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Air pollution effects on structure, proteins and flavonoids in pollen grains of *Thuja orientalis* L. (Cupressaceae)

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Abstract

Increase in the levels of air pollution due to the increase in industrial and agricultural technology has prompted investigation of mechanisms that contribute to air pollution tolerance in plants. Pollen grains of *Thuja orientalis* were collected from controlled (less polluted) and polluted areas (mainly SO₂, NO₂, CO, HC and APM). *Thuja* pollen is considered inaperturate and granulate. The exine is shed during rehydration, leaving the male gametophyte naked. The pollen grains collected from polluted areas are smaller and more fragile compared to control ones. The exine splitting is faster and higher in polluted pollen grains. SDS–PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis) pattern do not show significant differences in polluted pollen than those in the control group. HPLC (high pressure liquid chromatography) analysis demonstrates that air pollution induces flavonoids accumulation to significantly higher levels in polluted pollen than in controlled ones. These observations suggest that plants try to respond suitably by adjusting their metabolism so that minimum damage is done due to air pollutants. Their protective responses may include an increase in antioxidant enzymes and metabolites and induction of protection-related secondary metabolite genes especially flavonoids.

Keywords: *Exine, intine, phenolic fingerprints, SDS–PAGE pattern*

Modern industry has provided our environment with an almost indefinite number of agents. The most common of these components are SO₂, NO₂, CO, HC (Hydrocarbons) and APM (Atmospheric Particulate Matter) (Ruffin et al., 1986). The quality of the air is now monitored daily in sensitive areas by numerous organisations specialising in air quality control (Bortnick & Stetzer, 2002). However, the impact of the various atmospheric pollutants on living organisms remains a poorly known phenomenon. Observation of the reactions of a living organism exposed to air pollution under natural conditions is necessary and some compounds formed in these reactions may be used as bioindicators.

The role of pollen grains in the fertilisation and fertility of plants is one of the most important biological factors. Fertility decreases under different stresses due to the direct and indirect effects on reproductive apparatus. The effects of air pollutants on roots and aerial parts affect generative apparatus development

and subsequently plant fertility (Emberlin, 1998). Pollutants affect pollen structure, germination and pollen tube growth after anther dehiscence until reaching to stigma (Majd et al., 1996; Peltre, 1998; Emberlin, 1998; Behrendt & Becker, 2001). Like any other plant cells, pollen grains contain many different types of proteins, which are located in major domains: in the cytoplasm and at the surface of exine and intine. These molecules are strategically sited to participate as the male partner in intercellular recognition reactions with the female stigma (Knox & Suphiohlu, 1996; Dickinson et al., 2000). Helander et al. (1997) did not observe any significant difference between protein bands of pollen grains collected from polluted and control areas. Jilek et al. (1993) observed an increase of the major birch pollen allergen, Bet v 1, in areas where nitrogen loads are high, while Hjelmroos et al. (1994) and Parui et al. (1998) found a decrease in Bet v 1 concentration due to air pollution. Studies on *Lagerstroemia indica* L. showed

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the reduction of total protein amount and staining intensity of protein bands under air pollution (Rezanejad et al., 2003). However, studies on pollen proteins collected from polluted and unpolluted areas have shown contradictory results.

Among the chemical compounds in plants, secondary metabolites in particular the phenolic compounds (e.g. phenols and flavonoids) are of a great importance in plant-environment relationships (Haslam, 1989; Rhodes, 1994). These compounds are of a particular interest because of their involvement in the response of the plant to environmental stress, such as a deficit in nutrients, the impact of ultraviolet rays (UV) or air pollution (Vogt et al., 1994; Chaves et al., 1993; Muzika, 1993; Penuelas et al., 1996; Cooper-Driver & Bhattacharya, 1998; Pisani & Distel, 1998; Robles et al., 2003). Besides providing beautiful pigmentation in flower, fruit, seed and leaf, flavonoids have been proposed to play diverse roles in plant growth and development, including defense, symbiosis, pollen development and male fertility, pollen germination and tube growth, polar auxin transport, as pigments that attract pollinators and seed dispersers, protection against ultraviolet radiation and the regulation of the cell cycle (Murphy et al., 2000; Woo et al., 2005). A flavonoid deficient mutant of *Petunia* Juss is male sterile because its pollen fails to germinate (Woo et al., 2005). However, this is not true for *Arabidopsis* Heynh, where the lack of flavonoids in flowers and stamens do not affect male fertility (Burbulis et al., 1996). This suggests that flavonoids are not universally required for fertility. Dixon and Paiva (1995) observed the higher quercetin content in the leaves from the polluted site. They suggested that the content increase could be associated with the defensive role of flavonoids under conditions of environmental stress.

Our studies in literature showed that so little is known about the effect of air pollution on *Thuja orientalis*. Alternatively, because of air pollution increase result from the heavy car traffic in Tehran and since this species has a great distribution in parks and landscapes, our purpose was to characterise and compare structure, proteins and flavonoids in pollen of this species in control and polluted areas.

Material and methods

Preparation and examination of pollen grains

Pollen cones of *Thuja orientalis* were collected as they were beginning to shed pollen from tress planted in controlled (National Botanical Garden, Paykan-shahr, 30 km far from Tehran) and polluted areas with heavy traffic (Moddares Highway, Tehran). A total of 15 trees were randomly selected from each

site (control and polluted). We concentrated on evenly sized (size assumed to be an indicator of tree age) and healthy trees. Both sites are located in a geographically limited area with fairly homogeneous conditions. Reports by the air quality centre at the environmental protection agency showed the type and mean of air pollutants concentrations in controlled and polluted areas at the sampling sites based on monthly means (Table I). Pollen cones were held in loosely covered paper boxes for a period of two days to a week until the pollen grains were released. Pollen grains were sifted by a 1 mm mesh screen and dried at room temperature for two weeks. Pollen grains were stored at -20°C until the time of analysis. Pollen structure was studied by Scanning and Light Electron Microscopy. Pollen specimens were prepared for scanning electron microscopy observation by dusting them directly onto sticky-tape coated aluminum stubs and gold coating. Photographs were taken with a JEOL JSM 633 OF SEM. For light microscopy, Pollen grains were generally dusted onto a drop of water on a microscope slide. Specimens were viewed with an Olympus model Ah2 light microscopy connected to camera.

Pollen regularity was assessed based on the percentage of well-regulated and normal pollen grains. At least 100 pollen grains were studied. The size of pollen grains from the controlled and polluted areas was compared by measuring the diameter of 20 pollen grains on each slide randomly. The length of the pollen tubes was measured with an ocular micrometer. All experiments were repeated four times. The results were analysed using one-way ANOVA in SPSS (version 9.0) and Duncan's multiple range test to compare the significance ($p < 0.05$) of the treatments (Wang et al., 2006).

Protein analysis

The pollen extracts were prepared by incubating pollen grains collected from polluted and controlled areas in 0.1 M phosphate buffered saline, PBS, pH 7.4 in 15% ratio with stirring at $4-8^{\circ}\text{C}$ for 4 h. Suspensions were centrifuged at 10 000 g for 20 minutes at 4°C and supernatants were removed. Loading buffer (glycerol, sodium dodecylsulfate,

Table I. Mean of air pollutants concentration in control and polluted areas.

Type of air pollutant	SO ₂ (ppm)	NO ₂ (ppm)	CO (ppm)	HC (ppm)	APM ($\mu\text{g m}^{-3}$)
Month					
May (polluted area)	0.082	0.064	3.4	1.80	177
May (control area)	0.002	0.010	0.6	0.05	46

APM – airborne particulate material; ppm – part per million.

mercaptoethanol, bromophenol blue) was added to each sample, vortexed briefly, heated in a boiling water bath for 5 min in the presence of reducing agent 2-mercaptoethanol. The extracts (40 μ l) and a molecular weight ladder (8 μ l; Feinbiochemica GmbH and Co.), were run using discontinuous (4% stacking, 12% resolving) sodium dodecylsulfate-polyacrylamide gels (modified method of Laemmli, 1970). Proteins were stacked at 50 V and then resolved at 70 V overnight. Electrophoresis was performed in a vertical slab gel apparatus (Akhtarian, Ps-2000, Model 75, Iran) three times. The proteins were then stained with 0.2% Coomassie brilliant blue R250 and destained with mixture of methanol, acetic acid and water (1:1:8).

Flavonoids analysis

Some pollen grains were extracted with ethanol: water (1:1, v/v) in 10% ratio (0.5 g pollen was extracted with 5 ml EtOH: H₂O) for 4 hours in room temperature. Extraction was aided by use of vortexing in 30 minutes intervals. The resultant mix was centrifuged at 5 000 g for 20 minutes at room temperature and the supernatant was used for high pressure liquid chromatographic (HPLC) analysis (Agilent model 1100). Injection volume was 20 μ l and elution performed with a flow rate 0.8 ml min⁻¹. The column used was c18 column (Zorbax 300SB). The solvents comprised water adjusted to pH 2.5 with orthophosphoric acid (A) and acetonitrile (B) mixed with a linear gradient starting with 100% A, decreasing to 91% over the next 12 min to 87% over the next 8 min and to 67% over the next 10 min. After holding the solvent at this composition for 2 min, A was decreased to 57% over the next 10 min, and then held at this level until the end of the 60 min analysis. Peaks were detected at 340 nm (Campos et al., 1997). Experiments were done by duplicate.

Results

Results obtained by scanning electron microscopy indicate that the pollen grains are inaperturate, non-saccate, and the pollen surface (ectexine) is covered with granulate elements (granula, all diameters < 1 μ m). Granula are of different sizes and are irregularly distributed in the exine (Figure 1A–C). These very small and rounded elements have minute spinous excrescences and the region between the granula is coarse (Figure 1C). Studies of light microscopy showed that pollen grains are spherical in shape and there is no obvious aperture on their surface. When pollen grains are suspended in water, the intine swell and the exine burst (Figure 2A–C).

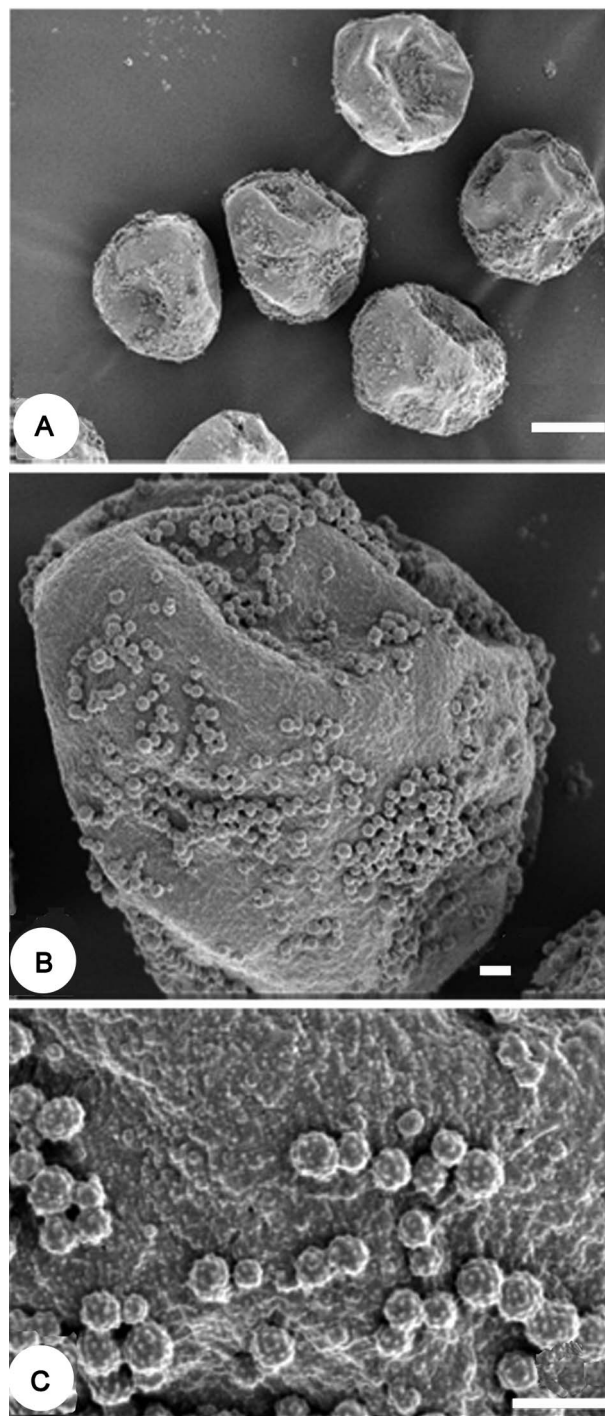
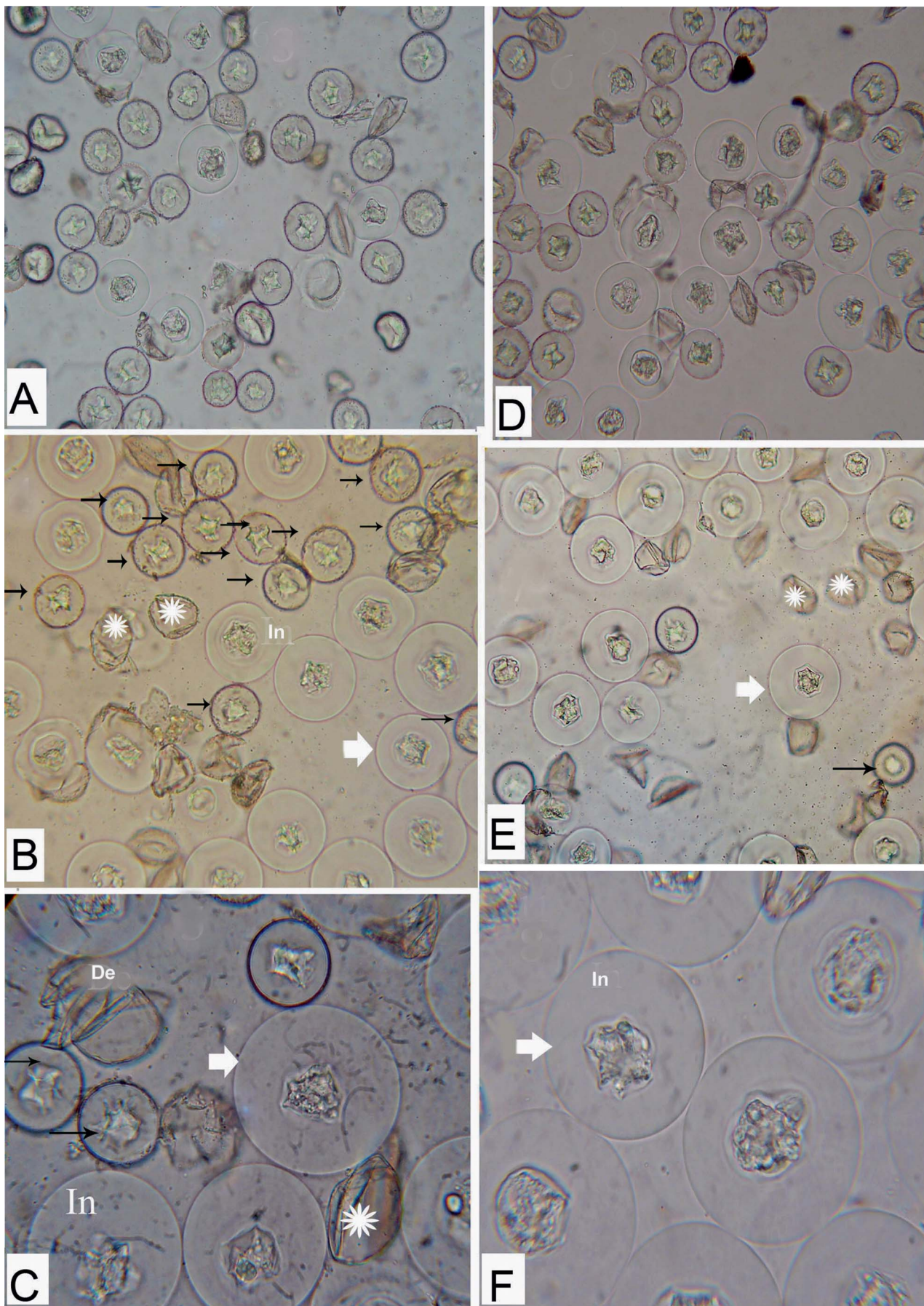


Figure 1. SEM micrographs of pollen from plants grown under controlled condition. The pollen grains are regular, note the granules attached (A–C) and their spines (C). Scale bars – 10 μ m (A); 1 μ m (B, C).

The results obtained from pollen structure showed that air pollution causes an increased number of irregular and fragile (breakable) pollen grains compared to the control samples (Figure 3). The splitting exine was faster and higher in polluted pollen than controlled ones when they were in water solution



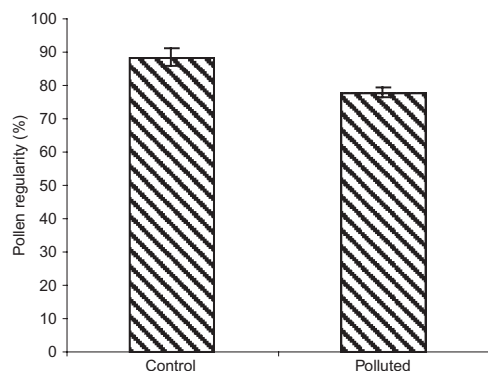


Figure 3. The pollen regularity mean (%) in control (i.e. unpolluted) and polluted area (mean $\pm$ standard error). One hundred pollen grains were studied. The experiment was performed by triplicate.

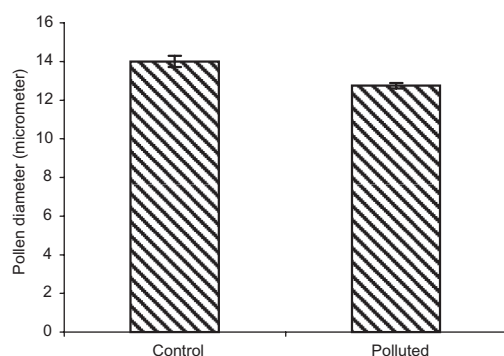


Figure 4. The pollen diameter mean (μm) in control and polluted area (mean $\pm$ standard error). Twenty pollen grains were measured randomly. The experiment was performed by triplicate.

(Figure 2D–F). Pollen size (mean) was reduced in polluted samples (Figure 4).

SDS-PAGE pattern of soluble proteins of pollen extracts does not show any obvious difference between polluted and control extracts (Figure 5).

Some of pollen grains were extracted with aqueous ethanol to produce an extract rich in phenolics for HPLC analysis. With solvent programming, the phenolics eluted with retention times (RT) between 1 and 60 min. The phenolic fingerprints (by comparing peaks in each retention time) were found to be sufficient to show the influence of air pollution on flavonoids of pollen grains in the present study. The results of this analysis show that in *Thuja orientalis*, the pollen grains exposed to air pollutants detect a 1.5 fold increase in peak of

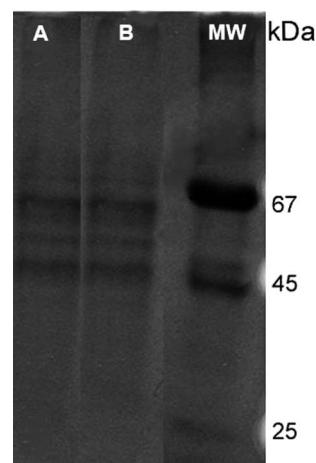


Figure 5. SDS-PAGE pattern of soluble proteins of pollen extracts. The pollen extracts of control (A) and polluted (B) area. MW – molecular weight marker.

retention time 33 (min) compared to the same peak in the controls (Figure 6).

Discussion

Exine is the first wall that is formed during pollen development, and normally persists until pollen tube emission. At dispersal stage, the pollen grains were dehydrated. During rehydration, the pollen intine absorbs moisture, expands and the exine is shed. Studies of Pacini et al. (1999) showed in *Juniperus* pollen, intine contains cellulose in the inner part and pectins in the thicker outer part. These polysaccharides are hygroscopic and probably responsible for the increase in intine volume observed during rehydration. This is very similar to natural condition for most gymnosperms, where pollen rehydration takes place in a liquid medium, i.e. the micropylar or pollination drop (a liquid secretion secreted by the ovule and exuded from micropyle). These hydrated pollen grains often detach their exine, and are surrounded by intine and the ghost of the half-opened exine (Duhoux, 1982; Southworth, 1988; Pacini, 1990; Nepi. et al., 2005). The detachment of the exine from the intine means that the two walls are not attached to each other, or loosely attached and able to separate relatively easily. Some researchers have shown that the pollen of the Cupressaceae is characterised by a pore with convex annulus, which plays key roles in the pollen hydration and exine breaking (Duhoux, 1982;

Figure 2. LM micrographs of pollen obtained from controlled (A–C) and polluted (D–F) material. A–C. Pollen grains 10 (A), 30 (B) and 120 (C) minutes after hydration. D–F. Polluted pollen grains 10 (D), 30 (E) and 120 (F) minutes after hydration. Pollen abnormality and exine fragility significantly cause detaching and removing precocious of exine in polluted pollen compared to control ones. Arrows, arrow-heads, and asterisks mark pollen proper, pollen with swollen intine (exine has detached) and detached exine respectively. De – detached exine, In – intine. Magnifications: $\times 400$ (A, B & D, E); $\times 1\,000$ (C, F).

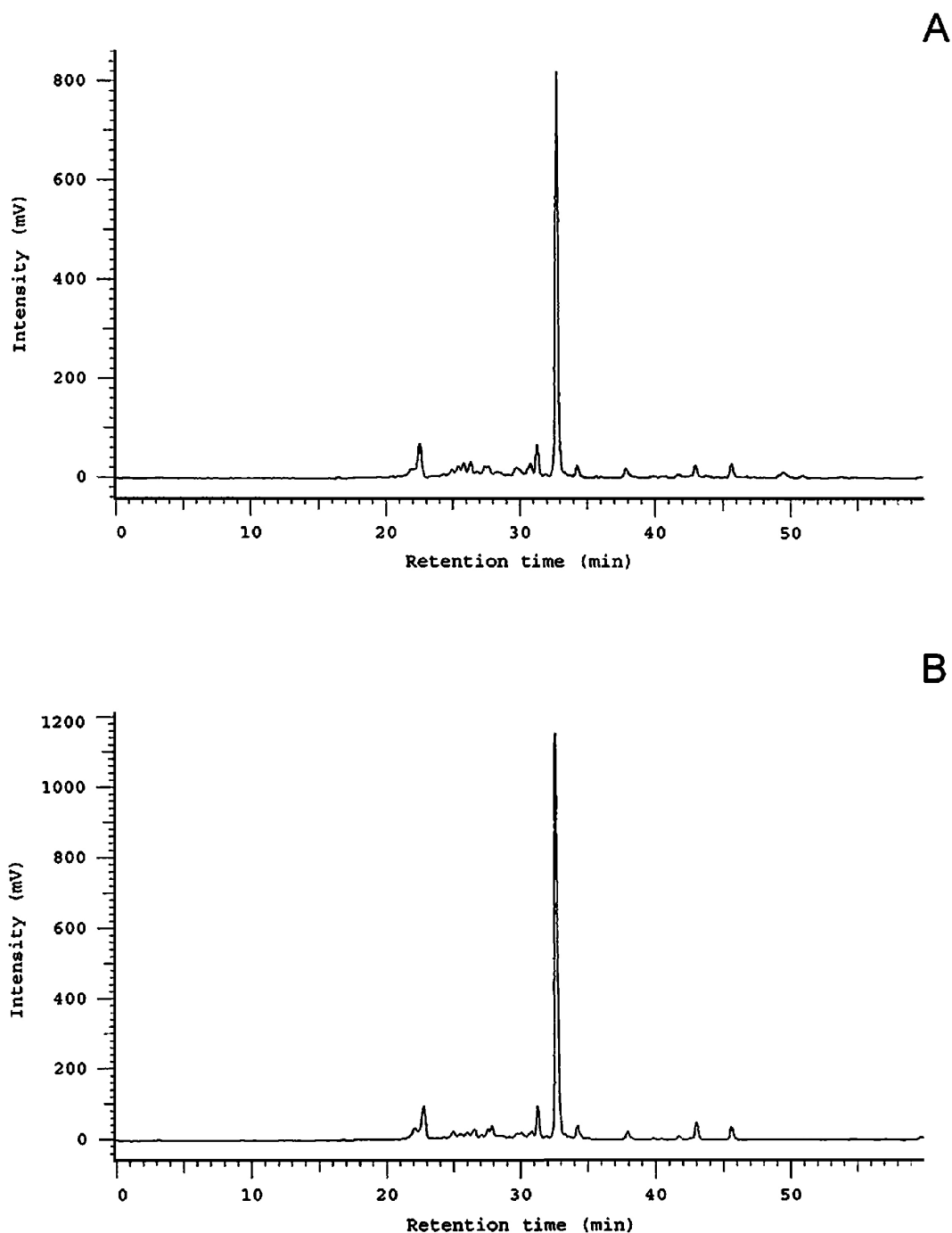


Figure 6. HPLC chromatograms of flavonoids from pollen grains of *Thuja orientalis*. The profiles of pollen extracts from polluted (A) and control (B) plants. Each experiment was performed twice.

Bortenschlager, 1990; Kurmann, 1994). Chichiricco and Pacini (2008) reported in specimens of *Cupressus arizonica*, only a minority either of living or dead pollen grains is provided with a germinal aperture consisting of an operculate pore with concave annulus.

Our specimens of *Thuja orientalis* showed the pollen grain of this species granulate (according to data cited in the 'Glossary of Pollen and Spore Terminology' (Punt et al., 2007)) and there is no obvious aperturate

on its surface. This is supported by the observations of Boucher et al. (2002) that explained Cupressaceae pollen is inaperturate. Also, according to the Thanikaimoni opinion (1986), only angiosperm pollen has true pores. The granula have different sizes and irregular distribution onto exine. Similar our results, studies of Andrew (1984) showed that pollen exine of *Cryptomeria japonica* (Cupressaceae) is covered with granula. Southworth (1986) observed that the pollen of *Juniperus*

communis is covered in stalked, spherical gemmae or granules which often become detached during fossilisation or preparation (cited by Thomas et al., 2007).

Air pollution caused shrinkage, thinning and fragility of pollen. These pollen grains lose their exine earlier compared to control ones when they are in contact to moisture. Indirect effects of air pollution on pollen grains are possible via the soil. If a plant grows in polluted soil, its physiologic functions may change and affect properties of the developing pollen grains (Helander et al., 1997). Also, injury of floral organs especially anthers can cause pollen abnormality and sterility, and consequently malfunction of fertilisation.

Results of SDS-PAGE of proteins showed no visible difference between protein bands of the pollen extracts collected from unpolluted (i.e. controlled) and polluted areas. Behrendt et al. (2001) reported that pollen freshly collected on rural meadows and near high-traffic roads demonstrates a striking difference according to origin, with higher allergen release rates from rural meadow pollen grains. Also, studies on *Lagerstroemia indica* showed total protein content and staining intensity of protein bands decrease under air pollution (Rezanejad et al., 2003). Similar to our results, Helander et al. (1997) did not observe any significant difference between protein bands in pollen grains from plants grown under polluted and control areas. Studies show contradictory results; therefore, it is possible that type of plant species, thinning of exine under air pollution or pseudo increase of pollen weight due to pollutants deposition on pollen surface caused these differences which need more studies.

In this report, the accumulation of high level of flavonoids is characterised in plants under study in response to air pollution. Our results are consistent with finding of Dixon and Paiva (1995) that observed the higher quercetin content of the plants in response to ozone. They suggested that the content increase could be associated with the defensive role of flavonoids under conditions of environmental stress. The studies of Robles et al. (2003) in *Pinus halepensis* leaves did not reveal any correlation between concentrations of SO₂ and of total phenols. However, significantly positive correlations between these two parameters are found for *Pinus sylvestris* L. (Giertych & Karolewski, 1993) and even for a distant species such as *Vicia faba* L. (Nandi et al., 1990). Similarly, the impact of sulfuric acid, in controlled environment, on *Pinus nigra* Arnold and *Pinus resinosa* Aiton engenders an increase in the phenolic compounds (Zobel & Nighswander, 1991). Robles et al. (2003) showed a decrease in total phenol concentrations with levels of nitrogen oxide pollution. However, our results showed an increase of flavonoids under air pollutants in natural environment. According to the results of this study, it is

proposed that all air pollutants together especially airborne particulate materials (APMs) have synergic effects and induce defense mechanisms such as flavonoids biosynthesis. Our studies in literature show no research about the effect of APMs on flavonoids. It is noteworthy that the concentration of these gaseous pollutants in polluted areas was 5–6 fold higher than controlled areas. The successful fertilisation is highly dependent on two factors: rapid germination and pollen tube growth. Thus, increased flavonoids levels under air pollutants, could be a defense mechanism against pollen damage because air pollutants increase pollen abnormality (Emberlin, 2000). Several lines of evidences indicate that most air pollutants enter the plant tissues and act primarily through the production of reactive oxygen species (ROS) also called oxidative-free radicals, so is the case with most other abiotic stresses (Alscher et al., 1997). Three important ROS, superoxide anion (O₂⁻), hydroxy-free radical (OH[•]) and H₂O₂ are highly toxic and cause changes in DNA, proteins and lipids and in membrane organisations. Several *in vitro* studies have demonstrated that flavonoids can directly scavenge ROS (Bors et al., 1994). This is apparently through the participation of flavonoids in peroxidase-mediated catabolism of H₂O₂, where flavonoids may act as electron donors (Takahama, 1989). Two major flavonoids, quercetin and kaempferol and their glycosides can be oxidised by H₂O₂ in the presence of horse radish peroxidase or in a cell-free extract of the leaves (Yamasaki, 1997). The leaf extract contains a peroxidase which could use flavonols as a reducing agent for the oxidation of H₂O₂. Thus, flavonoids content increase in response to air pollution. As these compounds can scavenge ROS and they could induce pollen development, pollen germination and pollen tube growth to enhance plant fecundity.

Conclusions

Air pollutants, such as those from industrial and vehicular emissions, interact with the pollen of plants causing alteration of physiology, ontogeny and morphology of pollen grains, both at the surface and within the pollen protoplasm. These changes to pollen grain structure can affect proteins and flavonoids of pollen grains. Increase in concentrations of total flavonoids, in pollen grains of *Thuja orientalis* may be considered related to changes in concentrations of air pollutants. Pollen grains, as bioindicators, provide particularly original and interesting information on the potential adverse effects of pollutants on living organisms because they accumulate high levels of secondary metabolites in particular the phenolic compounds (e.g. phenols and flavonoids). These observations suggest that flavonoids may be involved

as a defence element against abiotic stresses including air pollutants, although experiments involving air pollutants other than O₃ are lacking to support this suggestion.

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