to changes in cell membrane permeability (calcium ions are “substituted” with sodium ions), disturb the ionic balance, reduce the chlorophyll content and the activity of many enzymes, and consequently lead to a reduction in efficiency of the main life processes of the tree (photosynthesis and respiration) (Marschner 1995; Cekstere and al. 2008; Gałuszka et al. 2011).

Studies in Warsaw confirmed that even low soil salinity may cause necrosis at the edges of leaves and rapid wilting and dying of trees (Dmuchowski and Badurek 2004). The leaves of all tested street side linden trees contained higher amounts of Cl than the toxicity threshold of 0.60% quoted in the literature (Shortle and Rich 1970; Chmielewski et al. 1985; Pauleit 1988). Street side trees were characterized by a significantly higher content of Cl than were the control trees. Similar results were obtained by, among others, Breś (1997), Migaszewski (2004), Marosz and Nowak (2008), and Oleksyn et al. (2007).

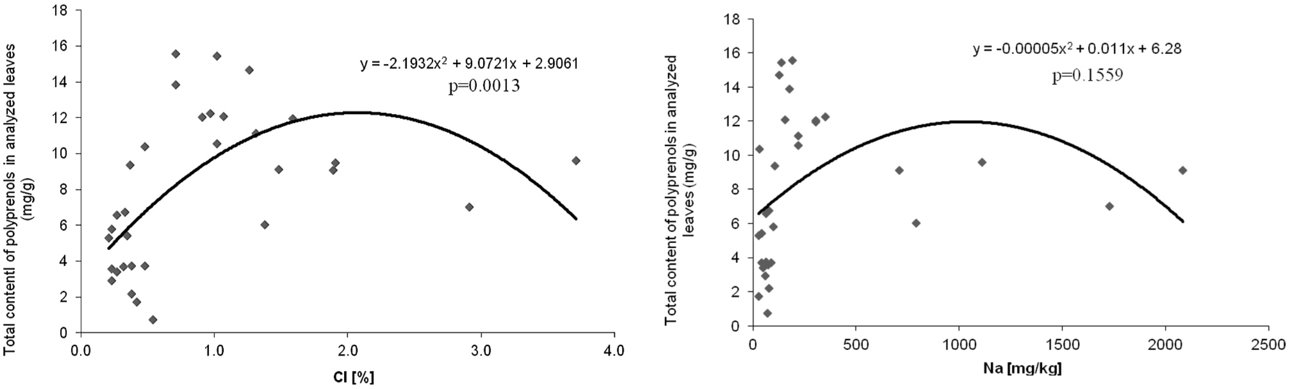

Fig. 4. Relationship between the concentration of Cl and Na in the leaves and the prenol lipids content

This study confirmed the statistically significant influence of the Cl content in the leaves on the deterioration of their health status. The leaves of healthy trees, without damage, were characterized by a lower content of Cl than were the "sick" trees with a high degree of damage to the leaf blade (over 40%). Most researchers believe that visual assessment of the degree of damage to leaves, crowns, trunks and the tree as a whole provide an important indication of the impact of stress factors affecting the tree (Hanisch and Kilz 1990; Skelly et al. 1990; Vollenweider and Gunthardt-Goerd 2005).

The Na content in the leaves of the studied lindens that were considered "sick" was characterized by very high variability, as evidenced by the high standard deviation. Trees with similar health status and similar Cl contents contained from 221 to 2083 mg/kg of Na. The toxic effects of Na on the woody vegetation were lower than those of Cl (Alaoui-Sosse et al. 1998). This was confirmed by, among others, research conducted by Kayama et al. (2003) on the effects of salinity on the vitality and health status of *Picea abies* and *Picea glehnii*. Interpreting the results of the Na content in the leaves of trees is difficult due to the lack of information in the literature about the thresholds of toxicity. Na is characterized by high lability in both soil and plants, and its excess mostly causes ionic imbalance rather than a direct toxic effect (Alaoui-Sosse 1998; Dmuchowski et al. 2011). This study found that the leaves of trees growing along a traffic artery were characterized by a higher concentration of Na than were the leaves of control trees. The results obtained are similar to those presented by Marosz (2011), who performed research on *Acer pseudoplatanus* and *Fraxinus excelsior*. Based on studies carried out on *Prunus salicina* by Ziska et al. (1991), it was shown that the movement of sodium ions occurs from the leaves to the shoots and roots. The main repository of sodium ions appears to be the timber tissue, not the leaf. The impact of Na on trees growing under conditions of salt stress has not been entirely explained in the literature. It is likely that the excess Cl has a greater impact on the tree health status than does the excess Na.

More and more information has appeared on the "protective" properties of prenyl lipids, which are postulated to modify the permeability of cell membranes, to exhibit antioxidant properties and to regulate intracellular transport (Loreto and Velikova 2001; Bergamini 2003; Swiezewska and Danikiewicz 2005; Surmacz and Swiezewska 2011). It is believed that polyisoprenoids, similarly to fatty acids, modulate the physicochemical properties of cell membranes by increasing their permeability, thus affecting the plant's ability to adapt to stress conditions (Upchurch 2008).

In this study, four prenyl lipids in linden (*Tilia* 'Euchlora') leaf were identified: prenol-9, -10, -11 and -12. Based on previous research, prenols 9–12 have also been identified in the families: *Euphorbiaceae* and *Moraceae* (Swiezewska et al. 1994) and *Laureaceae* (Roślińska et al. 2002). In accordance with studies conducted by Roślińska et al. (2002), the leaves of the studied linden trees accumulated prenol-10 in the highest amounts.

The content of prenyl lipids was highest in the "healthy" trees, lower in the "sick" trees, and lowest in the control trees, while the composition of the polyprenol mixture was identical in all the studied groups of plants. The street-adjacent trees growing under conditions of high environmental pressure and subjected to stressors were characterized by a higher content of these compounds than were the control trees. Skorupińska-Tudek et al. (2009) noted an increase in the dolichol content in the roots of *Coluria geoides* and *Cucumis sativus* in response to salinity, heavy metals, low temperature and lack of potassium and phosphorus in the culture medium. Furthermore, polyisoprenoids may have a protective effect during viral infection in *Nicotiana tabacum* plants; a 7-fold increase in the polyprenol content in the leaves of plants resistant to infection (Samsun NN) with tobacco mosaic virus was noted, while no such increase was observed in the leaves of the tobacco plants sensitive to viral infection (Samsun NN) (Bajda et al. 2009). The effect observed in the present study, *i.e.*, a much higher level of polyprenol accumulation in the leaves of the "healthy" trees and thus greater resistance to stressors, possibly suggests a similar molecular mechanism for polyisoprenoid involvement in plant adaptation to biotic and abiotic stress. The results obtained for the "sick" plants were consistent with this interpretation; the lower resistance of the "sick" linden trees to NaCl was correlated with a lower level of accumulation of polyprenols in the leaves, even though the polyprenol level was elevated in comparison to the leaves of control trees.

The results obtained suggest a protective role of prenol lipids in limiting the accumulation of Cl in leaves. This is evidenced by the high prenol lipid content in the leaves of trees classified as "healthy" but growing under conditions of increased soil salinity and by the lower prenol lipid content in the leaves of the "sick" and control trees.

It is advisable to further investigate the role of prenol lipids in the leaves of trees subjected to salt stress.

Conclusions

This study confirmed the negative effect of salinity on the health status of trees. The leaves of "healthy" trees, without damage, were characterized by a lower content of Cl and Na than were the "sick" trees (with severe damage to the leaf blade).

Four prenol lipids were identified in the leaves of Crimean linden: prenol-9, prenol-10, prenol-11 and prenol-12, with the highest content observed for prenol-10. The proportions of the individual components of the mixture of polyprenols did not change under different conditions of tree growth. There were statistically significant differences in the prenol lipid contents. The prenol lipid content was highest in the leaves of the "healthy" trees, lower in the "sick" trees, and lowest in the control group. This trend applied to all the identified prenols. The results obtained suggest a protective role of prenol lipids in limiting the accumulation of Cl in leaves.

Acknowledgments

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