Bioindication of anthropogenic pollution with *Plantago lanceolata* (*Plantaginaceae*): metal accumulation, morphological and stomatal leaf characteristics

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Abstract: Four metals (Zn, Cu, Pb, Cd) were determined by ICP-AES, as well as visual damages, length and width of leaf lamina, number and sizes of stomata on both surfaces of *Plantago lanceolata* leaves from four urban and non-ferrous industrial sites. The elements in the surface soils were analyzed. According to the obtained data, the leaves of *P. lanceolata* can be used as accumulative bioindicators not only for Zn and Pb, but also for Cd. There were found some leaf characteristics suitable for bioindication of industrial pollution: the length of leaf lamina, the number and sizes of the stomata. Visible leaf damages were found only in the area of non-ferrous industry. The sizes of leaf lamina and stomata were also sensitive characteristics of urban traffic pollution.

Key words: bioindication, leaf morphology, metal accumulation, *Plantago lanceolata*, pollution, stomata

Introduction

The industrial and urban-area pollution can be successfully assessed using wild plants with high accumulative capacity and other sensitive characteristics for bioindication. Schwanitz & Hahn (1954) reported *P. lanceolata* L. as a species showing races tolerant to Zn. Subsequently, this species was classified by Lambinon & Auquier (1964) and Antonovics & al. (1971) as an "indifferent pseudometallophyte", i.e. as being able to live on contaminated soil but without showing abundance, or particular vitality. Other authors (Horak & Huber 1974; Wu & Antonovics 1976) demonstrated that along busy motorways *P. lanceolata* evolved tolerance to lead. This species was mentioned by Nikolaevskij (1979) as a species of poor resistance in regions with high level of pollution. Nowadays *P. lanceolata* is still subject of investigations in areas with different sources of pollution (e.g. Öztürk & Türkan 1993; Kos & al. 1996; Klus & al. 2001; Yurukova 2004).

The necessity of studying the capacity of that widespread wild species for bioindication of anthropogenic pollution defines the aim of this study: to evaluate the leaf accumulative capacity of *P. lanceolata* for Zn, Cu, Pb and Cd, and to analyze some morphological and stomatal leaf characteristics in urban and industrial areas.

Material and methods

This study was carried out with specimens of *P. lanceolata* from an urban area, Plovdiv town (the second largest town in Bulgaria), and from an industrial area, the biggest non-ferrous smelting works in the country, near Plovdiv. Sampling of the aboveground phytomass (20 individuals per site) and the surface soil up to 10 cm depth (3 samples per site) was carried out at the peak of the growing season (July) of 1998 and 2001, at four sites. The first site, used as a control one, was located near an islet

on Maritsa River and away from the big sources of pollution. The second site was chosen near the smelter for lead and zinc, close to the administrative buildings. Site 3 was in the northern industrial part of the town and Site 4 in the centre of the town with high motor traffic (Table 1).

Table 1. Pollution on the studied sites

Site	Type of pollution	Main pollutants
1	Non-polluted urban area according to data supplied by the regional atmosphere-monitoring system	
2	Industrial: non-ferrous industry	dust, heavy metals (Zn, Cu, Pb, Cd) and gases (SO2, N_xO_x)
3	Urban: in the industrial part of the town, with a thermo-electric power station and other factories	dust, SO_2, NO_2, Cd- and Pb-aerosols
4	Urban: mainly by motor transport	dust, Pb-aerosols, N_xOx-gases

Three groups of analyses were applied:

– analysis of heavy metals in the soils and in the leaves of *P. lanceolata*;

– morphological analysis of the leaves;

– stomatal analysis of the leaf epidermis.

Approximately 1 g of a ground plant sample (dried at 85 °C) was treated with nitric acid overnight. Wet ashing continued in water bath after several portions of hydrogen peroxide were added. The volume of the obtained filtrate was diluted up to 50 ml.

The soil samples from surface layers (0–10 cm) were cleaned from mineral particles and organic waste, sifted through a fine steel sieve (1 mm) and dried at 85°C. One gram was treated with 15 ml mixture of perchloric and nitric acid in 3:1 ratio in a sand bath. The procedure was repeated with hydrochloric acid. The digested soil sample was finally filtrated and the filtrate diluted up to 50 ml and stored in a plastic flask. Duplicates of each plant and soil sample were prepared. The elements have been analyzed by atomic emission spectrometry with inductively coupled plasma (ICP-AES). Precision was ensured by replicating, blanks, plant reference material CRM 281 (ryegrass) and soil reference material CRM 142R (light sandy soil). In this study the toxic metals Zn, Cu, Pb, Cd (in $mg.kg^{-1}$ dry weight) were chosen, for which there are admissible concentration limits in the soils and foods in Bulgaria.

Soil acidity was determined with the help of Jenway pH-meter (England), after the sample had been in suspension (distilled water ratio in the soil was 1:2.5) for 18 hours.

Considering the advantages of using morphological leaf symptoms for bioindication (Manning & Feder 1980; Posthumus 1982; Schubert 1985), the visual damages and growth rate of the leaves were studied. The degree of damage was assessed on a five-point scale for the following characteristics: chloroses, necroses and dry leaves (Dimitrova & Ninova 1998). The scale included: I level –undamaged plant (0 % damaged leaf surface), II level – slightly damaged plant (up to 25 %); III level – averagely damaged plant (up to 50 %); IV level – considerably damaged plant (up to 75 %), and V level – strongly damaged plant (over 75 %), including dead plant. Visual damages were assessed for each individual and then the results were summarised for all studied plants (20 plants per site). To evaluate the process of leaf growth, the length and width of the leaf lamina were measured 100 times per site (five leaves from 20 plants), using plotting paper.

Ten leaves from 10 plants per each site were fixed in 70 % ethyl alcohol for stomatal analysis. The epidermal samples from the middle part of the leaf of both leaf surfaces were plated in glycerine medium and observed with light microscope Ergaval Carl Zeiss – Jena, ×750. The number of the stomata per 1 mm^2, the length and width of the stomata were analyzed with helical eyepiece micrometer. The number of the stomata was calculated per visible microscope field, than recalculated per 1 mm^2. The values of stomatal characteristics were estimated on the basis of 100 measurements per site. Light-microscope micrographs of the upper and lower leaf epidermis were made.

The differences found in morphometric and stomatal data between the control and other sites were assessed by Student's t-test. Correlative analysis of the analyzed elements in the leaves of *P. lanceolata* was performed.

Results

1. Content of heavy metals in soils and leaves

The mean concentrations of the elements Zn, Cu, Pb, Cd and acidity in the surface soil layers in the different sites are presented in Table 2. During the two years of investigation the concentrations of Cd varied most. They fluctuated within the range from 1.6 $mg.kg^{-1}$ up to 180 $mg.kg^{-1}$ (1998) and from 1.9 $mg.kg^{-1}$ up to 207 $mg.kg^{-1}$ (2001). The maximum value was found to exceed by 60 (69) times the admissible limit. The second in concentration element, Zn, was found to vary by 66 (67) times or up to 27 (28)

times above the admissible limit (Table 2). Lead varied by 45 (46) times and the values were between 60 (Site 1) and 2822 mg.kg-1 (Site 2). The highest concentration exceeded by 35 times the limit for soils in the country. Copper concentrations varied to a lesser extent (19–20 times): maximum 657–710 mg.kg-1 in Site 2 and minimum 33–38 mg.kg-1 in Site 1. The highest value exceeded by 2.5 –2.7 times the set limit.

Table 2. Element concentrations (±SD) in the surface soil layers (0–10 cm), mg.kg-1 dry weight

Year		Site 1	Site 2	Site 3	Site 4	Bulgarian admissible norms for pH = 7.0–7.5
1998	pH	7.3	7.0	7.6	7.6	
	Zn	142 ±12	9556 ± 42	373 ±25	176 ±13	340–360*
	Cu	33 ±5	657 ±11	252 ±9	82 ±8	260–270*
	Pb	62 ±2	2822 ±13	184 ±10	61 ± 4	80*
	Cd	2.2 ±0.5	180 ±11	3.6 ±1.0	1.6 ±0.8	3.0**
2001	pH	7.2	7.3	7.5	7.7	
	Zn	139 ±11	9240 ±38	380 ±20	169 ±12	340–360*
	Cu	33 ±5	657 ±11	252 ±9	82 ±8	260–270*
	Pb	62 ±2	2822 ±13	184 ±10	61 ± 4	80*
	Cd	1.9 ±0.7	207 ±12	3.8 ±0.9	2.3 ±0.6	3.0**

* – according to Regulation No 3, 1979
** – according to Regulation, 1997

The results for 1998 and 2001 of the elements analyzed in the leaves of *P. lanceolata* are shown in Table 3.

Table 3. Element concentrations (±SD) in the leaves of *P. lanceolata*, mg.kg-1 dry weight

Year		Site 1	Site 2	Site 3	Site 4	Bulgarian hygiene norms for dry edible plants*
1998	Zn	109 ±10	2531 ±22	128 ±11	100 ±9	50
	Cu	10 ±1	65 ±2	12 ±1	17 ±1	50
	Pb	5.6 ±1.0	327 ±18	7.0 ±1.5	33 ±3	4.0
	Cd	0.55 ±0.04	47 ± 4	1.4 ±0.5	0.85 ±0.07	0.30
2001	Zn	84 ±11	2384 ±18	119 ±10	108 ±10	50
	Cu	8 ±1	70 ±1	10 ±1	13 ±1	50
	Pb	5,8 ±1	338 ±14	6.8 ±1.2	30 ±2	4.0
	Cd	0.46 ±0.04	45 ±3	1.6 ±0.5	0.95 ±0.06	0.30

* norms for medicinal plants are missing

The difference between the maximum and the minimum concentrations was the highest for Cd (around 90 times), followed by Pb (around 60 times). Both heavy metals showed higher values in Site 2 (the non-ferrous smelter) and the lowest values in Site 1 (the control one). All concentrations obtained for these metals exceeded the Bulgarian hygiene norms for dry edible plants (Regulation No 5 1984): up to around 155 times for Cd and around 80 times for Pb. Concentrations of Zn exceeded the hygiene norm and varied within the range of 2384–2531 mg.kg-1 (Site 2) and 84–109 mg.kg-1 (Site 1). The variation of Cu was lower (6.5–8.8 times, respectively). Copper content was below the limit in the urban sites. Regarding the exceeding of hygiene norms, the elements in the leaves of *P. lanceolata* from the smelter area were found in the following descending order: Cd>Pb>Zn>Cu. In the urban site with industrial pollution Cd was in the lead, whereas in the urban site with high traffic the order changed to Pb>Cd>Zn>Cu. The order in the control site was: Zn>Cd>Pb>Cu. The thus established pattern of heavy metal accumulation in the leaves was valid for both years of investigation.

The correlation analysis of elements in the leaves of *P. lanceolata* from different sites showed positive correlations with sufficient statistical reliability (Table 4). As seen from Table 4, the heavy metals Zn, Pb, Cd and Cu had a remarkably high correlation coefficient between each other.

Table 4. Correlation coefficient of the elements in *P. lanceolata* leaves

Year		Cu	Pb	Cd
1998	Zn	0.866**	0.797*	0.970***
	Cu		0.990**	0.959***
	Pb			0.918***
2001	Zn	0.998***	0.998***	0.999***
	Cu		0.999***	0.997***
	Pb			0.997***

Significance levels: * $p \leq 5\%$, ** $p \leq 1\%$, *** $p \leq 0.1\%$

2. Morphological analysis of the leaves

Visual damages of the leaves were found in the area of the non-ferrous smelter (Site 2). Dry leaves and leaf chloroses and necroses were observed during both years of investigation. The necrosis started from the leaf tip and reached the middle part of the leaf lamina. The visual damages affected over 75 % of the leaf surface. According to the used scale, that stood for level five (strongly damaged plants). The plants from the other studied urban sites (including the control one) had no visual leaf damages.

The analysis of morphomertic data of the leaf lamina showed statistically significant smaller mean values of the length and width of the leaves of plants from Site 2 and Site 4 in comparison with those from the control site (Table 5). As seen from the data in the Table, changes in the leaf length were considerably greater than those in the leaf width (always with $P<0.001$). Data for this characteristic showed a statistically sig-

nificant deviation in plants from Site 3 in comparison to Site 1, but changes differed for each year of investigation.

Table 5. Length and width of *P. lanceolata* leaves (cm)

Year	Site	Length			Width		
		Mean ±SD	t	p	Mean ±SD	t	p
1998	1	13.59 ±2.36			1.17 ±0.25		
	2	10.24 ±1.49	11.96	<0.001	1.11 ±0.24	2.00	0.05
	3	14.79 ±4.10	2.55	0.01	1.24 ±0.61	1.00	ns
	4	11.42 ±2.97	5.71	<0.001	0.86 ±0.30	7.75	<0.001
2001	1	17.92 ±1.48			1.35 ±0.30		
	2	11.34 ±1.28	32.95	<0.001	1.18 ±0.22	4.25	<0.001
	3	14.55 ±3.30	9.36	<0.001	1.44 ±0.61	1.29	ns
	4	12.50 ±3.10	15.94	<0.001	1.05 ±0.24	7.50	<0.001

t – Students's t-test; **p** – probability; **ns** – non sighificant

3. Stomatal analysis

Histological analysis of the leaf epidermis confirmed the observation of Metcalfe & Chalk (1957) that stomata were present on both leaf surfaces in *P. lanceolata*. It was found that there were stomata surrounded by two (diacytic stomatal type) and three-four (anomocytic stomatal type) cells, whereas Metcalfe & Chalk (1957) have usually indicated the diacytic type for *P. lanceolata* (Plates I–VI).

Some anomalies were observed about the guard cells of the stomata: a smaller size or lack of one of the guard cells (Plate II, Fig. 2; Plate V, Fig. 2). In some cases there were deformed stomatal cells in the epidermis of leaves from Site 2 (Plate II, Fig. 1).

There were essential changes in the quantitative stomatal characteristics: the number of stomata and their size. During the first year of investigation the mean number of the stomata was 179.90/mm2 on the upper epidermis and 231.70/mm2 on the lower epidermis of the plants from the control site (Table 6). In the other sites there were a higher number of stomata on the upper epidermis, mostly on Site 3, followed by Site 2. In Site 4 the difference was insignificant as compared to the control one. The number of stomata on the lower leaf epidermis was significantly higher in the plants from Site 3 (284.90/mm2) and Site 2 (269.15/mm2), as compared to the control site (p<0.001). In Site 4 the number of stomata was lower, but the difference was statistically insignificant, as on the upper epidermis. Data on 2001 confirmed the tendency of an increasing stomatal number with industrial pollution (Site 2 and Site 3) and again showed statistically insignificant differences between the control plants and those from the site with high traffic (Table 6).

Table 6. Number of the stomata (mm-2) of *P. lanceolata* leaf epidermis

Year	Site	Upper epidermis			Lower epidermis		
		Mean ±SD	t	p	Mean ±SD	t	p
1998	1	179.90 ±35.35			231.70 ±49.35		
	2	205.45 ±43.05	4.58	<0.001	269.15 ±40.60	5.86	<0.001
	3	221.90 ±59.85	6.03	<0.001	284.90 ±82.95	5.51	<0.001
	4	185.85 ±76.65	0.70	ns	217.35 ±62.65	1.80	ns
2001	1	167.46 ±28.64			212.60 ±56.68		
	2	213.56 ±54.55	7.48	<0.001	278.54 ±42.55	9.30	<0.001
	3	209.18 ±87.45	4.53	<0.001	265.37 ±79.76	5.39	<0.001
	4	177.27 ±56.65	1.54	ns	205.75 ±67.45	0.79	ns

t – Students's t-test; **p** – probability; **ns** – non significant

The mean stomatal sizes (length – L and width – W) of the control plants were: L=22.09 μm and W=14.45 μm on the upper epidermis, L=21.39 μm and W=14.59 μm on the lower epidermis. Statistically significant differences (p<0.001) were found in the stomatal sizes between the control site and the remaining sites from polluted areas. The tendency of changes was different. In Site 2 and Site 3 the stomata had lower length and width, as compared to the control ones, whereas in Site 4 the stomata were larger. On the basis of measurements of the length and width of the stomata, it was found that the stomata from Site 4 were the largest (L=24.04 μm and W=16.84 μm on the upper epidermis, L=22.07 μm and W=15.79 μm on the lower epidermis) and those from Site 3 were the smallest (L=19.98 μm and W=14.06 μm on the upper epidermis, L=19.19 μm and W=13.99 μm for the lower epidermis; Fig. 1, Plates I–VI). As seen from the chart, the results for 2001 confirm the trends in stomata size changes.

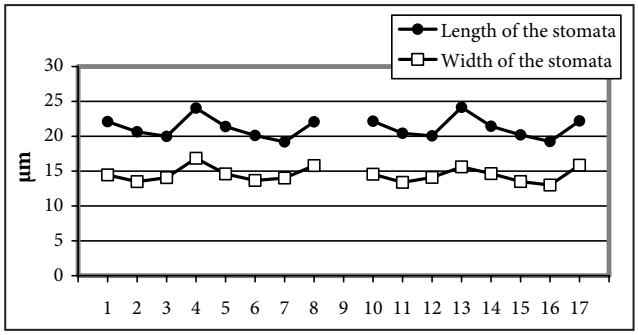
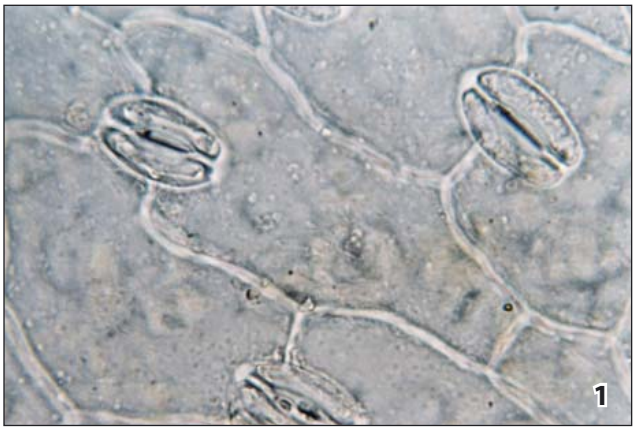
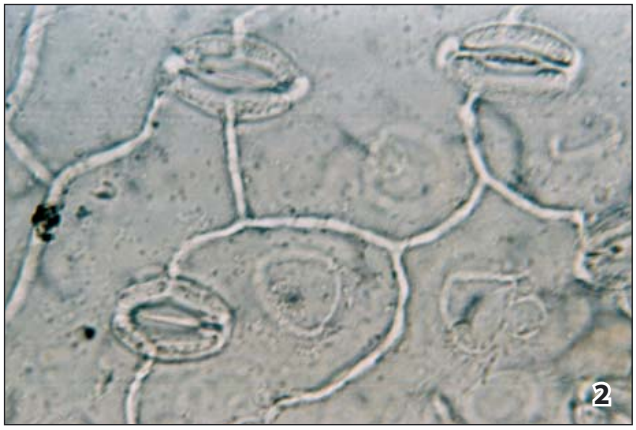


Fig. 1. Length and width of stomata in *P. lanceolata* (mean values):
– 1998 (1-8), 2001 (10-17);
– upper leaf epidermis (1-4, 10-13), lower leaf epidermis (5-8, 14-17);
– Site 1 (1, 5, 10, 14), Site2 (2, 6, 11, 15), Site 3 (3, 7, 12, 16), Site 4 (4, 8, 13, 17).

Plate I

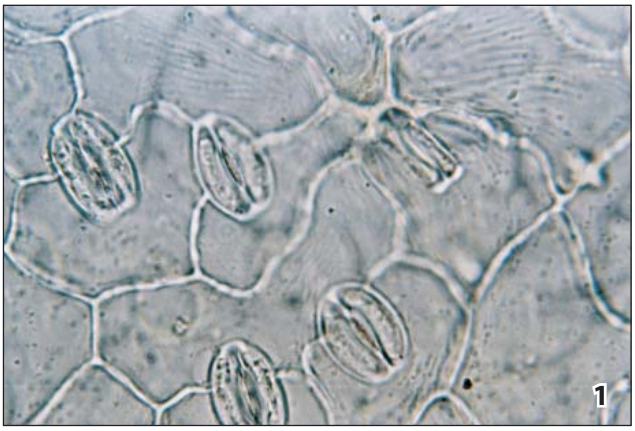


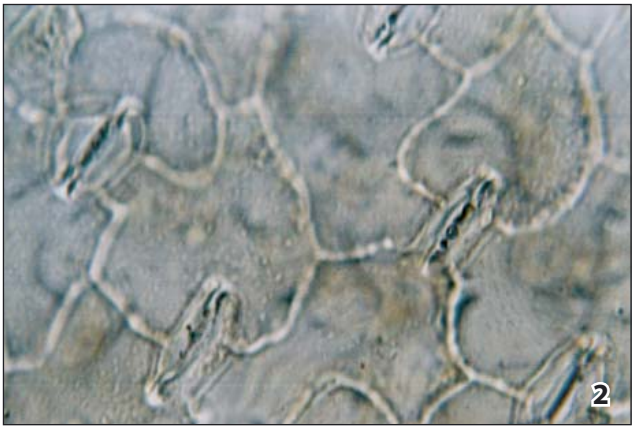


Figs 1-2. Light micrographs of the leaf epidermis in *P. lanceolata*. Site 1:

1, upper surface (×750); **2**, lower surface (×750). Bar scale 33 mm = 25 μm

Plate II

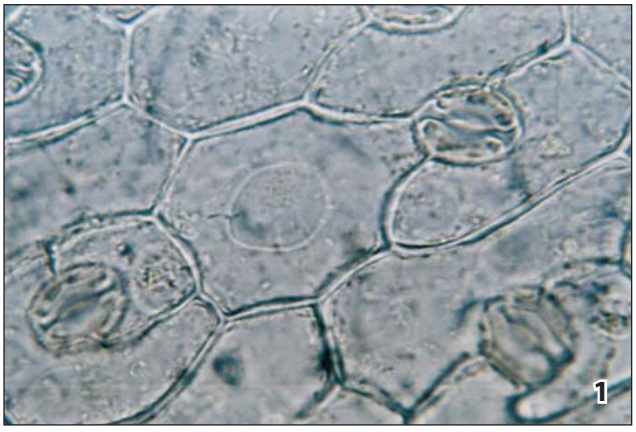


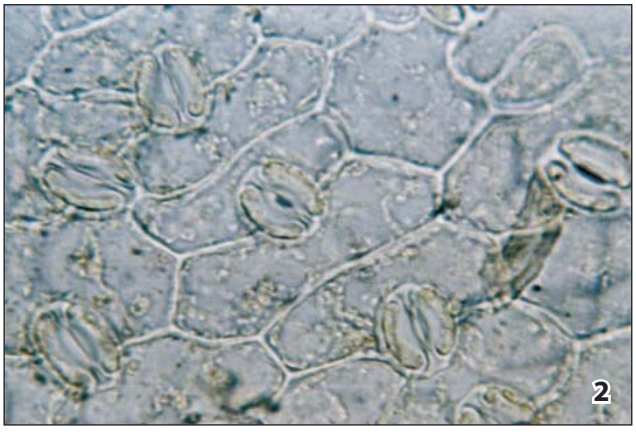


Figs 1-2. Light micrographs of the leaf epidermis in *P. lanceolata*. Site 2:

1, upper surface (×750); **2**, lower surface (×750). Bar scale 33 mm = 25 μm

Plate III

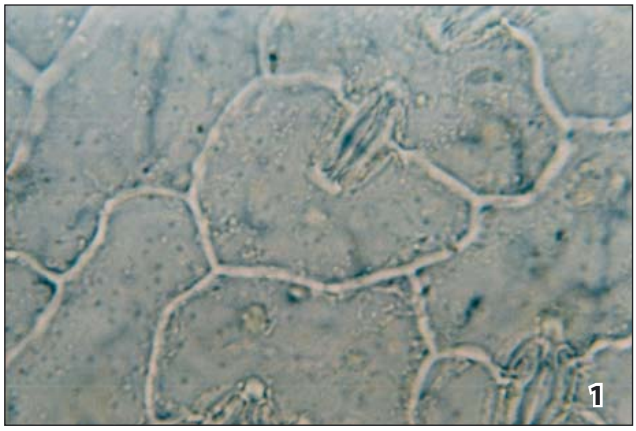


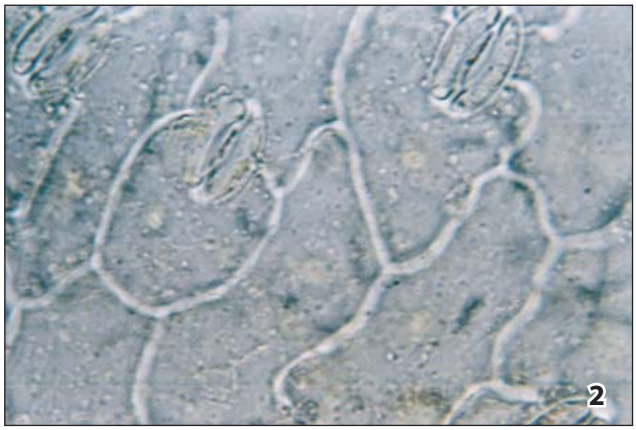


Figs 1-2. Light micrographs of the leaf epidermis in *P. lanceolata*. Site 3:

1, upper surface (×750); **2**, lower surface (×750). Bar scale 33 mm = 25 μm

Plate IV

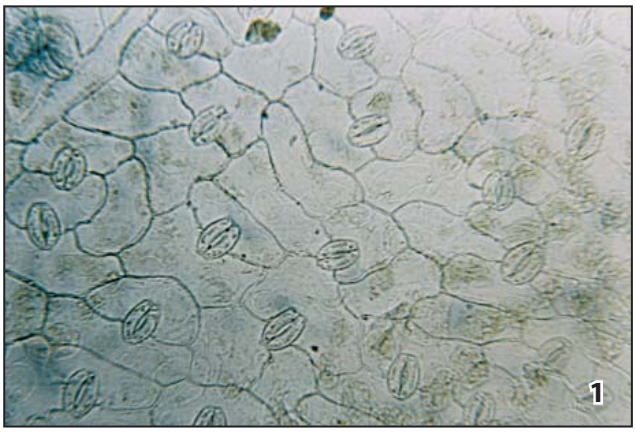


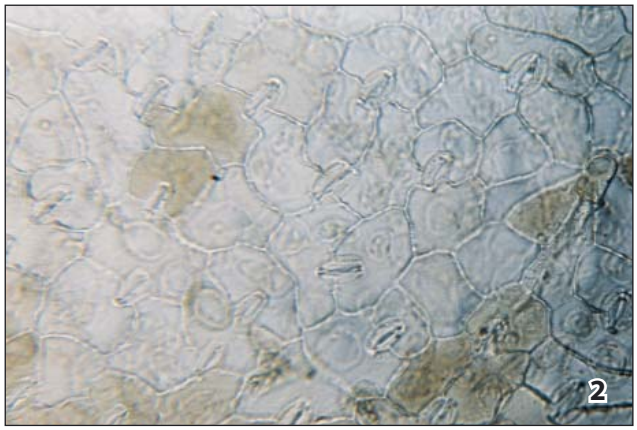


Figs 1-2. Light micrographs of the leaf epidermis in *P. lanceolata*. Site 4:

1, upper surface (×750); **2**, lower surface (×750). Bar sale 33 mm = 25 μm

Plate V

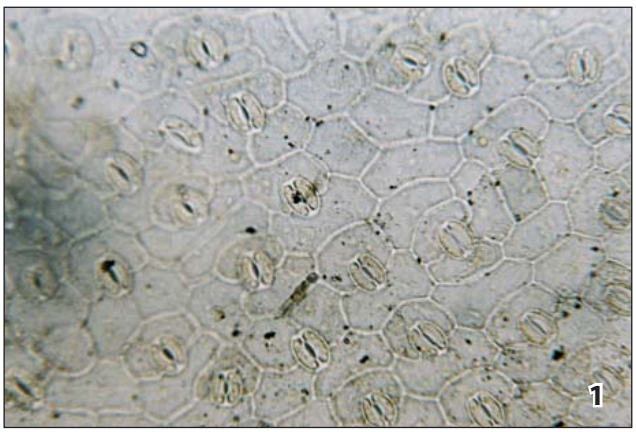


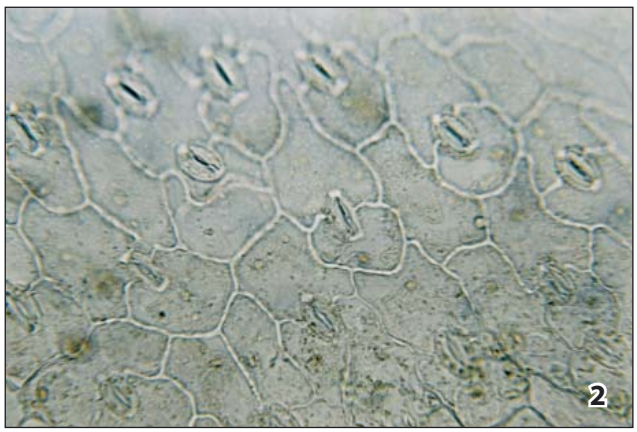


Figs 1-2. Light micrographs of the lower leaf epidermis in *P. lanceolata*:

1, Site 1 (×250); **2**, Site 2 (×250). Bar scale 11 mm = 25 μm

Plate VI





Figs 1-2. Light micrographs of *P. lanceolata* lower leaf epidermis:

1, Site 3 (×250); **2**, Site 4 (×250). Bar scale 11 mm = 25 μm

Discussion

The published studies about element concentrations in *P. lanceolata* from different urban and industrial areas and the results of the present research call for discussion.

Romero & Elejalde (1985) assessed the level of pollution using five wild plants (including *P. lanceolata*) from several industrial, urban and rural places of Biscay, Spain. The mean values determined by AAS method were: Ni 3.7, Cd 2.1, Cu 27, Zn 112, Pb 49, and Cr 1.8 ppm. The present research has shown similar mean concentrations of Cu, but the mean values of Cd, Zn and Pb are higher: 8, 7 and 3 times, respectively.

P. lanceolata was used as an accumulative bioindicator in the vicinity of a lead smelter in Budapest district (Kovacs & al. 1993). The authors have found 47 mg.kg^{-1} of Pb in the species leaves, whereas the maximum value found in the Plovdiv area was 338 mg.kg^{-1}, i.e. seven times higher. The surface soil layers (0–10 cm) of three profiles in Budapest accumulated on the average: Cd 6.5, Cu 548, Pb 626, and Zn 1499 mg.kg^{-1}. In this study the following mean concentrations were found in the surface layers from four sites: Cd 50, Cu – 263, Pb 763, and Zn 2522 mg.kg^{-1}, i.e. the values are up to 8 times higher for Cd. The applied analytical method in both investigations was ICP-AES, but the sampling in Hungary was done early in summer.

Wu & Antonovics (1976) determined the accumulation of 313 ppm Pb in *P. lanceolata* at the main street in Durham, North Carolina. The maximum value of Pb in the town of Plovdiv was 33 mg.kg^{-1} (in an area with high traffic), i.e. 9 times lower.

As Markert (1992) has generalised the information, the so-called inter-element relationships in the different species were of great interest. The present data contribute to the knowledge of inter-elemental correlations in the polluted areas.

The high concentrations of Cd, Pb and Zn in *P. lanceolata* determined in our study should alert one to the risk of collecting its leaves for medicinal use from urban and industrial areas.

It is well known that industrial pollutants (gases, heavy metals) have a negative effect on the plant species, e.g. leaf damages (Dässler 1976; Lacasse & Treshow 1976; Manning & Feder 1980; Schubert 1985) and/or poorer growth of the vegetative organs (Tingey & Reinert 1975; Ormord 1978; Dueck & al. 1985; Dimitrova & Ninova 1998). In the present study the pollutants from the non-ferrous smelter have caused visual leaf damages (chloroses, necroses, dry leaves) in *P. lanceolata*, as well as leaf growth suppression evidenced by the smaller size of the leaves. Length of the leaves was the more sensitive characteristic. It was established that the pollutants resulting from traffic have produced no visual damages on the leaves, but suppress their growth, as has already been shown for other species (Beavington 1973; Ormrod 1978).

Stomatal analysis showed that industrial pollution (Site 2, Site 3) was the cause for the increase of the number of stomata per 1 mm^2 and the decrease in their size. Salgare & Acharekar (1990), Dimitrova & Ninova (1994) mentioned similar changes of the stomatal characteristics for the herbaceous species in industrial areas and considered these characteristics very sensitive. The number of stomata per unit of area was listed first by Nikolaevskij (1989), among 10 morphological, anatomical, physiological, and biochemical characteristics for bioindication of pollution. This stomatal characteristic was included in the National Biomonitoring Program of Bulgaria (Ninova & al. 1999). Resistant species have leaves with xeromorphic characteristics, which probably help their adaptation to such stress factors as the heavy metals and gases (Ilkun 1978; Nikolaevskij 1979; Kutschera-Mitter & al. 1982). The tendency of an increase in the stomatal number found in *P. lanceolata* in Site 3 could be of adaptive nature, whereas for the plants from the territory of the smelter a positive correlation was confirmed between the number of stomata and visual leaf damages, found also by Nikolaevskij (1979) for the wild herbaceous species. Distortions of the stomatal guard cells were found, although seldom, in the industrially polluted site. Similar distortions were reported by other authors too (Stebbins & al. 1967; Patel & Inamdar 1971). A tendency for larger stomatal sizes was observed in plants growing in urban traffic pollution.

In conclusion, it should be noted down that the surface soil layers near the non-ferrous smelter have been heavily polluted with Zn, Pb, Cd, and Cu. *P. lanceolata* can be used as a good bioindicator for heavy metal accumulation in industrial and urban areas. Data on accumulative capacity allow us to recommend this species not only for Zn and Pb, but for indication of Cd, too. Some leaf characteristics have also proved suitable for bioindication of industrial pollution: the length of leaf lamina, the number and sizes of the stomata. Visible leaf damages have been found only in the area of non-ferrous industry. The sizes of the leaf lamina and stomata are also sensitive characteristics of urban traffic pollution.

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