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STRUCTURAL MODIFICATIONS IN BLACK PINE NEEDLES AS POTENTIAL BIOMARKERS OF ENVIRONMENTAL POLLUTION

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Abstract: This study presents micro-morphological characters and anatomical modifications detected in black pine needles collected from urban polluted areas in order to propose new biomarkers useful in air pollution biomonitoring. The plant samples were collected during the July 2021 from 10 different polluted sites from Bucharest and analyzed using light and scanning electron microscopy. When compared with the samples collected from less polluted sites, needle micromorphology and structure have showed significant alterations such as injured stomatal complex, eroded forms of tubular wax type and complete degradation of wax crystalline structure, raised peristomatal areas, early wax plugs perforations and abnormal epidermal teeth. The loads of heavy metal of samples were assessed by X-ray fluorescence spectrometry and the results showed a positive correlation between structural alteration incidence and needle heavy metal surface depositions.

Keywords: air pollution, *Pinus nigra*, structural alterations, biomonitoring, biomarkers

1. INTRODUCTION

Presently, urban ecosystems face an intensive air pollution mainly from anthropogenic activities, including transportation, domestic use of fossil fuels and industrialization but also from unknown sources. Due to the mixtures of known and unknown pollutants, chemical monitoring may not be always satisfactory (Hanson et al., 2013). A reliable alternative is to use biological indicators such as modification in plant morphology, structure and physiology. This approach is relatively new but increasingly used by scientists to measure the level of air pollution. The method has the advantage that is cost effective, and also that the effect is evident on plants before it can be harmful to humans (Mitra & Reddy, 2014).

Plant leaves are vital organs conditioning growth, adaptation and survival but also, serve as the main contact and exchange interface with the environment. Therefore, in a highly anthropized environment like urban areas, leaves are the most sensitive and responsive organs of plants. A range of leaf traits like wax structure, epidermis morphology, stomatal and trichomes structure were subject of investigations and, based on it, several biomarkers of environmental pollution were detected and reported (Rai et al., 2010, Mitra & Reddy, 2014, Areington et al., 2015, Mukherjee et al., 2019, Kozłowski et al., 2019). Among plant species investigated in this respect, conifer tree seems to be the most suitable, due to their perennial leaves facilitating a prolonged exposure period of time and consequently providing more obvious responses. The most investigated conifer species to describe biomarkers is Scots pine (*Pinus sylvestris* L.), in opposite, there are only a few reports on black pine and most of them refer to its bark as potential biomarker of air pollution (Chiarantini et al., 2016, Rimondi et al., 2020).

Black pine (*Pinus nigra* J. F. Arnold) is a hardy coniferous species withstanding drought, heat and pollution thus widely distributed in urban environments along roads, in parks, gardens and green landscapes. Besides from its ubiquity in urban areas, the black pine needles are persistent from 4 to 7 years and this prolonged perenniality make them a very good investigation material to describe air pollution biomarkers.

European Environment Agency (EEA), ranks European cities according to their average levels of fine particulate matter, over the past two full calendar years. According to EEA, Bucharest rank is 263 from a total of 323 of European cities, with an air quality categorized as poor (EEA, 2021). With levels of fine particulate matter ($PM_{2.5}$) above the guideline value of the World Health Organization, Bucharest is currently one of the most polluted European cities.

The main purpose of this study was to identify some structural traits with biomarker value in order to use black pine as bioindicator of air pollution in urban areas.

2. MATERIALS AND METHODS

2.1. Sample collection and study area

Needle material were collected on full vegetation stage (July 2021) from 10 different sites from Bucharest located along roadsides with intense traffic. For an acurate evaluation of pollutans effect we have also analysed samples collected from sites less polluted sellected from Campina city (Prahova county). The geographical coordinates of the sampling sites are shown in Table 1 and each site locations is pinned in a map shown in Figure 1.

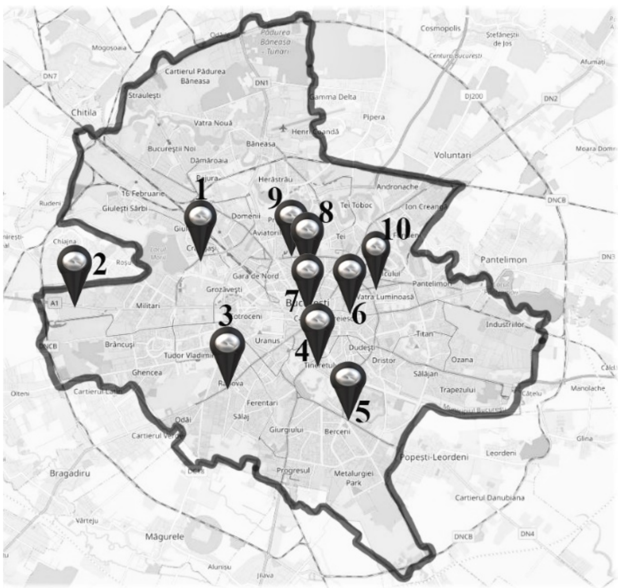

Figure 1. Location of the sampling sites

Pairs of 1- and 2-year-old needles were collected from each site from branches about 1.5 – 2 m above ground level, which is recognized as the average height where humans breathe.

Fresh collected needle samples were closed in paper envelopes and preserved at 4 ºC for further investigations.

Table 1. Geographical coordinates of sampling sites

Sample site	Geographical coordinates	Site address
P1	44°27'05.9"N 26°02'45.9"E	Șos. Virtuții
P2	44°26'05.4"N 25°59'07.0"E	Bd. Iuliu Maniu
P3	44°24'04.3"N 26°03'09.1"E	Intrarea Ghimeș
P4	44°24'15.1"N 26°05'46.9"E	Bd. Pieptănari
P5	44°23'36.4"N 26°07'20.2"E	Șos Olteniței
P6	44°25'34.2"N 26°07'04.8"E	Bd. Unirii
P7	44°25'39.9"N 26°06'12.9"E	Piața Unirii/Parc Unirii
P8	44°26'13.0"N 26°06'07.3"E	Bd. Nicolae Bălcescu
P9	44°27'09.8"N 26°05'11.1"E	Piața Victoriei/ Cotroceni
P10	44°26'29.2"N 26°08'01.5"E	Piața Iancului
P.ctrl Câmpina	45°08'29.3"N 25°46'38.6"E	Str. Zăpodie

2.2. Histological analysis

For the histological investigation, fresh fragments of about 1 cm in length were taken from needle tip, middle and base. The samples were cut using a hand microtome (Euromex MT. 5500) and the obtained cross sections (15-20 µm thick) were transferred onto glass slides. Histological observations were made using optical microscopy under a Nikon Eclipse E200 microscope and micrographs were recorded using a Nikon Coolpix 5400 digital camera.

2.3. Scanning electron microscopy studies

For scanning electron microscopy (SEM) investigations the needles were cut in 2-3 cm long pieces including the tip, middle and base and mounted on aluminium stubs with double-sided adhesive electroconductive tape, in order to expose both the adaxial and abaxial surfaces. The mounted specimens were coated with a thin layer of gold (99.9% purity) using the Jeol JFC 1300 Auto Sputter Coater. Coated samples were placed into the microscope vacuum chamber and examined using the Jeol JSM 66-10 LV scanning electron microscope at an accelerating voltage between 10kV and 20kV, at the magnification range of 80-8,000×.

2.4. X-ray fluorescence spectrometry

Total metal content of samples was measured with the X-ray fluorescence spectrometer Rigaku ZSX100e. In order to discriminate between particulate matter deposition and metals accumulation, samples of both washed and unwashed needle tissue were analyzed. The washed samples were obtained by soaking the needles in distilled water for 60 min followed by a gentile brushing and three times rinsing with distilled water. Both washed and unwashed plant material samples were oven dried at 60 °C until constant weight and then grounded onto a fine powder. The sample preparation for powder (Loose powder method) have been used in the atmosphere of He 99.99% purity. 5 g of the plant powder from each sample were placed in the sample cells of 30 mm readable diameter (Cat. No. CH 1530) covered on both sides with polypropylene foil of 6μm thickness from Rigaku (cat. no. 3399G003) and fixed with snap-on ring. The spectrometer readings were assessed by the software package ZSX, version 5.40 from Rigaku. For quantitative analyses, the software converts the intensity peak in mass percentage. The results were generated in oxide percentage of the detected elements (Young et al., 2016). The final results were expressed in mg of metal in particulate matter deposition per kg of dry matter according to formula 1.

$$PMD = M_{uw} - M_{w} \quad (1)$$

Where PMD is metal content of the particulate matter deposition, M_{uw} and M_{w} is the metal content of unwashed and washed samples, respectively.

3. RESULTS

SEM observation (Fig. 2) when compared with the samples from less polluted areas (as shown in A1-3 and D1) showed that all the 2-year old samples collected from Bucharest have strong evidence of air pollutants damage including: complete alteration of crystalline wax structures (B3); Florin rings-like structures (B1); raised abnormal epidermal cells (B1); irregular leaf surfaces due to collapse of underlying epidermal cells (D2); damaged unfunctional stomatal complex (B1, 2, 3; C1, 2, 3; E1, 2); stomatal wax plugs erosion with channel-like perforations (C3); and abnormal serrations (D2, 3). In addition to micromorphological alteration SEM investigation showed a wide range of surface depositions from crystalline matters (C1) to microspheres composed from iron oxide or glass (E1, 2). Moreover, in most of the samples occurred an intensive surface fungal colonization (E3).

Optical microscopic observation upon needle cross sections (Fig. 3) showed structural modifications such as: numerous resin canals, collapsed phloem tissue, hypertrophied Strasburger cells, and cells with phenolic droplets.

Metal composition of particulate matter (PM) depositions showed that *P. nigra* needle surface could retain various type of metals; among these iron and potassium were retained in highest concentration (Fig. 4).

4. DISCUSSION

Environmental pollution alters the morphology and structure of leaf and thus their properties (Niu et al., 2020). In conifer tree species changes in epicuticular wax structures have been successfully used as biomarker of air pollution (Grodzińska-Jurczak, 1998) being described mostly in Norway spruce (*Picea abies* (L.) H. Karst.) and Scots pine (*Pinus sylvestris* L.), but none in black pine. Our results confirm the presence of similar wax alteration as previously described in other species, like: different degrees of fusion of wax tubes, accelerated erosion of stomatal waxes leading to stomatal occlusion and early wax plugs formation. In addition to previously described wax changes, we have found that in condition of urban pollution wax plugs from 2-years old needles shows signs of erosions like heavy perforations and multiple channels, unlike the needles developed in unpolluted areas where wax -plugs are functional at least six years. Wax plugs are completely developed in 2-yers-old needle and are involved in: reduction of water loss, protection against pathogens, preventing entry of water into stomatal pores and preventing the formation of a water film on leaves (Mohammadian et al., 2007). After the growing process is completed the epicuticular waxes cannot be resynthesized (Grodzińska-Jurczak, 1998), thus any alteration in wax plugs is irreversible and is often a sign of air pollution. In this respect channels-like perforation of wax plugs in 2-years-old black pine needles, could be considered a reliable biomarker of air pollution.

Another micromorphological feature observed for the first time in polluted air exposed needles, is the presence of Florin rings-like structures. Florin rings are circular thickening formed by the cells surrounding the stomata of pine needles, first described by Florin (1931). Six different types of Florin rings have been described for the genus *Pinus*, and *P. nigra* was described as having a type A Florin ring, meaning absent or barely visible (Yoshie & Sakai, 1985). Our observations showed needles with evident Florin ring-like thickenings (Fig. B1) mostly in samples from P1, P4, P7 and P8 sites. This modification of peristomatal micromorphology could be interpreted as a stress induced adaptation when stomatal function is altered. The outer stomatal ledges prevent wide opening of the stomatal pore, reduce water loss (Pautov et al., 2017) and could change the direction of growth of fungal hyphae and decrease the chance of internal fungal infections through stomatal pore (Mohammadian et al., 2009).

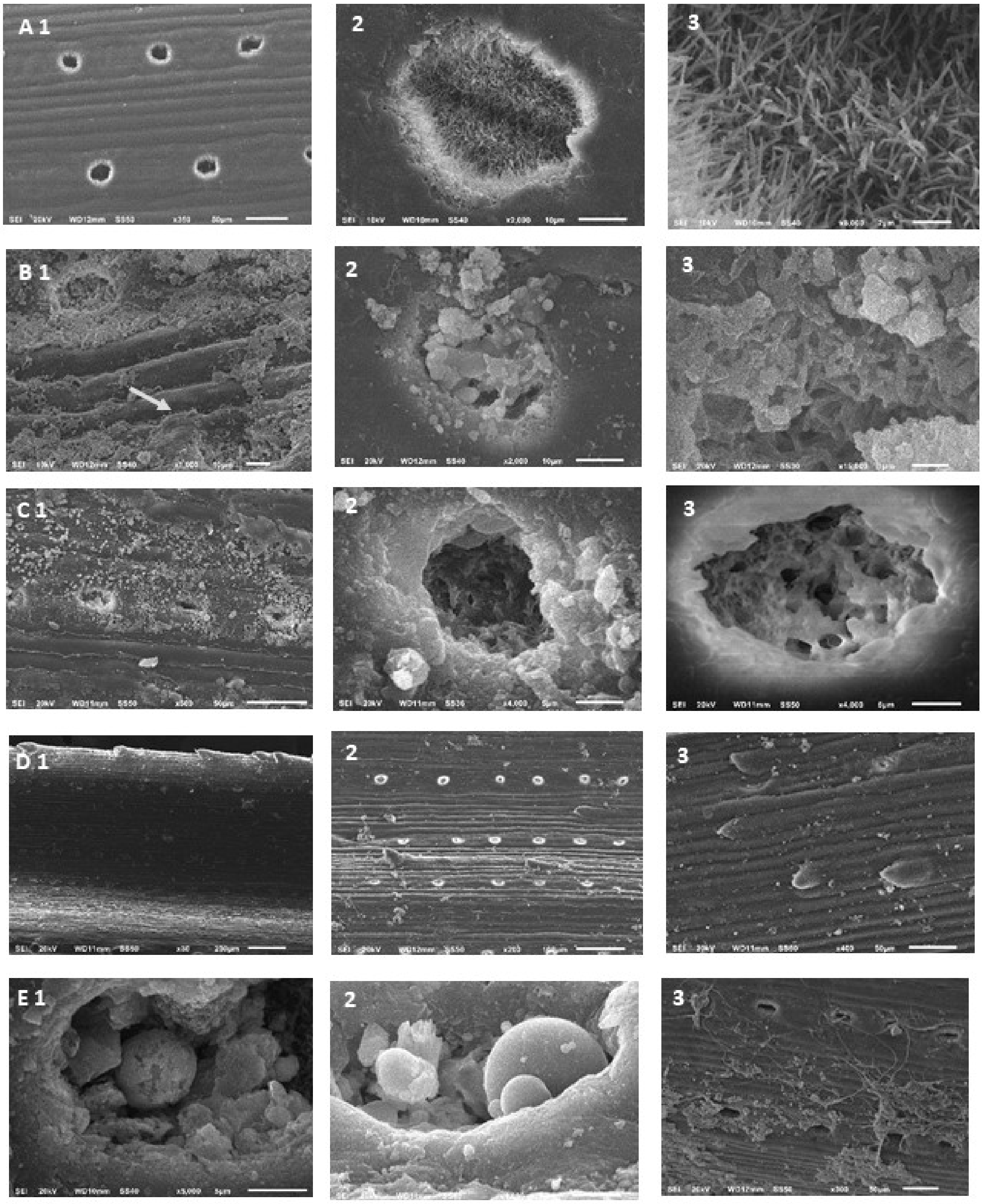

Figure 2 - SEM micrographs: A - sample from clean areas – **1** - general view, **2** - detail of undamaged stomatal complex, **3** - detail of tubular wax tubes; **B** - **1** - sample from P1 locality showing irregular surface with crust depositions and Florin-like ring (arrow), **2** - clogged stomata and raised epidermal cells, **3** - detail of deposition and subsequent wax tubes fusion; **C** - **1** - sample from P2 locality showing crystallized depositions and (**2**) - complete amorphized wax tubes, **3** - sample from P3 locality showing channel-like perforations; **D - 1** - sample from clean areas showing marginal normal serration, **2** - sample from P3 locality and **3 -** from P7 locality with abnormal middle serrations; **E - 1, 2 -** samples showing microspheres in stomatal pores, composed of iron oxide (E1 - from P5 locality) and of glass (E2 - from P8 locality), **3** - fungal colonization (from P1 locality).

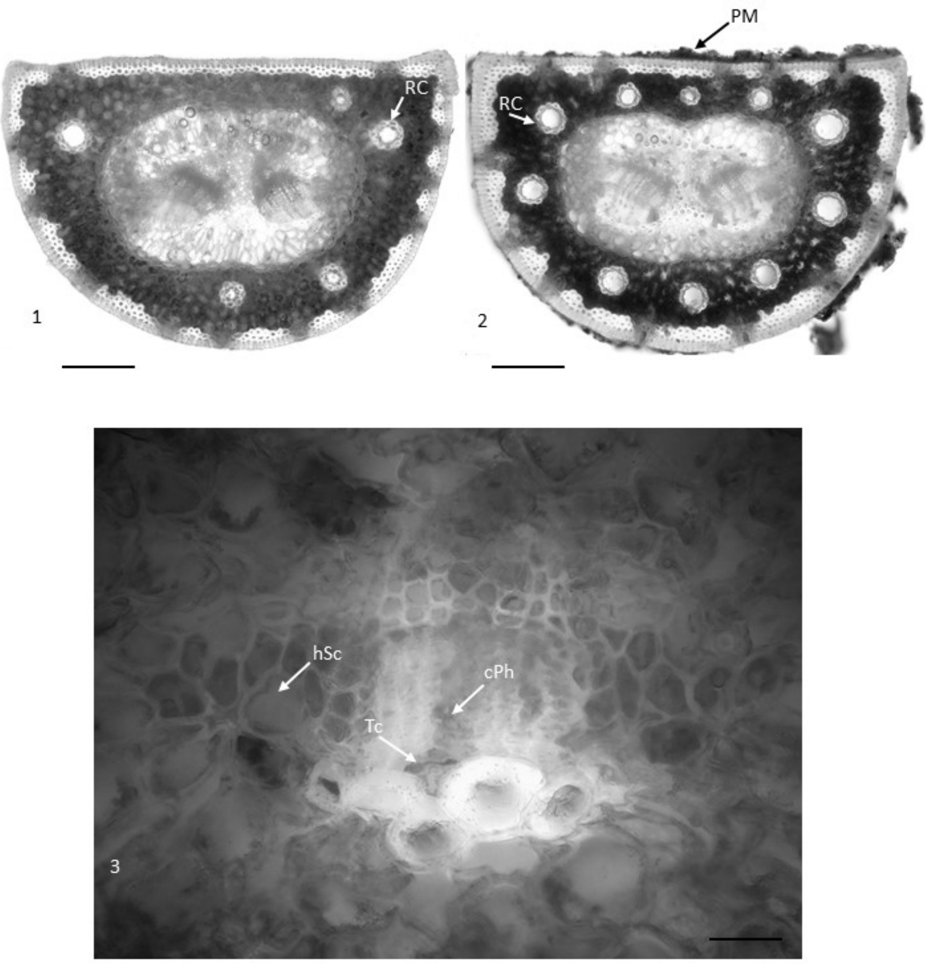

Figure 3 - Cross sections observations: **1** - sample from clean areas, **2** - sample from polluted area showing numerous resin canals (RC) and particulate matter deposition (PM); **3** - detail of sample from polluted area showing collapsed phloem tissue (cPh), hypertrophied Strasburger cells (hSc), and cells with phenolic droplets (Tc). Bar scale 200 μm (1 and 2) and 20 μm (3).

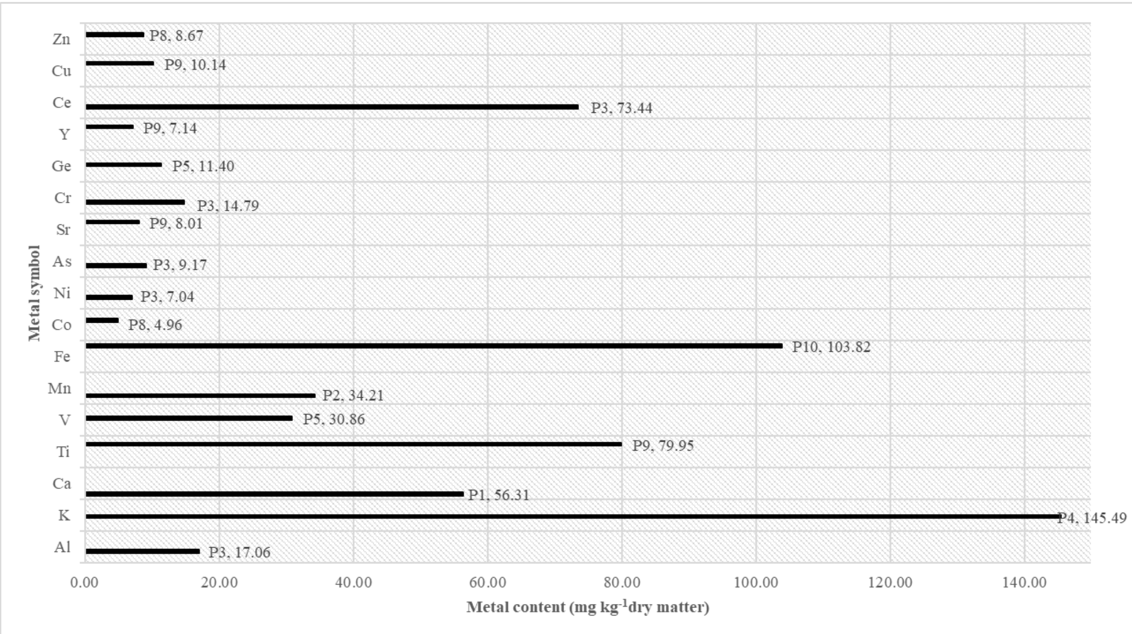

Figure 4. Metal content of particulate matters (PM) deposition in needles of *Pinus nigra* from contaminated sites

The most intriguing structural feature observed in samples from P4, P5, P6, P7, P8 and P9 sites, was an abnormal median serration made from scattered epidermal tooth (Fig. 2, D 2,3). Black pine normal structure shows a fine serrate needle edge made by uniseriate epidermal tooth (Fig. 2, D1). It was suggested a correlation between climate and leaf-margins characteristics, thus tree species from cool temperate forests generally have leaves with toothed or lobed margins (Baker-Brosh & Peet, 1997). Moreover, was reported that toothed margins are more active in photosynthesis and transpiration than untoothed margins (Royer & Wilf, 2006). Since epidermal teeth enhance the rate of carbon uptake, it is reasonable to assume that supplemental epidermal tooth could be interpreted as structural adaptation compensatory to the stomatal altered function. Thus, abnormal epidermal scattered tooth could be used as biomarkers of air pollution.

In cross-section the structural modifications were clearly visible. In all samples from polluted areas were observed supplementary resin channels (Fig. 3.2), between 10 and 12, unlike the normal structure that shows between 3 and 6 resin channels (Fig. 3.1). The supplementary resin channels could be interpreted as structural adaptation to particulate matters deposition, since pine needle resins serve as stress defense response. It was reported that different stages of wax degradation are correlated with an increase wettability (Wang et al., 2013). When wax barrier is damaged, structures show different signs of altered water balance and transport of assimilates, mainly in tissues related with water and nutrient transport, such as phloem, which showed partly collapsed tissue, Strasburger cells, which are hypertrophied and parenchymatic cells with phenolic droplets (Fig. 3.3); all these modifications could serve as structural markers of air pollution.

Airborne particles in urban areas are originated mainly from traffic (Hietikko et al., 2018) and is documented that vehicle exhaust affects both anatomical structures and physiological traits of plants (Qin et al., 2014). While the main source of air pollution in the sampling area was traffic, we can assume that the described morphological and structural modifications are related with this type of anthropogenic emissions. Traffic related metals such as nickel (Ni), chromium (Cr), copper (Cu), zinc (Zn) and iron (Fe) were identified in particulate matters deposition, with the highest concentrations in samples from P3, P8, P9 and P10 sites (Fig. 4). As consequence, the most affected needle structure and morphology was observed mainly onto samples from these sites. Apart from known related metals, we have identified in needle depositions, other metals which are not strictly related with traffic such as cobalt (Co), titanium (Ti), germanium (Ge), manganese (Mn), vanadium (V), strontium (Sr), etc., probably resulted from other anthropogenic activities. Other depositions are correlate with calcium high content as revealed from XRF analyses, in samples from site P2, where compact crust depositions are present.

Another “sign” of epicuticular wax degradation in air polluted environments, is fungal colonization of needle surface, which shows a poor/vulnerable quality class of wax (Grodzińska-Jurczak, 1998). Different stages of fungal colonization were observed onto needle surfaces from all sampling sites, meaning that wax structure was altered.

A particular type of traffic related airborne particulate matters with health hazard are microspheres (size under 2 μm). These particles have undergone fragmentation and melting inside vehicles engines, which take in primary dust particles and exhaust round-shape microparticles (Li et al., 2016). Our observations showed that needles from all sampling sites retain spherical particles with size between 10 μm and 0.5 μm.

Due to their epicuticular consistent wax, needle surface in conditions of elevated temperatures of hot summers, act like natural “traps”, for a range of air pollutants including those difficult to trap such as microspheres with size under 2 μm, known as respiratory hazard (Fig. 2, E1,2). This capacity combined with species ubiquity and resilience in urban environments recommend *P. nigra* for air pollutants assessment. Moreover, by trapping a large range of airborne particles black pine could act like natural green filters when planted along roadsides with intense traffic.

5. CONCLUSIONS

Our investigations showed that *P. nigra* needle structure is sensitive to air pollutants and could be used to discriminate biomarkers of environmental pollution. The newly described leaf modifications such as Florin rings-like thickening, wax plugs channels and abnormal middle serrations, could be successfully used as reliable indicators of air pollution in urban environments. The newly described biomarkers are clearly distinguishable and because are stress adaptations, could be observable in heavy polluted urban environments where other ornamental plant species hardly survive. Moreover, only simply evaluation of needle depositions could discriminate hazard air pollutants, since most of the particles from needle depositions are respirable dust, thus potential harmful for human health.

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