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Research Article

The Effect of Air Pollution on Microsporogenesis, Pollen Development and Soluble Pollen Proteins in *Spartium junceum* L. (Fabaceae)

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Abstract: Air pollution induces abnormality in anthers, reduction of pollen grains' umbers and male sterility. Anthers of *Spartium junceum* L. (Spanish broom) were collected at different stages of development from control (less polluted) and polluted areas (mainly SO_2, NO_2, CO, hydrocarbons (HCs) and airborne particulate material (APM)). Structure and development of anther walls and pollen grains were studied and compared. Groups of the control pollen grains were exposed to the polluted air of Tehran for 10 and 20 days. Under pollution stress some anthers became abnormal and shrunken. The tapetum layer had a precocious growth in the microspore mother cell stage and a precocious digestion at the tetrad stage. In some anthers pollen grains were smaller, attached together, shrunken and deformed compared to control ones. The pollen grains collected from polluted areas as well as the ones exposed to gaseous pollutants showed collapse and thinning of the exine. Agglomeration of polluted particles on the pollen surface induced cellular material release under humid conditions. Total soluble protein content and SDS-PAGE patterns did not show significant difference in polluted pollen grains compared to control ones.

Key Words: Air pollution, anther development, cellular material release, microsporogenesis, pollen structure, soluble proteins, *Spartium junceum*

Introduction

Pollen grains are essential for correct fertilisation and therefore plant fertility. Fertility decreases under various stresses due to the direct and indirect effects on the reproductive apparatus. One of the problems that we encounter today is air pollution caused by increasing human population and technology. The harmful effects on higher plants by chemical pollutants do not only include mutations and chromosomal damage but also a wide range of morphological and physiological alterations. These abnormalities depend on environmental and genetic factors and may ultimately lead to change in reproductive capacity (Pfahler, 1981). For example, pollutants entering the soil and altering its pH can cause the subsequent mobilisation of trace elements, leading to root injury (Emberlin, 1998, 2000). In addition, pollutants may interfere with the growth of aerial plant tissues through different processes. These include gaseous pollutants entering plants via their stomata and disrupting metabolic pathways of respiration and photosynthesis. Studies by Kaiser et al. (1993) showed that SO_2 enters mesophyll cells, where it reacts with water to form sulphuric acid, which inhibits photosynthesis in the chloroplasts. The combined effects of pollutants on roots and aerial parts affect generative apparatus development and subsequently plant fertility (Emberlin, 1998). Pollutants affect pollen structure, viability, germination and pollen tube growth on stigmas (Majd & Mohamadi, 1992; Emberlin, 1998). These effects cause pollen sterility and fertilisation reduction. In Tehran, air pollution is mostly caused by heavy traffic. In addition, anthers are highly sensitive to different stresses, e.g., heat, cold, drought and moisture (Ahmed et al., 1992; Saini 1997; Saini & Westgate, 2000; Suzuki & Tajeda, 2001). Since *Spartium junceum* is widely distributed in Tehran, the aim of this project was to study the effect of air pollution on anther development and microsporogenesis in this species.

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It has been demonstrated that gaseous and particulate pollutants might influence pollen allergenic proteins in molecular structure, quantity and release (Behrendt & Becker, 2001). Researchers showed that air pollution could cause collapse and thinning of the exine, and changes in total protein content and electrophoretic profile (Majd & Mohamadi, 1992; Behrendt et al., 1997; Pelter, 1998; Emberlin, 1998, 2000). Assaying pollen proteins affected by air pollution is important because proteins of the wall and coatings are important in adhesion to both vectors and components of the stigma. Moreover, they are effective in protection against UV radiation, prevention of desiccation and retention of signalling molecules that participate in pollen-stigma interaction (Dickinson, 2000). Another purpose to study these proteins is their allergenecity in humans.

Materials and Methods

Preparation of microscopic slides

Samples were collected from plants of *Spartium junceum* grown in a control area (National Botanical Garden, Paykanshahr, 30 km from Tehran) and from plants grown in a polluted area with heavy traffic (the city centre) in June 2002. The climatic and edaphic conditions in the 2 regions were the same. The collection of samples started when buds initiated and continued at close intervals until flowering. Samples were fixed in FAA (formalin: acetic acid: alcohol ethyl 96º 2:1:17) dehydrated in a graded alcohol series and embedded in paraffin. Serial sections of 8-12 µm were prepared and examined by light microscopy (LM).

Preparation of pollen grains

Mature pollen grains were collected from the control and polluted areas after dehiscence and desiccated at room temperature and then sifted.

Examination of pollen structure

Groups of the control pollen were placed inside plates and exposed to ambient pollutants of the heavy traffic area for 10 and 20 days in July 2002. Reports by the air quality centre at the environment protection agency show the type and mean of air pollutant concentrations in the control and polluted areas at the sampling sites of buds and flowers (June) as well as in the place of pollen exposure to air pollution (July) (Table 1). During this period some days were exceptional because short time peaks (3 h) of air pollutants were reported as 0.1 ppm (SO_2), 0.2 ppm (NO_2), 13.7 ppm (CO), 8.1 ppm (HC) and 191 μgm^{-3} (APM). Structure agglomeration of APM and cellular material release were examined by LM and SEM. The regularity and size of pollen grains were determined inside mature anthers (before dehiscence) as well as after dehiscence. Some pollen grains were fixed by the acetolysis method and studied. Pollen regularity was assessed based on the percentage of well regulated and normal pollen grains. At least 100 pollen grains were studied. The comparison of the pollen size in the control and polluted areas was performed by measuring the diameter of 20 pollen grains on each slide. All experiments were repeated 4 times.

Protein Analysis

Pollen extracts were prepared by incubating pollen grains in 0.1 M phosphate buffered saline (PBS) pH 7.4 in 15% proportion with stirring at 4-8 ºC for 4-8 h. Suspensions were centrifuged at 10,000 ×g for 40 min and the supernatants were removed. The protein content of different extracts was estimated in 595 nm as described in the Bradford (1976) method. Proteins from samples and standard protein were separated using SDS-PAGE at 80 V constant voltage for 1-2 h at 15 ºC (modified method of Laemmli 1970). Proteins were detected with 0.2% Coomassie brilliant blue R250 and destained using a methanol:acetic acid:distilled water (1:1:8) mixture. Statistical analyses were performed using ANOVA, Duncan's test.

Table 1. Mean of air pollutant concentrations in control and polluted areas.

Type of air pollutant / Month	SO_2, ppm	NO_2, ppm	CO, ppm	HC, ppm	APM, μg^{-3}
June (polluted area)	0.063	0.06	9.1	2.8	162
July (polluted area)	0.07	0.1	8.4	2.7	154
June (control area)	0.002	0.01	0.6	0.1	54
July (control area)	0.003	0.01	0.65	0.12	60

Results

Anther development under normal conditions

In each 4 corners of the young anther, a subepidermal layer and some dividing sporogenous cells are observed (Figure 1a). Each subepidermal layer divides periclinally, forming an endothecial layer, 2 middle layers and a tapetal layer (Figure 1b). The tapetal layer shows high activity and stainability. Primary sporogenous cells give rise to microspore mother cells (Mmcs) by mostly transverse and a few longitudinal divisions and cell enlargement (Figure 1b). Microspore mother cells start to round and separate from each other and show prophase *I* (Figures 1b, 1c). At this time point, the locular cavity is enlarged and the tapetal cells are disconnected from each other and from the middle layer. Microspore mother cells undergo meiosis with simultaneous cytokinesis (Figures 1d-1f). At the end of meiosis *II*, tetradic cells and then tetraspores are formed (Figures 1e, 1f). During these phases, tapetal cells attain their largest size by enlarging radially toward the locule centre, and distinct spaces are visible among adjacent tapetal cells (Figures 1e, 1f). After the dissolution of the callose wall, the vegetative and generative cells are formed (Figure 2a). Shrinkage and degeneration of the tapetal cells become evident during microspore development (Figures 2a-2c). The thickened bars develop along walls of endothecial cells and the middle layer degenerates (Figures 2a-2c). By this time, differentiating microspores show irregular forms (Figures 2a, 2b), and finally, with the increase in size and the wall differentiation, they differentiate to regular mature pollen grains (Figure 2c). Results obtained by LM (Figure 2e) and SEM (Figures 3a-3d) show a regular pollen structure and exine after dehiscence. Pollen grains appear to be tricolporate (Figures 3a-3c). Figures 9 and 10 show the regularity and size (diameter) of pollen grains before and after dehiscence.

Anther development under conditions of air pollution

From the microsporocyte stage, some anthers show structural abnormality (Figures 4a, 4b). Precocious growth of the tapetum at this stage (Figure 4c) and its precocious degeneration at the tetrad stage are observed (Figures 4d-4f). In abnormal anthers, sporogenenous tissue (Figures 4b, 4c), tetrads (Figures 4d-4f) and developing microspores (Figures 5a, 5b) are abnormal. Pollen abnormalities are seen as irregularity, shrinkage, thinning and breakage of the exine (Figures 5a-5c). Cellular material release is induced also (Figure 5d). Under air pollutant conditions, pollen grains are found to be smaller as well as more irregular (Figures 9 and 10).

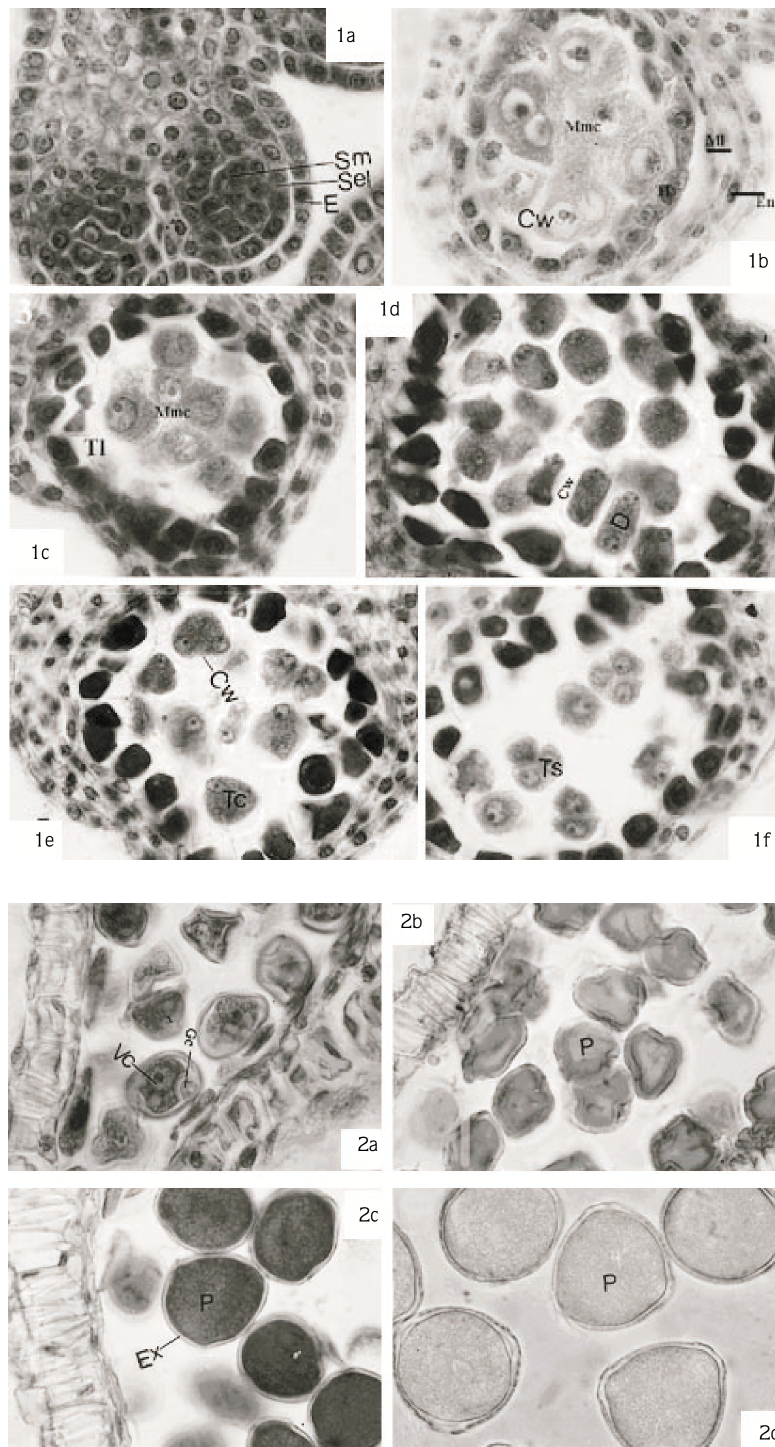
The study of pollen structure by SEM in the samples collected from the polluted area shows a marked degree of APM agglomeration on their surface compared to control pollen (Figures 6a-7d). Moreover, these pollen show abnormality in form (Figures 6c, 6d), touching (connecting with) each other (Figure 6c), precocious formation of pollen tube and cellular material release (Figure 6d) and irregularity and shrinkage of the exine (Figures 6e, 6f). Apertures appear closed and shrunken in the samples collected from the polluted area compared to the non-polluted ones (Figures 6a, 6b). In addition, micropores of exine sculpturings are bigger and more fragile than the control ones (Figures 6e, 6f). Pollen grains exposed to air pollution for 10 days (Figures 7a, 7b) and 20 days (Figures 7c, 7d) have irregularity in pollen shape and cellular material release.

The total protein content in pollen extracts collected from the polluted area and the area exposed to air pollution is lower compared to control samples although these differences are statistically non-significant (Table 2).

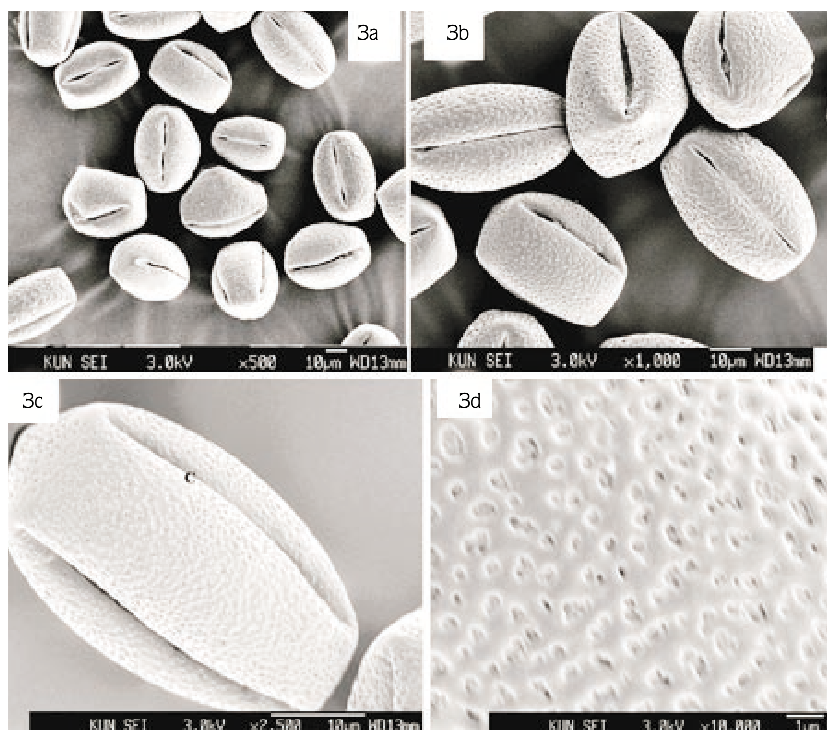
SDS-PAGE patterns of soluble proteins of pollen extracts do not show any apparent difference between extracts from the polluted and control areas (Figure 8).

Discussion

In the early stages of anther development sporogenous tissue comprises a few attached and stainable cells surrounded by a subepidermal layer. This layer through division and differentiation forms anther walls, of which the tapetum is the innermost layer. Microspore mother cells arise from the sporogenous mass after their division and enlargement. At prophase stages, a callose wall surrounds microspore mother cells until tetrad formation. This wall protects developing cells from interaction with other cells. The formation and persistence of this wall in *Spartium junceum* is in agreement with the findings of other researchers working on Angiosperms (Ahmed et al., 1992; Majd & Mohamadi, 1992; Chichiricco, 1999; Hermann & Palester, 2000; Suzuki & Tajeda, 2001). At the same



Figures. 1a-2c. Light micrograph of cross section of anther development under normal conditions (less polluted) (X1000). 1a. Young anther. 1b. Wall layers, microspore mother cells and callose wall are obvious. 1c. Tapetal cells and Mmcs are separating from each other and from adjacent tissues. 1d. The early meiosis II. 1e. Meiosis II stage and tetradic cells formation. 1f. Tetraspores formation. Figure 2a. Microspore mitosis (some cells show binucleate stage) and formation of vegetative cell and Generative cell. Tapetum degeneration is obvious. 2b. Maturing pollen. 2c. Mature pollen. 2d. Mature pollen after anther dehiscense. Callose wall = Cw, Endothecium = En, Epidermis = E, Exine = Ex, Generative cell = Gc, Microspore mother cell = Mmc, Pollen = P, Subepidermic layer = Sel, Sporogen mass = Sm, Tapetum layer = Tl, Tetradic cells = Tc, Tetraspores = Ts, Vegetative cell = Vc.



Figures 3a-3d. Electron micrographs of pollen structure of the plants grown under control condition, the pollen grains and exine are regular. Scale bars show the magnification. Colporous = C.

time, concomitant with microspore mother cells' differentiation and the beginning of meiosis, tapetal cells are enlarged and separated from each other and form the middle layer. Then the locular cavity increases and, by this time, the tapetal cytoplasm is completely dense and stainable. Subsequently these cells are shrunken and eventually after the binucleoli pollen stage degenerated. These characteristics of the tapetal layer show its nutritive role in the development of pollen. The middle layer is ephemeral and degenerated soon after. The studies by Hardy (2000) showed that the middle layer(s) is ephemeral in Leguminosae. Air pollution changes both the structure and development of anthers, leading to an increased number of deformed pollen grains compared to control samples. Apetures are closed and micropores of exine sculpturings are bigger and more fragile. Some researchers reported that airborne particle materials adhere to the pollen surface, causing the collapse and degradation of the exine surface, and shrinkage and abnormality of pollen (Majd & Mohamadi, 1992; Emberlin, 1998, 2000; Parui et al., 1998; Pelter, 1998). Pfahler (1981) showed that high pollution induces mutagenicity and physiologic changes. Therefore, high concentrations of air pollutants, especially APM, which most days is higher than standard (heavy traffic area in the city centre), cause abnormality in microspore mother cells, tapetum, developing pollen, and the whole anther. In addition, structural abnormality of the tapetum induces pollen irregularity and sterility. The reduction in total protein content in pollen extracts is not statistically significant, but the pollen-particle interaction induces cellular material release such as proteins under moisture conditions. The cellular material release affects fertilisation because pollen proteins interfere in the adhering of pollen to vectors and the pistil, pollen protection against UV radiation, prevention of desiccation and retention of signalling molecules that participate in the pollen-stigma interaction (Pacini & Francchi, 1993; Dickinson, 2000). Therefore, release of these proteins affects pollen structure and consequently fertility. Results obtained from SDS-PAGE of pollen proteins in the control and air pollution conditions are consistent with results by

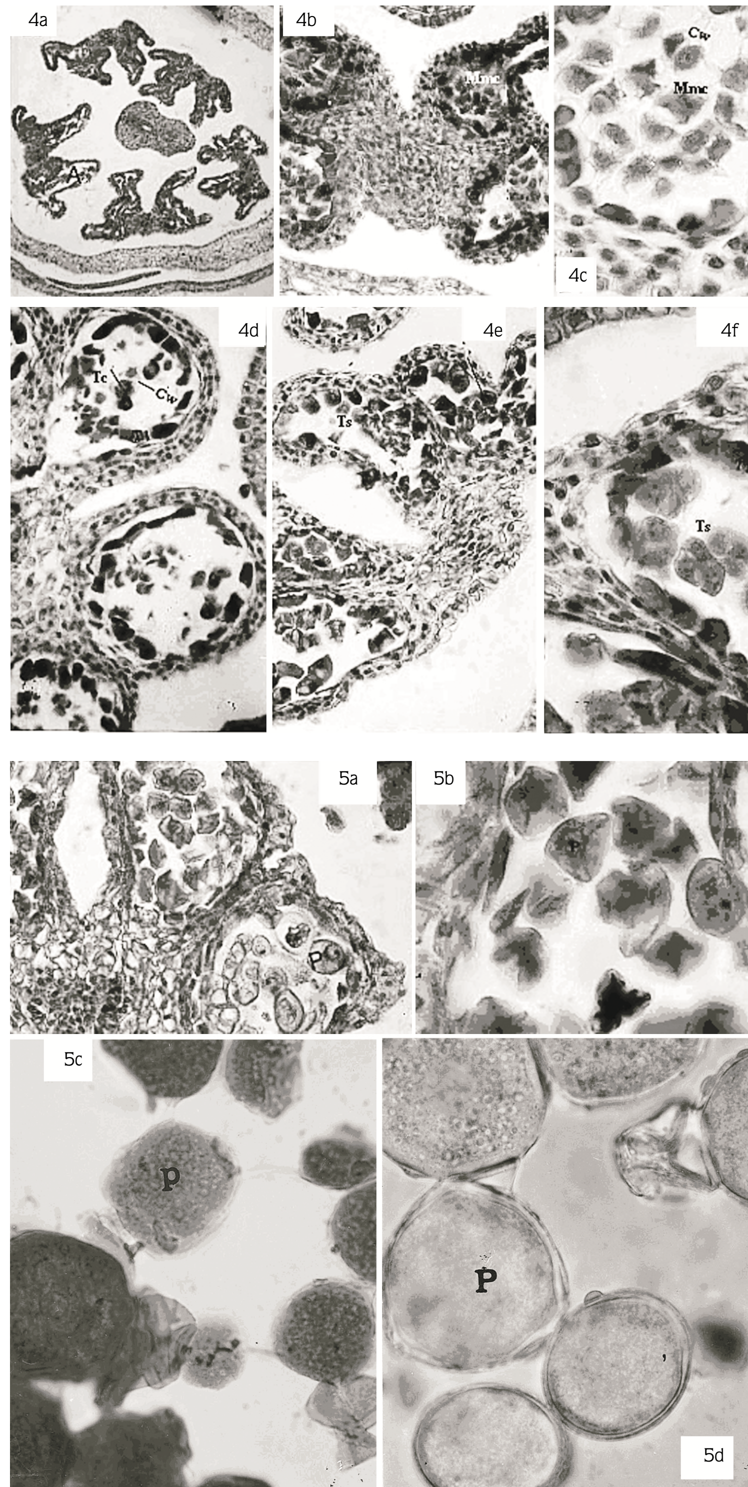
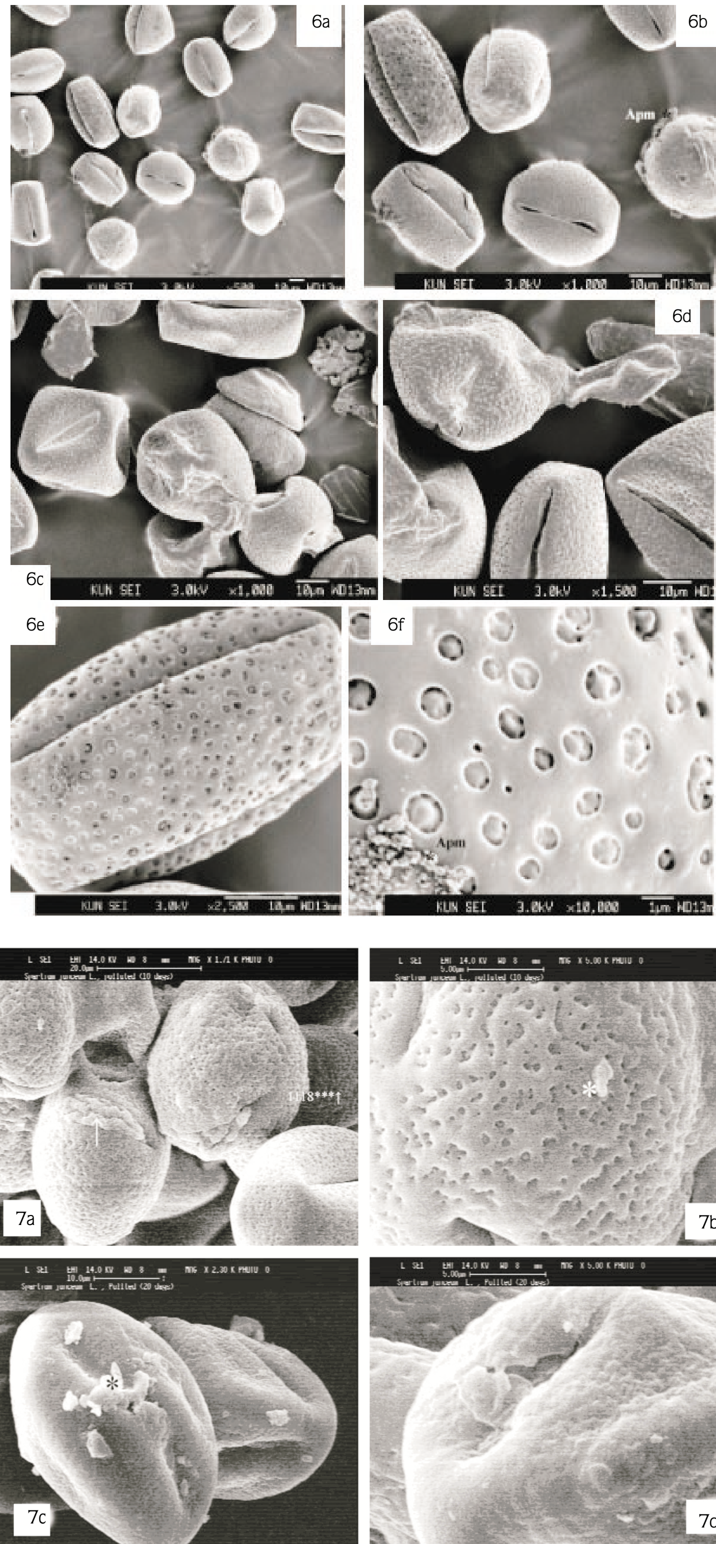


Figure 4a-5d. Light micrograph of cross section of anther development under air pollution conditions. 4a, 4b. Abnormal anther(s), respectively X100 and X400. 4c. microspore mother cells and tapetum are abnormal, X1000. 4d. Abnormal tetradic cells. Tapetum has premature growth, X400. 4e, 4f, Tetraspores abnormal, touching each other and degenerating, respectively X400 and X100. Figures 5a, 5b. Abnormal and irregular microspores, note the abnormal anther, respectively X400 and X1000. 5c. Mature pollen, breakage and thinning of exine, some pollen grains touching each other, X1000. 5d. The mature pollen after anther dehiscence, note the thin and breakable exine as well as cellular material release compared to control ones, X1000. Callose wall = Cw, Endothecium = En, Epidermis = E, Microspore mother cell = Mmc, Pollen = P, Subepidermic layer = Sel, Sporogen mass = Sm, Tapetum layer = Tl, Tetradic cells = Tc, Tetraspores = Ts.



Figures 6a-7d. Electron micrographs of the pollen grains of plants grown under air pollution conditions. In Figures 6a-6f, the pollen collected from polluted area. In Figures 7a, 7b, the pollen exposed to air pollution for 10 days. In Figures 7c, 7d, the pollen exposed to air pollution for 20 days. Scale bars show magnification. Arrows show cellular materials release. Asterisks show airborne particulate matter agglomeration, Airborne Particulate Material = APM.

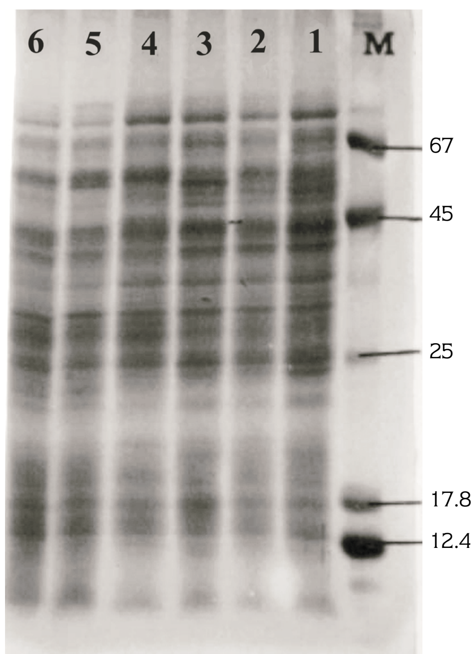


Figure 8. SDS-PAGE pattern of soluble proteins of pollen extracts, 1-6 respectively the pollen extracts of control, polluted area, 10 days exposed to control air, 10 days exposed to polluted air, 20 days exposed to control air and 20 days exposed to polluted air, Marker (Standard protein) = M.

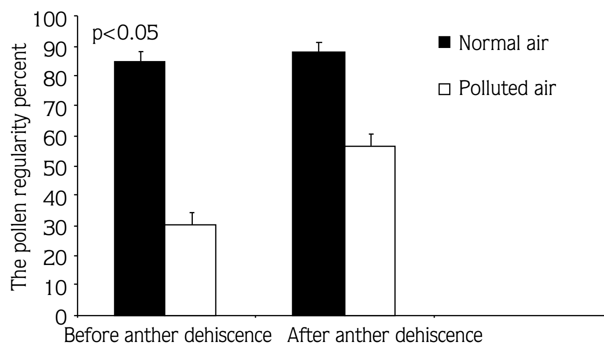


Figure 9. The effect of air pollution on pollen regularity (%).

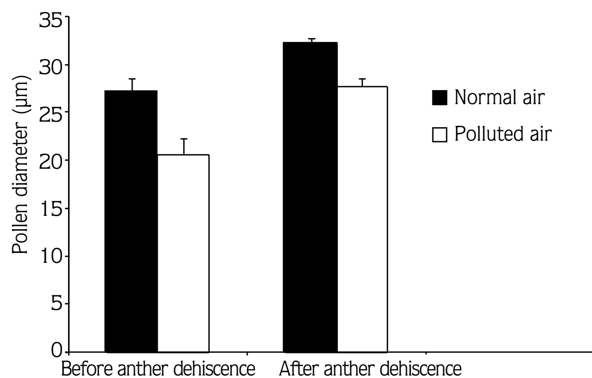


Figure 10. The effect of air pollution on pollen diameter (µm).

Table 2. Protein content of pollen extracts in different conditions (mg protein per gram pollen (dry weight), mgg^{-1}) (mean ± standard error).

Collected from control area	Collected from polluted area	Exposed to control air (10 d)	Exposed to polluted air (10 d)	Exposed to control air (20 d)	Exposed to polluted air (20 d)
6.5 ± 0.12	6.1 ± 0.16	6.2 ± 0.8	6.3 ± 0.10	6.4 ± 0.15	5.7 ± 0.1

Helender et al. (1997), who did not observe any significant difference between protein bands of polluted and control areas. However, studies by Behrendt et al. (1997) showed a dose-dependent shift in the intensity of IgE binding reactivity to lower molecular weight sites. In addition, Hjelmroos et al. (1994) and Parui et al. (1998) found a decrease in Bet v 1 concentration under air pollution. Studies on *Lagerestroemia indica* showed a reduction in total protein content and a subsequent lower staining intensity of proteins under air pollution conditions (Rezanejad et al., 2003). Taken together, studies on pollen protein show contradictory results. It is possible that the type of plant species, thinning of the exine under air pollution or a pseudo increase in pollen weight due to pollutant deposition on its surface could easily be the cause of these differences and therefore this matter needs to be studied further.

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