The European Journal of Plant Science and Biotechnology ***©2008 Global Science Books***

Pollution-Induced Histochemical and Chemical Adaptation in *Pinus* Needles from the Palermo Area (Italy)

Maria Grazia Alaimo[*] • Daniela Vizzì

Department of Botany, University of Palermo, Via Archirafi 38, 90123 Palermo, Sicily, Italy
***Corresponding author*: * mg.alaimo@unipa.it mariagrazia.alaimo@tin.it**

ABSTRACT

We examined adaptations in histochemical composition and chemical element content in pine needles caused by pollution stress. Phenol content and trace element concentrations were measured in mature needles of *Pinus halepensis* Mill. and *Pinus pinea* L. from an urban ecosystem (the city of Palermo, Italy) and compared with samples collected in periurban sites where air pollution is presumably lower. Macro-, micro- and toxic element concentrations in needles are affected by urban pollution, seasonal conditions and by the passage of time: pollutant capture rates and phenol accumulation remain high in bioindicator plants; this indicates a sub-pathological reactivity to persistent stress factors that activates the plants' detoxification mechanism, prompting secondary metabolite production. Phenol content is positively correlated with some toxic elements (trace metals). Although this manuscript summarizes the results of a study conducted during a specific period, our findings can be considered representative and generalizable. The plants chosen for our investigations, *P. halepensis* and *P. pinea*, are widely used as ornamental plants in many cities, including Palermo, where it is also employed as a biomonitor to check the quality of the environment.

Keywords: biomonitor, phenols, stress, trace elements

INTRODUCTION

It is well-known that the air in urban and periurban areas is often polluted by a variety of harmful agents such as organic micropollutants, nitrogen and CO_x compounds and trace-element-enriched fine particulate. Fine particulate consist of a mixture of solid and liquid particles, including the metal lead, and zinc, nickel, copper, sulphates, nitrates, ashes, coal dust, etc. and are often involuntarily transported by pollen or intercepted by leaves. The leaf surface is the first zone of impact of air pollutants on plants. The leaf is selective in trapping and/or retaining particles. The nature of the plant surface has an influence on the retention behaviour (Smith and Jones 2000). Pollution often causes visible symptoms of foliar injury and the injury is associated with the increase in the accessibility of toxic elements to plants. The chemical composition of leaves is frequently used to monitor environmental pollution. The aerial part of same plants can collect metals from the air, as well as from the soil (Bergkrist *et al*. 1989; Starr and Ukonmaanaho 1999).

As part of an ongoing research project on the bioindication and biomonitoring properties of herbaceous and arboreal plant species in urban and remote (periurban) areas, we measured macro-, micro- and toxic element (trace element) concentrations in samples of *Pinus halepensis* Mill. and *Pinus pinea* L. (2002-2003) and assessed histochemical properties and stress-induced adaptations. Our investigations were carried out on *P. halepensis* and *P. pinea*, which are differently distributed in the urban and periurban areas of Palermo. We found that the two species show the same response to pollution both at a morphological and cytohistological level, and for this reason we refer to *Pinus* spp. when showing results throughout the paper.

Our aim was to detect and measure trace elements and determine their impact in passive biological indicators in the Palermo area (Sicily, Italy) in order to correlate histochemical properties with trace element content. Histochemical staining to detect phenols, conducted in different periods, allowed us to assess secondary metabolite accumulation in the vacuoles of needle mesophyll and epidermis cells. We can then verify if solubilized phenols in the cytoplasm will, when hyper-produced, impregnate the external walls of the epidermis to become localized, like an opaque coat, in the space between the epidermis external walls and cuticles. This process (Zobel and Nighswander 1991; Alaimo *et al.* 2000c) is a mechanism of plant protection and detoxification which evolves in the needles and produces modifications in them that can be detected by comparing them with control samples from unpolluted sites. We chose *Pinus* to monitor atmospheric pollution because it is widely used as an ornamental and forest plant in and around the city of Palermo, and because it has been the object of many of our studies (Alaimo *et al*. 2000a, 2000b, 2000c; Lombardo *et al*. 2001) and of others (Karolewski and Giertych 1995; Giertych *et al*. 1997; Giertych *et al*. 1999; Hiatt 1999). In these studies we demonstrated that the pine's adaptive response to urban pollution or anthropogenic stress, varies according to seasonal conditions and different environmental factors. Pollutant aggregation is facilitated by the needles' abundant secretions (Bargagli *et al*. 1991; Bargagli 1993; Dmuchowski and Bytnerowicz 1995) and epicuticular waxes.

We have sampled pine needles in and around the city of Palermo since 1998 (Alaimo *et al*. 2000a) in order to study air aerosol trace metals and determine the stress-induced effects adaptations in needle histochemistry, morphology and histology. Air pollution frequently causes visible needle injury symptoms. Injury is often associated with an increase in the plant's exposure to toxic elements (Angoletta *et al*. 1993). We investigated the distribution of trace elements in pine needles growing at unpolluted control sites and in polluted areas. Chemical composition of needles is often used to monitor environmental pollution: needle chlorosis or necrosis, cell structure alterations and increased phenols content are observed where there is an accumulation of toxic elements (Alaimo *et al.* 2003; Melati *et al.* 2006).

These results confirm the suitability of the method for

Received: 11 March, 2008. Accepted: 30 April, 2008.

Original Research Paper

Table 1 Site of collection of leaves and pollen samples in the urban and periurban sites of Palermo (2002-2003).

Acronym / Site	Site	Urban / Periurban Site	Vegetable entity	Date
PA7	Ponte Ammiraglio	Urban	*Pinus pinea* L.	February 2002
PI9	Piazza Indipendenza	Urban	*Pinus halepensis* Mill.	February 2002
ADG3	Via A.De Gasperi	Urban	*Pinus pinea* L.	February 2002
VB21	Via Belgio	Urban	*Pinus pinea* L.	February 2002
IF15	Isola delle Femmine west	Periurban	*Pinus pinea* L.	February 2002
IF5	Isola delle Femmine east	Periurban	*Pinus pinea* L.	February 2002
VFC29	Via F.Crispi	Urban	*Pinus halepensis* Mill.	February 2002
RLD6	Via L.Da Vinci north (Rotonda)	Urban	*Pinus halepensis* Mill.	February 2002
RLD8	Via L.Da Vinci sud (Rotonda)	Urban	*Pinus halepensis* Mill.	February 2002
SFB20	Sferracavallo	Periurban	*Pinus halepensis* Mill.	February 2002
FDM24	Fiera del Mediterraneo	Urban	*Pinus pinea* L.	February 2002
OB10	Orto Botanico	Urban	*Pinus halepensis* Mill.	February 2002
TPUD	Piazza Unità d'Italia	Urban	*Pinus halepensis* Mill.	June 2003
VFC29	Via F.Crispi	Urban	*Pinus halepensis* Mill.	June 2003
IF15	Isola delle Femmine west	Periurban	*Pinus pinea* L.	June 2003
SFB20	Sferracavallo	Periurban	*Pinus halepensis* Mill.	June 2003
PI9	Piazza Indipendenza	Urban	*Pinus halepensis* Mill.	June 2003
VB21	Via Belgio	Urban	*Pinus pinea* L.	June 2003
PIP	Piazza Indipendenza	Urban	*Pinus halepensis* Mill. pollen	February 2003
OBP	Orto Botanico	Urban	*Pinus halepensis* Mill. pollen	February 2002
ADGP	Via A. De Gasperi	Urban	*Pinus pinea* L. pollen	February 2002

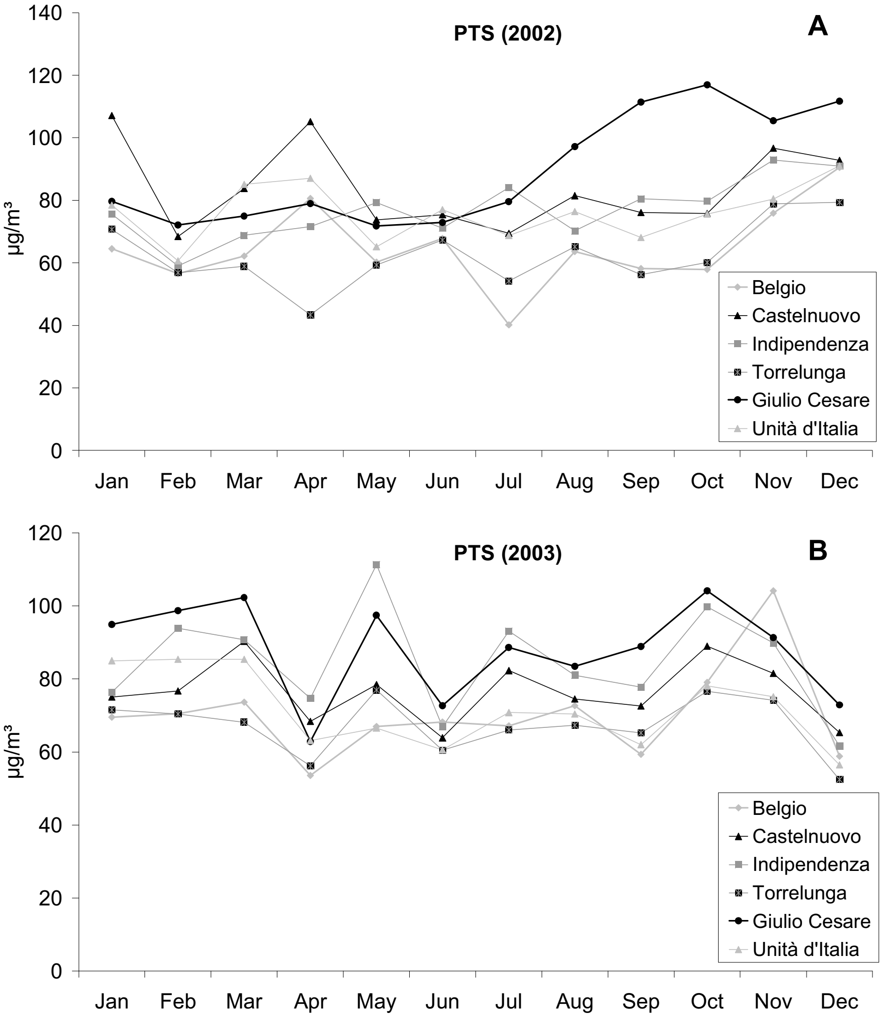


Fig. 1 Mean monthly values of TSD (Total Suspended Dusts) measured in the urban area of Palermo during 2002 (A) and 2003 (B). The values are expressed in µg/m^3 (Anonymous 2004, 2005).

the biomonitoring of urban areas. Our data indicate a different presence of pollutants depending on the season (winter/summer) and this can be referred to different metereological conditions and human activity, in particular urban traffic which decreases strongly in summer.

MATERIALS AND METHODS

Needles and pollen samples of two *Pinus* spp. (*P. pinea*, *P. halepensis*) were collected at several urban and periurban sites (listed in **Table 1**) in Palermo (Sicily) in which vascular plants are subjected to different environmental stress conditions. Needles and pollen, for the sake of comparison, were taken randomly from all the plant's exposed parts, selecting only fully-grown needles (sampled twice in February 2002 and June 2003).

Data on the quality of the air in Palermo and surrounding areas in 2002 and in 2003 are reported in **Fig. 1A, 1B** (Anonymous 2004, 2005). These data refer to monthly average values of total suspended dusts that can stick to the leaves of plants and pollen, and which contain inorganic and organic biological and chemical substances.

Needle and pollen samples were prepared for histological, histochemical and chemical analyses. Biological analysis began with a morphological assessment carried out *in situ* during sampling. Batches of at least 10 mature samples were collected. The tests were repeated five times. Later, in the laboratory, the needles were cryosectioned at the proximal and distal portions and stained with 0.08% Fast Blue BB solution, at pH 6.5 in 0.2 acetate buffer, they were incubated at room temperature for 30 min to assess polyphenols following Gahan's test (Gahan 1984). Dark, red-brown deposits indicate polyphenols, absent in the controls collected in unpolluted sites. Microscope observations were conducted by optical microscope (Orthoplan, Leica) and by SEM (Cambridge-Stereoscan 360). Samples were oven-dried at 80°C for 48 h before mounting for SEM observations. The dried needles were mounted on alluminium stubs with an acrylic adhesive ("Fotobond", Agfa-Gevaert Ltd.) and were double-coated with carbon and then gold prior to viewing in a scanning electron microscope at 20 kV (Woclkcrling 1988).

Both leaf and pollen samples were dried between glass slides, rather than dehydrated with ethanol, to highlight particulate sticking to the plant structures for the SEM evaluation.

Determining trace elements, mineralization of 0.5 g of needles, previously furnace-dried at a temperature of 80°C (pollen at 34°C) for one night and then reduced to very small fragments with an ordinary mill, were weighed out. Needles and pollen were treated with 6 ml HNO_3 at 65% and 1 ml H_2O_2 at 30% and put into a microwave mineralizer connected to a unit which prevents vapour stagnating inside the furnace. This mineralizer model supplies non-pulsed power, allowing for excellent control of the oxidization processes of organic samples. Volume was adjusted by adding distilled water to the clear solutions, now free of organic substances.

Analyses were carried out with an atomic absorption spectrometer (Perkin-Elmer 2380 USA) and flame atomisation (for Cu, Mn, Zn). Blanks were systematically measured; the total amount of Pb in the samples was in all cases negligible. All tests on both standards Mg-chloride, Cu-chloride and Zn-chloride (BDH Chemicals Ltd.) and samples were repeated three times to minimize the risk of errors. Major and trace elements were determined in a part of each sample with instrumental neutron activation analysis INAA (Ba, Br, Ca, Cr, Fe, K, Mo, Na, Sb, Sc, Sm) and ICP-MS (Al, Mg, P, Sr, Mn, Cu, Pb, Zn), using the standards NBS 1572 and 1632 B for reference (Alaimo *et al*. 2003).

Several replicates yielded a precision of 20-30% for minor elements and 5-10% for major elements (Actlab, Ontario, Canada). Concentrations are expressed in ppm or µg/g dry weight (Soto Gonzales *et al*. 1996). The tests were repeated five times. On account of biological, chemical and physical variables, homogenous within each class, but not between classes, we did not subject the data to parametrical statistical analysis, but to observations to map the presence or absence of plant injury. Given the uniformity of our findings, comparable to previous observations, we did not resort to sequential plans, which would have provided unequivocal evidence of the correlation between pollution and the injuries.

RESULTS

Chlorosis, necrosis at tips and in other parts of the needle, and tendency towards sclerophylly in often curling pine needles are measures of needle damage with consequential histochemical alterations. We interpreted ppm concentrations of trace elements and phenolic accumulations in needles as indicators of stress pollution.

Deviations from the normal type are regarded as reactions to suboptimal or damaging quantities or intensities of environmental factors, situations for which we broadly use the term stress.

Stress as such, of course, cannot be measured as it is only effective in the interaction between environmental factors and organisms. However, the strain, as caused by the action of stress on an organism, here *Pinus* spp., can be measured. The most frequently employed methods for quantification of damage after stress treatment are:

a) To highlight the presence of necrotic areas after stress application; plant tissue, for example, leaf, exposed to a defined stress shows necrotic brown areas. Necrotic areas are mainly due to plasmolysis of the cell of the mesophyll.

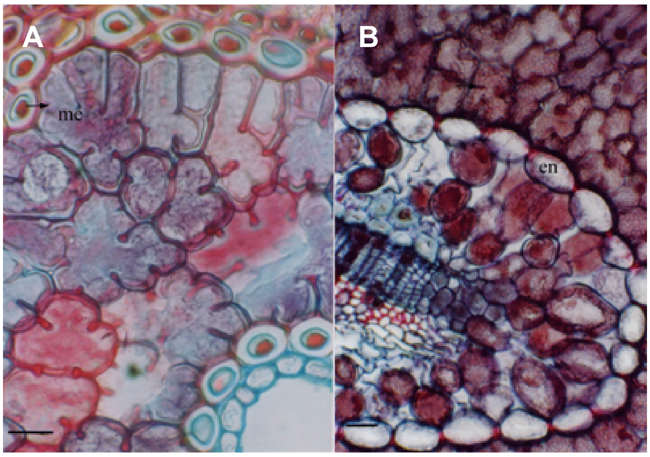


Fig. 2 (A, B) Cross sections of *Pinus halepensis* Mill. needle sample from a control site, showing the appearance of the cells in the absence of phenols. The mesophyll cells (me) exhibit a normal enlargement and the endodermis (en) is monostratified.

b) Absorption and accumulation of neutral red in the vacuole of undamaged cells. This is generally accompanied by a change in colour of the indicator, because the vacuolar pH is slightly acidic.

c) To highlight the increase and accumulation of phenols inside the vacuoles by the fast blue BB colorimetric reaction which show dark-red or brown accumulation (Schulze *et al*. 2005).

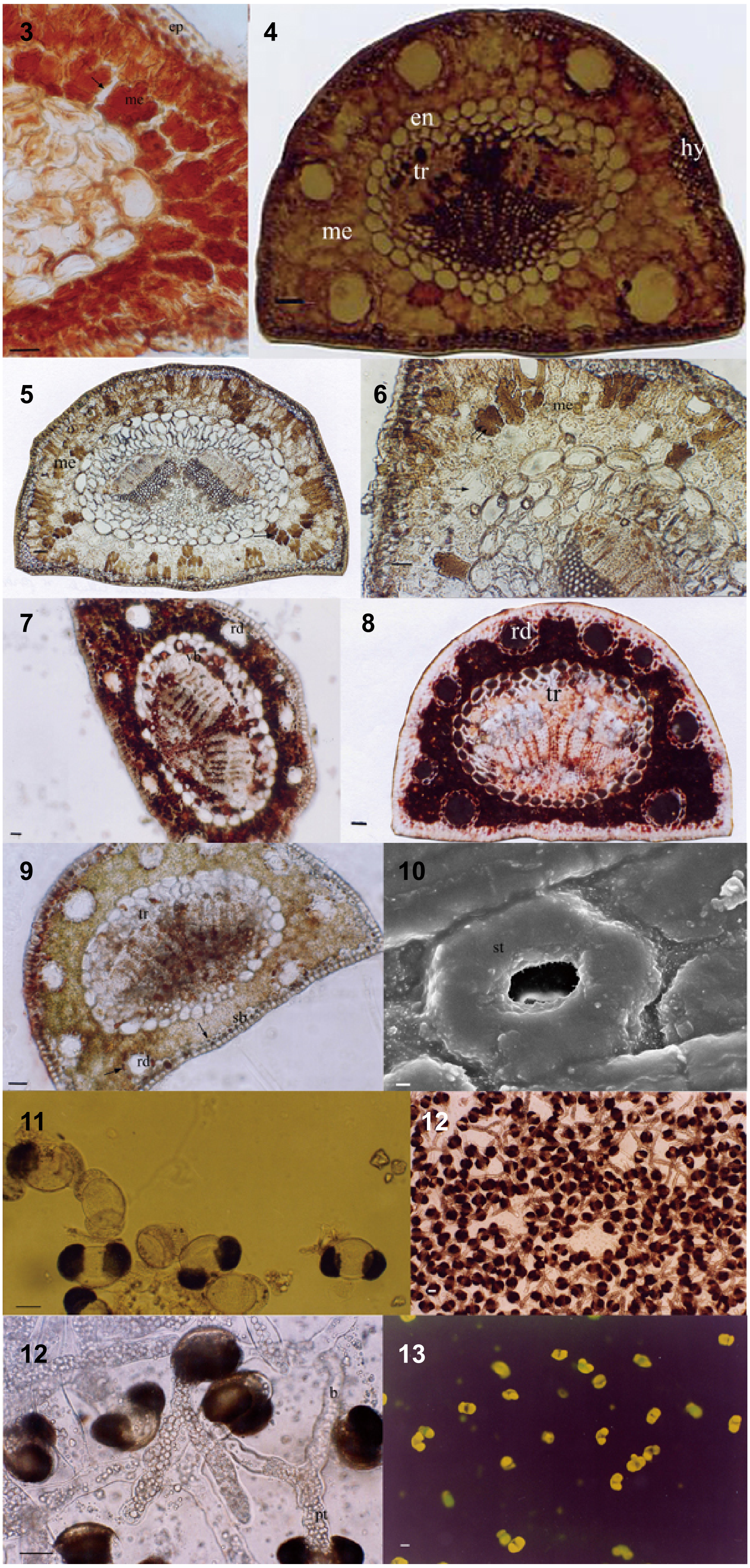
Polyphenol accumulation was regarded as an indicator of histochemical changes. Needle structures of the 2002-2003 samples were compared: transversal cryosections were stained with Fast blue BB to highlight phenols and cell contours.

Comparing needles from polluted sites with controls (**Figs. 2A-2B**) from unpolluted areas, the following stress-induced adaptations or injuries were observed in *Pinus* spp.: structural damage, cuticular and epidermal cell alterations, cell mesophyll dysplasias, necrosis, resin duct alterations, proliferation and disorganization of endoderm or conduction bands, polyphenol accumulations in needles (**Figs 3-9**). Several morphological and cytohistological injuries can be observed in the needles, involving distal portions, cuticles, mesophyll, endoderm (**Figs. 3-6**) resin ducts and conduction bands (**Fig. 4**), with collapse of the mesophyll. Secondary metabolites are produced in the needles, which have deformed cells. The mesophyll cells of polluted samples appear displasic (**Fig. 3**), full of abundant phenol. The phenols, later solubilized, are distributed in the cytoplasm (**Figs 3-4**). Endoderm proliferation, or band swelling and mesophyll structure reduction can be seen in needles from urban areas (**Figs 7-8**) compared with needles from more sheltered areas (**Fig. 9**), and **Fig. 10** also shows the collapse of stomata.

We carried out a chemical analytical evaluation of trace elements in needles with more extensive stress-induced injuries, and compared results with samples from denser urban sites or from urban sites with lighter traffic and less household heating pollution.

Trace element levels (macro-, micro- and toxic elements) recorded in February 2002 and in June 2003 in needle samples of *Pinus* spp. collected in various parts of the city of Palermo are reported in **Tables 2-5**.

For the sake of comparison, in parallel experiments we examined pollen for structural modifications (split exine, extruded matter) and changes in pollen quality (decrease in pollen vitality and germinability). The pollen are mixed; some grains are immature and small, others are mature, missing one of their air sacs or with a split central body exine (**Fig. 11**). Pollen vitality and germinability from urban stations with heavy traffic is good (**Figs. 12-13**).



Figs. 3-13. Fig. 3 Cross section of *Pinus halepensis* Mill. needle's basal portion from a site with intense traffic. Phenols fill the mesophyll (me) and the epidermis (ep) cells. The cells are dysplasic, stained dark red, and separated by wider intercellular spaces (arrow). **Fig. 4** Cross section of *Pinus pinea* L. needle sample from an urban area with moderate traffic. Figure shows accumulation of phenols in transfusion (tr) and hypodermic cells (hy), in some mesophyll cells (me), and around resin ducts (rd). The endoderm (en) appears bistratified. **Fig. 5** Cross section of *P. halepensis* needle sample from an urban area with moderate (1000/1200 cars per hour at peak hours 9-11, 17-19) traffic. It shows the gradual accumulation of phenols in the cells of mesophyll (me). **Fig. 6** Cross section of *P. halepensis* needle's distal portion. Figure reveals greater increase of necrotic areas (arrow) in the cells of mesophyll (me). Phenols are evident (double arrow) as a thin ribbon and scattered particles with darkened cytoplasm. **Fig. 7** Image of *P. halepensis* needle sample from an urban area with intense (2200/2500 cars per hour at peak hours 9-11, 17-19) traffic. Dense red droplets from phenols are located around the resin ducts (rd) and in the vascular bundles (vb), mesophyll (me) structure reduction is present. **Fig. 8** Cross section of *P. pinea* needle's basal portion. All the subepidermic cells, the resin ducts (rd) and some cells in the endoderm (en) and transfusion tissue (tr) are heavily stained red-brown, after fast blue testing to highlight phenols. Cytoplasm has disappeared, or is dimly discerned as a thin mass between phenols and cell walls. **Fig. 9** Cross section of *P. halepensis* needle's basal portion. Gradual red accumulation of phenols in undeformed cells, from control site, in the subepidermic layer (sb), in a few cells around the resin ducts (rd) and in transfusion tissue (tr). **Fig. 10** Scanning electron micrographs showing a collapsed stomata (st) of *P. pinea* needle from a polluted site. **Fig. 11** Mature pollen mixed with degenerate *P. halepensis* pollen material and particulate from a site with heavy traffic. **Fig. 12** Image to microscope of *P. halepensis* pollen grains. (**A**) Germinability of pollens at different magnifications. (**B**) Different shapes and lengths of the tube (pt), with protrusions and numerous branches (b) which will not become actual tubes. **Fig. 13** Fluorescent coarctated *P. halepensis* pollen grains with normal viability. Although the pollen grains appear morphologically deformed their vitality, as tested with fluorescein diacetate (Melati *et al*. 2004) is normal.

The results reported in **Tables 2-5** below indicate that the elements that are generally the most abundant in soil and rocks are also the most abundant in pine leaves. In particular, Al, Ca, Fe, Mg, Na and P have the highest weight percentages and Ca, Mg and K are the most predominant.

Zinc and copper appear to be slightly enriched with respect to soil; it must be borne in mind that both these elements are essential for plants. Zinc is an essential element in all organisms and plays an important role in biosynthesis of enzymes and same proteins. Normal concentrations of Zn in plants are in the range of 2 to 20 ppm (Kabata-Pendias and Piotrowska 1984).

Table 2 Macroelement concentrations (Feb 2002) in *Pinus* needles from different polluted or unpolluted sites.

SITE	Al	Ca	Mg	Na	P
PA7	300	15400	3300	1500	950
PI9	300	12900	4800	400	970
ADG3	200	7300	5200	2400	600
VB21	300	5000	3800	4800	890
IF15	200	5400	4800	6800	730
IF5	300	9700	2700	5000	1010
VFC29	200	7400	5700	6000	770
RLD6	300	4800	4200	4600	830
RLD8	300	16400	3900	1600	690
SFB20	500	15200	3500	3400	1060
FDM24	200	6500	5100	1100	910
OB10	800	3200	5300	3800	550
SD	171	4687	929	2057	163
Average	325	9100	4358	3450	830

Data are in ppm (dry weight).

Table 3 Microelement concentrations (Feb 2002) in *Pinus* needles from different polluted or unpolluted sites.

SITE	Cu	Mn	Pb	Zn	Mo	Fe	Cr	Sd
PA7	10	44	12	69	-0.1	540	1.2	1,1
PI9	11	37	14	56	-0.1	440	2.1	1,6
ADG3	11	31	14	60	-0.1	460	2	1.7
VB21	9	18	13	576	1.2	480	1.9	1.3
IF15	9	35	28	60	-0.1	370	1.3	0.6
IF5	8	34	21	65	-0.1	350	2.1	0.4
VFC29	18	20	16	75	1.5	620	2.2	2.8
RLD6	18	18	13	23	0.81	230	2	2.4
RLD8	17	28	18	20	1.6	560	3	3.4
SFB20	8	39	11	20	0.71	480	1.8	1.2
FDM24	14	46	12	18	0.86	360	1.4	2.3
OB10	5	41	8	18	0.73	190	0.7	0.6
SD	4	10	5	23	0.63	129	0.59	0.9
Average	11	33	15	45	0.6	423	1.08	4

Data are in ppm (dry weight). Negative values indicate less than the reporting limit.

Table 4 Macroelements concentrations (June 2003) in *Pinus* leaves from different polluted or unpolluted sites.

SITES	Al	Ca	Mg	Na	P
TPUD	500	14400	2700	1600	810
VFC29	300	5800	3500	4500	820
IF15	300	10500	2800	4200	920
SFB20	800	11700	2000	1200	1030
PI9	400	13900	3300	800	830
VB21	300	14100	2700	1100	890
SD	197	3290	528	1662	84
Average	433	11733	2833	2233	883

Data are in ppm (dry weight).

Table 5 Microelement concentrations (June 2003) in *Pinus* needles from different polluted or unpolluted sites.

SITES	Cu	Mn	Pb	Zn	Mo	Fe	Cr	Sb
TPUD	17	25	6	39	0.81	600	2.6	2.4
VFC29	23	18	6	46	1	800	2.6	2.7
IF15	16	41	7	27	0.33	400	1.5	0.9
SFB20	13	40	-3	30	0.33	400	0.9	0.6
PI9	15	52	5	21	0.71	500	2.4	2.2
VB21	28	47	8	32	1.6	100	4	5.9
SD	6	13	4	9	0.48	234	1.1	1.9
Average	19	37	5	32	1	467	2	2

Data are in ppm (dry weight). Negative values indicate less than the reporting limit.

Normal concentration of Pb in plants are < 10 ppm (Kabata-Pendias and Piotrowska 1984). Allen *et al.* (1974) and Kabata-Pendias and Piotrowska (1984) considered a much lower value of 3 ppm as a normal natural level for plants. Kabata-Pendias and Piotrowska (1984) considered 30 ppm as an excessive or toxic level of this element.

Copper is similar to Zn, i.e. it is a microelement essential for all organisms and is an important constituent of many enzymes of oxidation-reduction reactions (Raven and Johnson 1986). According to Kabata-Pendias and Piotrowska (1984), the normal concentration of Cu in plants ranges from 2 to 20 ppm, but in most plants the normal Cu concentrations are in a narrower range of 4-12 ppm.

The highest average concentrations of Al were recorded at site OB10 (800 ppm) in February 2002, and at site SFB20 (800 ppm) in June 2003. Mo concentrations are consistently very low, less than 2 ppm. At site VFC29 (February 2002) the highest average concentrations of Zn (75 ppm), Cu (18 ppm) and Cr (2.2 ppm) were recorded; only at site RLD8 is the level of Cr slightly higher (3 ppm), while Cu has the same value of 18 ppm at site RLD6 in the same area. In June 2003, the highest levels of Cu (28 ppm) and Cr (4 ppm) were found at site VB21, and the highest level of Zn was recorded at site VFC29 (46 ppm).

Cu and Zn levels are higher than those indicated by Kabata-Pendias and Pendias (1993). In February 2002 the highest average concentration of Pb was recorded at site IF15 (28 ppm), while in June 2003 Pb level was highest (8 ppm) at site VB21.

In February 2002, the highest average concentration of Mg was found at site VFC29 (5700 ppm); in June 2003 levels were again highest at site VFC29 (3500 ppm). Mn levels were 46 ppm at site FDM24 in February 2002, and 52 ppm at site P19 in June 2003.

Fe concentrations are lowest at site OB10 (190 ppm) in February 2002 and at site VB21 (100 ppm) in June 2003.

DISCUSSION

The surfaces of leaves on aerosol-contaminated plants contain numerous trace elements, in quantities that are determined by environmental conditions, season and leaf characteristics (Smith *et al.* 1978). Trace elements are easier to detect in urban and industrial sites; some stick to leaf surfaces, while others, such as Zn and Cd (Little and Martin 1972), reach the plant's internal tissues (Haghiri 1973). A few studies conducted on unwashed lettuce leaves collected in urban areas have drawn attention to high concentrations of some trace elements (Beavington 1975a, 1975b); other research has identified the presence of trace elements in rainwater (Hallsworth and Adams 1973). Numerous works have confirmed the presence of trace elements in leaves from urban sites (Smith 1972; 1973). Air contaminants stick to the plant and some trace elements can then be absorbed (Haghiri 1973). A part of Pb remains on the leaf surface (Zimdahl 1976). Some authors consider leaf absorption of some trace elements to be limited (Schuck and Locke 1970), while others have established – for Pb for example – that insoluble salts remain on the outside of the leaf epidermis (Smith 1970) while soluble salts (for example Pb, Cl, Br) or salts that become soluble after impact, seep into the internal tissues of the plant through the stomata and other entrances. Only a part of Pb will, after a determinate amount of time (about two weeks), penetrate the cuticle (Arvik and Zimdahl 1974). Water washing removes particulate, more easily from smooth leaf surfaces than from sticky, resinous surfaces like the pine, or hairy surfaces (Smith and Jones 2000). However, on pine needles and conifers in general, atmospheric pollutants attach to epicuticular waxes and washing tends to be ineffective, unless chloroform is used. In this case, washing for 1 minute removes all the surface contaminants and the elementary composition of the needles of different ages from different sites becomes uniform (Wyttenbach *et al.* 1992). For this reason we propose to conduct new research on pine in the near future, using chloroform for our experiments. In this study, our plant needles were simply cleaned under a stereoscopic microscope to remove foreign bodies, then dried in an oven to prevent fungus and bacteria growing, and avoid elements being lost or contaminated. The subsequent phases were mineralization and chemical measurement. We examined needle stress-induced adaptations and measured trace element concentrations in samples of *Pinus* spp. collected in urban and periurban sites of the city of Palermo (Italy). The needle adaptations we observed involve external morphology, as well as cytohistological structure: the incidence of malformations has decreased since our study began, while secondary metabolite (phenols) production has remained high, confirming the excellent bioindication and biomonitoring properties of the pine, and the accumulative properties of other plants growing at the same sites, such as pellitory. We can currently observe a decrease in some trace element incorporation in the pine, and a high production of polyphenols, triggered as a defence mechanism. We continued to find modifications in the 2003 pollen samples, consisting mainly in a decrease in vitality but not germinability, or hyper-production of pollen in inflorescences which are bigger than normal (Melati *et al.* 2004). Metal content in leaves is affected by natural processes (earth dusts, sea spray) and by anthropogenic activities (fossil fuel consumption, household heating and industry). The level of Ca is undoubtedly influenced by the city's calcareous substratum, and Al by the soil's clay component. Other elements, such as Pb, Cu, Cr, Zn and Sb come from human sources. Cd and Zn are used in car batteries and carburettors (Alaimo *et al*. 2000c, 2003).We compared winter samples (February 2002) with summer samples (June 2003) and observed, for example, that Pb concentration reaches 15 ppm in the winter compared to 5 ppm in the summer. Levels of Zn also are higher in the winter (45 ppm) compared to the summer (32 ppm in June 2003). On the contrary, Cr levels are 2 ppm in June 2003 and 1 ppm in February 2002. Sb levels remain constant at 2.2 ppm. Cu, Pb and Mo levels are higher in the summer. Taking Fe as an example, we can see that levels drop from 423 ppm in 2002 and then rise to 467 ppm in 2003, proving that concentrations cannot be attributed to a simple accumulative effect of pollutants on the leaves. The elements Cu and Zn are essential for normal plant growth. In winter 2002, Cu concentrations range from 5 to 18 ppm, and Zn concentrations from 18 to 75 ppm: this Zn level diverges greatly from the normal levels of 2-20 ppm indicated by Pendias and Piotrowaska (1984). In summer 2003 Cu levels went up, ranging from 13 to 28, and Zn levels fall, ranging from 21 to 46 ppm. Higher Pb concentrations were recorded in areas with heavier traffic, namely at sites RLD6 (13 ppm), UFC29 (16 ppm), ADG3 (14 ppm) and P19 (14 ppm) in February 2002. Levels were also high at sites IF15 (28 ppm), IF5 (21 ppm). Concentrations were lower in June 2003. Cu is essential for plants. Concentrations do not exceed 4 ppm (June 2003); in winter (February 2002) they remain below 2.3 ppm. Cu is produced by the wear and tear of brake pads and tyres, and Fe by the wear and tear of car mechanical parts. Our results demonstrate that *Pinus* spp. absorbs Al, Cu, Mg, Mn, Pb and Zn. This phenomenon of absorption (Smith and Jones 2000) is regulated more by seasonal and climactic factors than by type of site. Even the sites chosen as controls (white) do not appear to be untouched by pollution, probably due to pollutant re-suspension (Melati *et al.* 2006). Applying the label "biological indicator of resistance" to pollutants four level of damages to plants can be identified; *Pinus* spp. is rather sensitive to chronic damage caused by toxic gases, compared to other plants which can be classified as relatively resistant or not very sensitive (Levitt 1980; Lorenzini 1999) while *Ficus* is less sensitive, and *Pittosporum* and *Parietaria* (Alaimo *et al.* 2005) can be classified as insensitive. This research confirms that trace element pollution is still present and that we can easily detect its effects in bioindicator and biomonitor plants (Smith and Jones 2000), such as species belonging to the genus *Pinus*, by examining the plants' structural adaptations and histochemical reactivity, and measuring macro- and micro-element content in leaves.

Although the structural data have not been subjected to any statistical analysis, the probability that they are indicative of atmospheric pollution linked to the presence of trace elements is high.

The structural modifications in the polluted plants are uniform and recurrent, and therefore merit careful consideration.

The increase in secondary metabolite production is probably connected to the role that these compounds play in respiration, as conveyers of electrons along the oxidative chain. The phenomenon must also be related to an increase in lignin production, a defence mechanism of the plant to provide a barrier to block the entry of pollutants (Strack *et al.* 1988).

In stress conditions secondary metabolites stabilize the membranes, increasing cohesion between the two layers and preventing membrane destruction. However, when the production of secondary metabolites is abnormal, they are discharged into the cytoplasm, damaging cell structures and causing necrotic areas to form.

The plant samples were collected in different sites at different times of year. Histochemical characteristics and trace element concentrations varied in relation to sampling period and less in relation to the site chosen for sampling.

ACKNOWLEDGEMENTS

We wish to thank Prof. Maria Rita Melati for her advice and guidance and we are grateful to Prof. Maurizio Sajeva for helpful discussion and revision of the manuscript.

REFERENCES

Alaimo MG, Lipani B, Lombardo M, Orecchio S, Turano M, Melati MR (2000a) The mapping of stress in the predominant plants in the city of Palermo by heavy metal dosage. *International Journal of Aerobiology* **16**, 47-54

Alaimo MG, Lipani B, Lombardo M, Orecchio S, Turano M, Melati MR (2000b) Indice biologico di resistenza al carico di immissioni di inquinanti. *Informatore Botanico Italiano* **32**, 73-76

Alaimo MG, Dongarrà G, Melati MR, Monna F, Varrica D (2000c) Recognition of environmental trace metal contamination using pine needles as bioindicators – The urban area of Palermo (Italy). *Environmental Geology* **39**, 914-924

Alaimo MG, Colombo P, Firetto A, Trapani S, Vizzì D, Melati MR (2003) Stress-induced injuries and trace element concentrations in vascular leaf plants from an urban environment (Palermo, Italy). *Journal of Trace Elements in Medicine and Biology* **17**, 65-74

Alaimo MG, Vizzì D, Melati MR (2005) Histochemical properties and trace element concentrations in *Parietaria* plants from urban sites (Palermo, Italy). *Aerobiologia* **21**, 21-31

Allen S, Grimshaw HM, Parkinson JA, Quarmby C (1974) *Chemical Analysis of Ecological Materials*, Blackwell Scientific Publications, Osney Mead, Oxford, UK, 565 pp

Angoletta M, Bentivoglio A, Giusto D (1993) Accumulo di metalli pesanti in piante erbacee. *Inquinamento* **5**, 74-79

Anonymous (2004) *Il rilevamento dell'inquinamento atmosferico e acustico nel comune di Palermo.* 4^a relazione 2001-2002. AMIA SpA, Palermo, 319 pp

Anonymous (2005) *Il rilevamento dell'inquinamento atmosferico e acustico nel comune di Palermo.* 5^a relazione 2002-2003. AMIA SpA, Palermo, 319 pp

Arvik WH, Zimdahl RL (1974) Barriers to foliar uptake of lead. *Journal of Environmental Quality* **3**, 369-370

Bargagli R, Marchigiani C, Siegel BZ, Siegel SM (1991) Trace metals anomalies in surface soils and vegetation on two active islands volcanoes: Stromboli and Vulcano (Italy), *Science of the Total Environmental* **102**, 209-222

Bargagli R (1993) Plant leaves and lichens as biomonitors of natural or anthropogenic emissions of mercury. In: Markert B (Ed) *Plant as Biomonitors. Indicators for Heavy Metals in the Terrestrial Environment*, VHC, Weinheim, pp 461-484

Beavington F (1975a) Some aspects of contamination of herbage with copper, zinc and iron. *Environmental Pollution* **8**, 65-71

Beavington F (1975b) Heavy-metal contamination of vegetables and soil in domestic gardens around a smelting complex. *Environmental Pollution* **9**, 211-217

Bergkrist B, Folkeson L, Berggren D (1989) Fluxes of Cu, Zn, Pb, Cd, Cr and Ni in temperate forest ecosystem. A literature review. *Water, Air and Soil Pollution* **47**, 217-289

Dmuchowski W, Bytnerowicz A (1995) Monitoring environmental pollution in Poland by chemical analysis of scots pine (*Pinus sylvestris* L.) needles. *Environmental Pollution* **87**, 87-104

Gahan PB (1984) *Plant Histochemistry and Cytochemistry*, Academic Press, London, 301 pp

Giertych MJ, de Temmerman LO, Rachwal L (1997) Distribution of elements along the length of Scots pine needles in a heavily polluted and a control environment. *Tree Physiology* **17**, 697-703

Giertych MJ, Karolewski P, de Temmerman LO (1999) Foliage age and pollution alter content of phenolic compounds and chemical elements in *Pinus nigra* needles. *Water, Air, Soil Pollution* **110**, 363-377

Haghiri F (1973) Cadmium uptake by plants. *Journal of Environmental Quality* **2**, 93-96

Hallsworth EG, Adams WA (1973) The heavy metal content of rainfall in the East Midlands. *Environmental Pollution* **4**, 231-235

Hiatt MH (1999) Leaves as an indicator of exposure to airborne volatile organic compounds. *Environmental Science and Technology* **33**, 4126-4133

Kabata-Pendias A, Pendias H (1993) Trace elements in soil and plants. PWN, Warsaw, pp 89-319 (in Polish)

Kabata-Pendias A, Piotrowska M (1984*) Zanieczyszczenie Gleb i Roslin Uprawnych Pierwiastkami Sladowymi*. CBR-opracowanie problemowe, Warszaw, Poland, 420 pp

Karolewski P, Giertych MJ (1995) Changes in the level of phenols during needle polluted development in Scots pine populations in a control and environment. *European Journal of Forest Pathology* **25**, 297-306

Levitt J (1980) *Response of Plants to Environmental Stresses*, Academic Press. New York, 607 pp

Little P, Martin MH (1972) A survey of zinc, lead and cadmium in soil and natural vegetation around a smelting complex. *Environmental Pollution* **3**, 241-254

Lombardo M, Melati MR, Orecchio S (2001) Assessment of the quality of the air in the city of Palermo through chemical and cell analyses on *Pinus* needles. *Atmospheric Environment* **35**, 6435-6445

Lorenzini G (1999) *Le Piante e l'Inquinamento dell'Aria*, Edagricole, Bologna, Italy, 335 pp

Melati MR, Alaimo MG, Mazzola A, Vizzì D (2004) A study on the quality of the environment using pollens. Investigations in urban and periurban areas of Palermo (Sicily, Italy). *Recent Research Developments in Environmental Biology* **1**, 379-394

Melati MR, Alaimo MG, Vizzì D, Firetto A, Mazzola A (2006) Agricolture: Monitoring the effects on humans and various agricultural plants of air pollution by particulate with trace metals. *Recent Research Developments Agronomy and Horticulture* **2**, 95-115

Raven PH, Johnson GB (1986) *Biology*, Times Mirror/Mosby College Publishing, St. Louis, 1198 pp

Schuck EA, Locke JK (1970) Relationship of automotive lead particulates to certain consumer crops. *Environmental Science and Technology* **4**, 324-330

Schulze ED, Beck E, Müller-Hohenstein K (2005) *Plant Ecology*, Springer-Verlag, Berlin, 702 pp

Smith WH (1970) Salt contamination of white pine planted adjacent to on interstate highway. *Plant Disease Reporter* **54**, 1021-5

Smith WH (1972) Lead and mercury burden of urban woody plants. *Science* **170**, 1237-1239

Smith WH (1973) Metal contamination of urban woody plants. *Environmental Science and Technology* **7**, 631-636

Smith WH, Stakawicz BJ, Harkov RS (1978) Trace-metal pollutants and urban-tree leaf pathogens. *Transaction of the British Mycological Society* **70**, 29-33

Smith KEC, Jones KC (2000) Particles and vegetation: implications for the transfer of particle-bound organic contaminants to vegetation. *Science of the Total Environment* **246**, 207-236

Soto Gonzales E, Alonso Rodriguez E, Lopez Mahìa P, Mumiategui LS, Prada Rodriguez D (1996) Determination of trace elements in tree leaves. *Annali di Chimica* **86**, 181-191

Starr M, Ukonmaanaho L (1999) Trace metal budgets for a small forested catchment in eastern Finland. *Proceedings of Extended Abstracts: 5th International Conference on the Biogeochemistry of Trace Elements*, July 11-15 Vienna, Austria, pp 680-681

Strack D, Heilemann J, Momken M, Wray V (1988) Cell wall-conjugated phenolics from coniferae leaves. *Phytochemistry* **27**, 3517-3521

Wjttenbach A, Bajo S, Tobler L, Adam M, Zottl HW (1992) Elemental concentrations in spruce needles, how to obtain and interpret the results. IAEA – SM – 325/185, IAEA, Vienna, pp 535-545

Woclkcrling WJ (1988) The coralline Red Algae: An analysis of the Genera and Subfamilies of Norgeniculate Corallinaceae. *British Museum (Natural History) and Oxford University Press, London and Oxford*, 226 pp

Zimdahl RL (1976) Entry and movement in vegetation of lead derived from air and soil sources. *Journal of the Air Pollution Control Association* **26**, 655-660

Zobel A, Nighswander JE (1991) Accumulation of phenolic compounds in the necrotic areas of Austrian and red pine needles after spraying with sulphuric acid: a possible bioindicator of air pollution. *New Phytologist* **117**, 565-574