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Histochemical Observations on the Needles of Norway Spruce Trees affected by Cement Dust Pollution

By

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With 4 figures

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Summary

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The callose deposition, phenolics and starch grains appearance as well as the position and organisation of Ca-oxalate crystals in current-year spruce (*Picea abies*, *Pinaceae*) needles affected by cement dust were compared with not affected ones. Although without chlorotic symptoms, previously reported lowering of chlorophyll content indicated the altered physiological status of affected needles. Direct deposition of cement dust on the needle surface caused the crystalline crusts formation. Necrosis of mesophyll cells was, mostly, present near the stomata what is connected with dissolving of crusts caused by moisture giving the calcium hydroxide solution generally penetrating through the stomata. The abundant callose deposits detected with aniline blue were present in hypodermal layers of cells as grain-like forms in different size making a coat around the needle mesophyll as well as in cell walls of mesophyll cells proximate to hypodermis plugging the plasmodesmata. In correlation with callose deposition numerous Ca-oxalate crystals were observed in affected needles mostly appeared as druses when treated with sulphuric acid in fresh hand-made

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sections. Electron microscopy showed massive crystal aggregates placed between sclerotic parts of cell walls. Detection of polyphenolic substances with Toluidine blue O stain revealed abundant massive granulated tannin deposits in the vacuoles of mesophyll cells in affected needles. The occurrence of tannins in mesophyll cells near the necrotic areas was in the form of thick ribbon or the thin one, as well as a homogenous mass indicating more advanced stage of cell injury. Dust affected needles had bigger and more abundant starch grains. In conclusion, the observed changes in callose deposition should be considered as the protective event, while other histochemical alterations indicated the stress situation caused by a direct deposition of the alkaline dust.

Zusammenfassung

Cesar V., Lepeduš H. & Ljubešić N. 2004. Histochemische Untersuchungen an zementstaubbelasteten Fichtennadeln. – Phyton (Horn, Austria) 44 (2): 203–214, 4 Abbildungen. – Englisch mit deutscher Zusammenfassung.

In diesjährigen zementstaubbelasteten Nadeln von *Picea abies* (*Pinaceae*) wurden Callose-Ablagerungen, phenolische Substanzen und das Auftreten von Stärkekörnern sowie die Lage und Bildung von Kalziumoxalatkristallen untersucht und mit unbelasteten Nadeln verglichen. Obwohl keine chlorotischen Symptome beobachtet werden konnten, weist der verminderte Chlorophyllgehalt, über den schon früher berichtet wurde, darauf hin, dass der physiologische Zustand in den belasteten Nadeln verändert ist. Die Deposition von Zementstaub auf der Nadeloberfläche hat die Bildung kristalliner Krusten zur Folge. Nekrosen von Mesophyllzellen sind – meistens – in der Nähe der Stomata zu beobachten, was mit der Auflösung der Krusten durch Feuchtigkeit, wobei sich eine Kalziumhydroxidlösung bildet, in Verbindung zu bringen ist. Diese Lösung dringt dann durch die Stomata in die Nadeln ein. Die mit Anilinblau reichlich nachgewiesenen Callose-Ablagerungen können in hypodermalen Schichten in Form von Körnern verschiedener Größe beobachtet werden. Dadurch bilden sie einen Mantel um das Nadelmesophyll, sie verstopfen aber auch in den Zellwänden des Mesophylls die Plasmodesmen, welche an die Hypodermis angrenzen. Zusammen mit der Callose-Ablagerung können auch zahlreiche Kalziumoxalatkristalle in belasteten Nadeln beobachtet werden. Wenn die Kristalle mit Schwefelsäure in frischen Handschnitten versetzt wurden, erscheinen sie als Drüsen. Die Elektronenmikroskopie zeigt massive Kristallaggregate zwischen den Zellwänden. Der Nachweis von phenolischen Substanzen mit Toluidin-Blau 0 deutet auf reichliche Ablagerungen von Tanningranula in den Vakuolen der Mesophyllzellen belasteter Nadeln hin. Die Tanninablagerungen nahe dem Bereich der nekrotischen Bezirke im Mesophyll erfolgen in dicken oder dünnen Bändern, aber auch als homogene Masse. Letzteres weist auf einen fortgeschrittenen Schädigungsgrad hin. Staubbelastete Nadeln besitzen größere und mehr Stärkekörner. Zusammenfassend kann gesagt werden, dass die Callose-Ablagerungen als Schutzreaktion betrachtet werden können, die anderen histochemischen Veränderungen hingegen als Stresssituation, die durch den alkalischen Staub verursacht wird.

Introduction

Cement dust is one of air pollutants that greatly affect the surround vegetation and soil. The investigations on the effect of dust emitted from the cement industry on plants were comprehensively reviewed by FARMER 1993. It has been pointed out that such investigations never reached the same attention compared to the other air pollutants. The direct precipitation of cement dust on conifer needles was shown to change epicuticular wax organisation (GRILL & GOLOB 1983, BAČIĆ & al. 1999), induce mesophyll necrosis (CZAJA 1961, 1962) and decrease the activity of guaiacol peroxidases (CESAR & LEPEDUŠ 2001). On the other hand, the indirect influence was expressed by changing the soil chemistry with reflection to the mineral nutrition and chloroplast pigments content (MANDRE & TUULMETS 1997, LEPEDUŠ & al. 2003b) as well as on the vascular bundle anatomy (CESAR & LEPEDUŠ 2001).

The structural and histochemical changes of spruce needles were demonstrated to be very effective tool for discrimination the action of different air pollutants in both, controlled experiments and the field investigations (SOIKKELI 1981, RUETZE & al. 1988, FINK 1989, 1993, PUECH & MEHNE-JAKOBS 1997). It has been shown that the individual cells in the highly complex tissue of needles are specifically changed when influenced by some abiotic environmental factor, in respect to their usual shape and function (FINK 1993, 1999). For example, the main target cells for gaseous pollutants appeared to be the mesophyll cells. Epidermal cells were demonstrated to be the main target cells for acid precipitation, while sieve cells were first reacting when Mg and K deficiency was present.

The absence of systematic research of such problematic in Croatia, concerning the cytological and histochemical aspects, inspired us to investigate the effect of alkaline dust produced from the cement factory in Našice (Croatia) on the needles of surrounding Norway spruce (*Picea abies* L. Karst.) trees. Our previous investigations pointed the dual impact of cement dust on spruce needles. A direct influence caused by deposition on needle surface and input through the stomata lowered the peroxidase activity because of the alkaline microenvironment. An indirect impact was visible in sieve cell distortion and a decreased amount of chlorophyll, indicating the Mg deficiency (CESAR & LEPEDUŠ 2001). It was, also, shown that the decline of chlorophyll *a* content in current – year needles exposed to cement dust began after a longer period of time than the decline in chlorophyll *b*. The decline of all measured pigments in previous – year needles showed no dramatic progress during the second year of exposure. At the end of that period the level of carotenoids was nearly the same in affected and unaffected needles suggesting that spruce needles could posses a biosynthetic capacity sufficient for acclimatisation to the conditions of an alkaline microenvironment (LEPEDUŠ & al. 2003b). In this study we compared the callose deposition, the appearance of tannins and starch grains appearance as well as the position and organisation of Ca-oxalate crystals in spruce needles that were affected and not affected with cement dust.

Materials and Methods

The samples were collected from two localities (Fig. 1). The first locality was in the vicinity (inside 3 km) of cement factory (Našice, Croatia) and the other one (control) was at distance of about 50 km (Osijek, Croatia). In Croatia, the quarter of cement is produced in Našicecement company. In the year 2000 company sold 751.000t of cement. Both sample plots were in urban area with very similar climate conditions and water supply on see level between 100 and 200 m. In determination of control locations few criteria were applied: in the surrounding, at least 5 km, of each sampled tree there was no industry what is considered as pollution source; regarding to the traffic pollution peaceful parts of the town with family houses and gardens were chosen; trees were growing on similar soil types: clay or loamy-clay (Tab. 1); all vegetation, not only spruce trees, on chosen locations could be described as healthy and very nice. At each sample plot the current – year needles were harvested from five 15-30 years old cultivated Norway spruce [*Picea abies* (L.) Karst.] trees. Branches bearing needles were harvested from the middle crown of each tree. Every time, the sampling was done between 8^{AM} and 9^{AM}. Twigs were put in the nylon bag, placed on the ice and darkened. The transport to the laboratory was done within one hour. The sampling was carried out in April 2000.

Table 1

Soil parameters of sampling plots in Našice (SAMPLE 1) and Osijek (SAMPLE 2); P(t) – percent of similarity; NS – not significant.

PARAMETER	SAMPLE 1	SAMPLE 2	P(t)
% $CaCO_3$	9.55 ± 0.54	4.36 ± 0.07	<1%
pH	8.40 ± 0.00	8.35 ± 0.07	NS
soil type	loamy clay	loamy clay or clay	

For light microscopic investigations about 2-mm-long segments were cut from the middle of each current-year needle and fixed for 24 hours at +4°C in 6% glutaraldehyde in 0.05 M phosphate buffer (pH = 6.8). The specimens were then dehydrated in 2-metoxyethanol, ethanol, n-propanol and n-butanol (two changes in each) and embedded in methacrylate resin (Historesin, Leica). Three µm thin sections were stained with 0.05% Toluidine blue O in benzoate buffer (pH = 4.4). The Lugol reagent was applied to demonstrate starch grains. To demonstrate the presence of callose in fresh hand-made sections, 0.05% aniline blue in Hepes buffer (pH = 9.25) was used (Feder & O'Brien 1968, O'Brien & McCully 1981). The Ca-oxalate crystals were also demonstrated in fresh hand-made sections with 20% sulphate acid.

For electron microscopy, material was fixed in 1% glutaraldehyde in cacodylate buffer (pH=7.2) at +4°C, postfixed with 1% OsO_4, dehydrated in ethanol and embedded in Spurr's resin. Ultrathin sections (50-80 nm) were contrasted with lead citrate and uranyl acetate, and examined with electron microscope Zeiss EM 10A.

Results and Discussion

Macroscopic observations showed dust layers covering the needles from Našice without any visible symptoms of yellowing (Figs. 2A, 2B). The

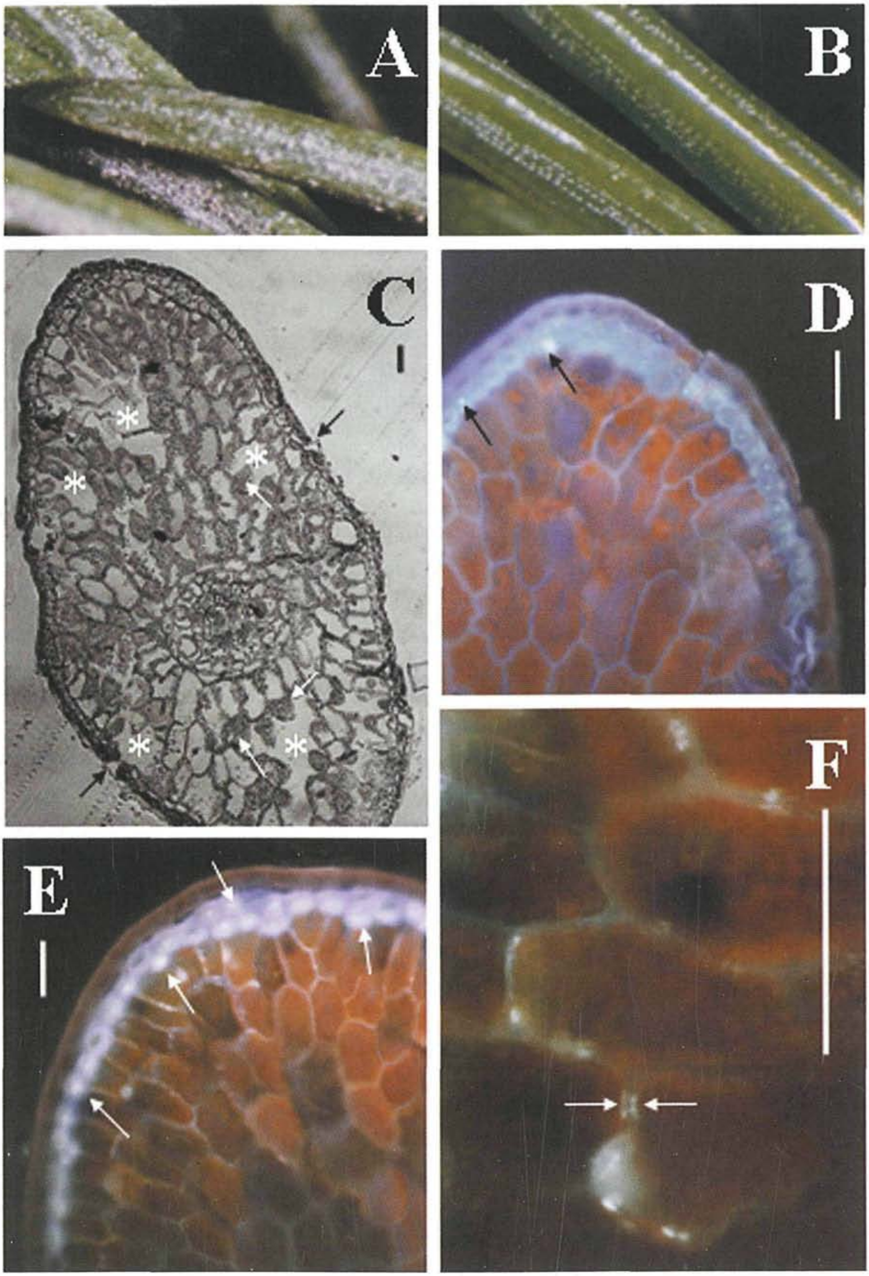
Fig. 1. The map of Croatia with the surrounding countries. The position of Našice (1), where the cement factory is located and Osijek (2) is marked.

absence of yellowing was observed in non-affected needles, too. Although without chlorotic symptoms, the lowering of chlorophyll content was reported for cement dust affected needles (MANDRE & TUULMETS 1997, CESAR & LEPEDUŠ 2001, LEPEDUŠ & al. 2003b), indicating altered physiological status of those needles.

Histological and histochemical analysis showed differences in needle mesophyll structural integrity between two investigated samples. The control needles from Osijek were characterized by intact epidermis and turgid mesophyll cells (photograph not shown). In contrast, dust affected needles from Našice appeared to have the partially necrotic mesophyll (Fig. 2C). Necrotic areas were mostly placed peripheral, near the stomata. Damaged areas also appeared in inner parts of mesophyll, but the analysis of serially cut sections showed that they were always connected with stomata. Direct deposition of cement dust on the needle surface caused the crystalline crusts formation. In a consequence of interaction with moisture, crusts dissolve and release the solution of calcium hydroxide through the stomata into intracellular spaces. This causes cell plasmolysis and death, consequently (CZAJA 1962). Our findings of mesophyll necrotic areas (Fig. 2C) near the stomata are in concordance with that hypothesis. Described spreading of cell damage could be categorized as slight to medium, according to SOIKKELI 1981. In that paper the damage was described in five phases corresponding with spreading of necrosis. Slight damage was characterised with injured cells near the stomata (phase I) and/or near the endodermis (phase II), medium damage by more advanced cell injury of inner parts of mesophyll, while severe or very severe damage (phases IV and V) corresponded with few or none healthy looking cells in the corners of needle cross-sections.

Poorly abundant callose deposits were detected in hypodermal cells of needle samples from Osijek (Fig. 2D). In needle samples from cement factory neighbourhood the callose was present in hypodermal layer of cells as grain-like forms in different size making a coat around the needle mesophyll (Fig. 2E). The bright white fluorescence of hypodermal cell walls indicates the presence of callose in those structures, too. The callose was also detected in the cell walls close to hypodermis, plugging the plasmodesmata between two neighbour mesophyll cells (Fig. 2F). In transmission electron micrographs callose deposits are seen lining the plasmodesm (Fig. 3A) but also covering the region of plasmodesmata (Fig. 3B). Similar results were presented by BÄCK & al. 1993 showing the callose deposition in plasmodesmata region as a rapid process induced by inappropriate conditions like acid rain and cold treatment. In the needle samples from Osijek, callose was totally absent in mesophyll cell walls (Fig. 2D). As nicely reviewed by FINK 1999 callose is considered to be the fastest response to any disturbance that increase the permeability of plasma membranes. BAČIĆ & al. 1999 demonstrated progressive epicuticular wax tubules erosion in needles of *Pinus halepensis* grown in the vicinity of the cement factory. Generally, gaseous pollutants, aerosols and various kinds of dust, particularly alka-

Fig. 2. A. Norway spruce needles from Našice covered with dust layers with no visible yellowing. B. The same needles pictured 8 months earlier when new borne needles were three months old. C. Necrotic areas (asterisk) in dust affected needles were placed, mostly, peripheral, near the stomata (black arrow) or inside the mesophyll but always connected with stomata. The residua of dead calls are marked (white arrow). D. Control needles had poorly abundant callose deposits in hypodermal cells (black arrow). E. Cement dust affected needles had callose present in hypodermal layer of cells as grain-like forms when indicated by aniline blue fluorescence (white arrow) making a coat around the mesophyll. F. Mesophyll cells close to hypodermis, in cement dust affected needles, had callose (white arrow) plugging the plasmodesmata of two neighbour cells. Bar 50 μm.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line ones, speed up the conversion of crystalline wax into amorphous form (GRILL & GOLOB 1983, SAUTER & VOSS 1986, SAUTER & al. 1987). That should greatly affect the needles wettabillity and rain retention (TURUNEN & HUTUNEN 1990). The increased needle wettabillity promotes cation exchange processes between apoplast and needles surface (TURUNEN 1996), what means than Ca^{2+}-ions are able to penetrate in needle inside not only through stomata, but at any place on the surface. High levels of Ca^{2+} could stimulate the synthesis and deposition of callose involved in long-term regulation of plasmodesmal closure (DING & al. 1999). The abundant callose deposits we detected in hypodermal cells of dust affected needles as well as in cell walls of mesophyll cells proximate to the hypodermis (Figs. 2E, 2F) are supporting this hypothesis. The intactness of the most of mesophyll cells close to hypodermis indicates that callose appearance was not only the answer to stress situation, but had a protective function against the progression of cell damage.

In correlation with callose deposition numerous Ca-oxalate crystals were observed in dust affected needles from Našice (Fig. 4A) showing the increased levels of calcium (BÄCK & al. 1993, KÖHLE & al. 1985). They mostly appeared as the druses when treated with sulphate acid in fresh hand-made sections. A very few smaller crystals were detected in the control needles from Osijek (Fig. 4B). Electron microscopy showed cell wall as the location of calcium oxalate crystals in dust affected needles (Fig. 3C). Numerous crystals were placed in cell walls of hypodermal region. Massive crystal aggregates were placed between sclerotic parts of the walls (Fig. 3D). The crystals of Ca-oxalate are the most prominently represented crystals in plants (ESAU 1977) with the detoxification function of excess Ca (FINK 1999). In his comprehensive study about the microscopic criteria for the diagnosis of abiotic injuries to spruce needles FINK 1993 showed the influence of different factors on Ca-oxalate crystals appearance. The results he obtained with two years old spruce needles from a damaged stand on calcareous soil and in ozone-fumigated material are going along with those presented here. RUETZE & al. 1988 reported the increased deposition of Ca-oxalate crystals in older needles of Norway spruce trees. So, the accumulation of the Ca-oxalate crystals that we have observed in dust affected needles (Figs. 3C, 3D, 4A) could be also considered as the premature aging symptom.

Detection of the polyphenolic substances with the Toluidine blue O stain, showed abundant tannin deposits in the vacuoles of mesophyll cells in dust affected needles (Fig. 4C), while no tannins were detected in control needles (Fig. 4D). Also, different appearance of tannins was observed. Most of the cells that were shown to have massive granulated tannin deposits in their vacuoles (Fig. 4C). In some other cells, tannins occur as a thick ribbon or the thin one, but also as a homogenous mass (Fig. 3D). Such appearance

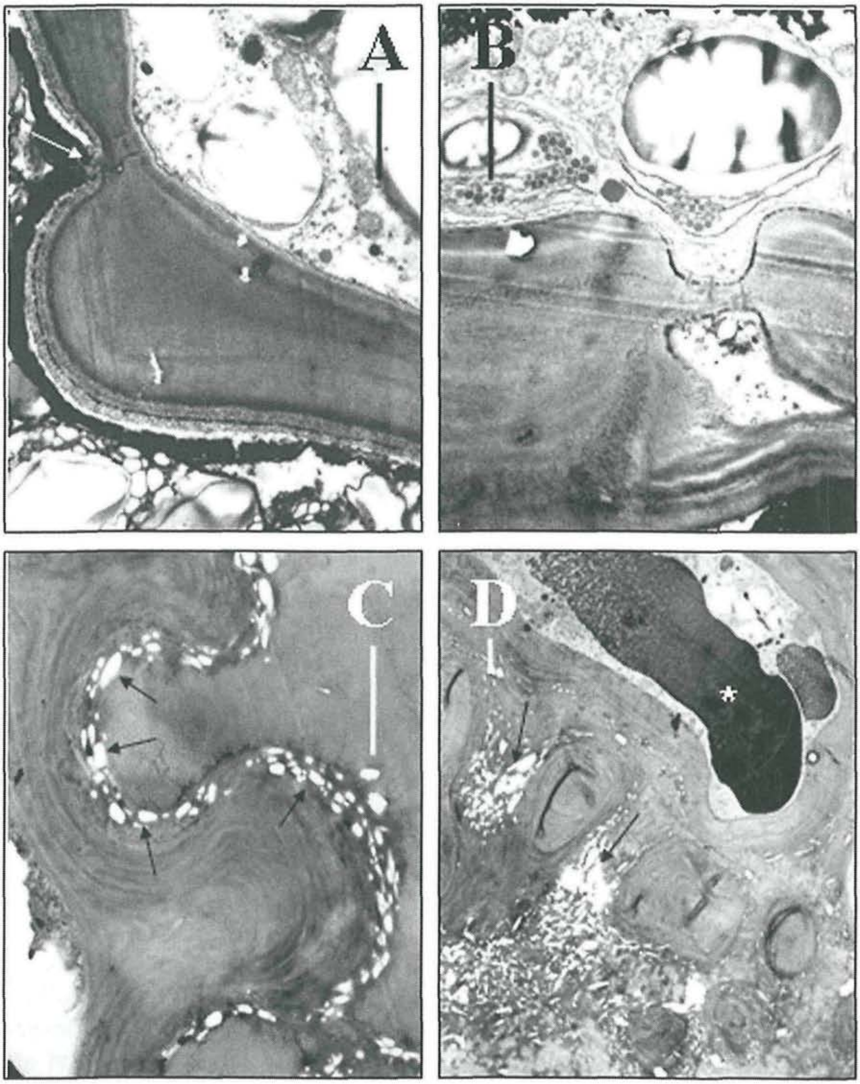

Fig. 3. Electron micrographs showing the deposition of callose and Ca-oxalate crystals in the cell walls of hypodermal region in needles exposed to cement dust pollution. Bar = 2 µm. A. Callose deposits lining the plasmodesmata (arrow). B. Overlapped plasmodesmata region. Chloroplasts having big starch grains, poorly developed thylakoid system and numerous plastoglobuli were also present. C. Calcium oxalate crystals (arrow) embedded in cell wall material. D. Massive oxalate crystal aggregates placed between sclerotic parts (arrow) of cell walls. Polyphenols in the form of homogenous mass (asterisk).

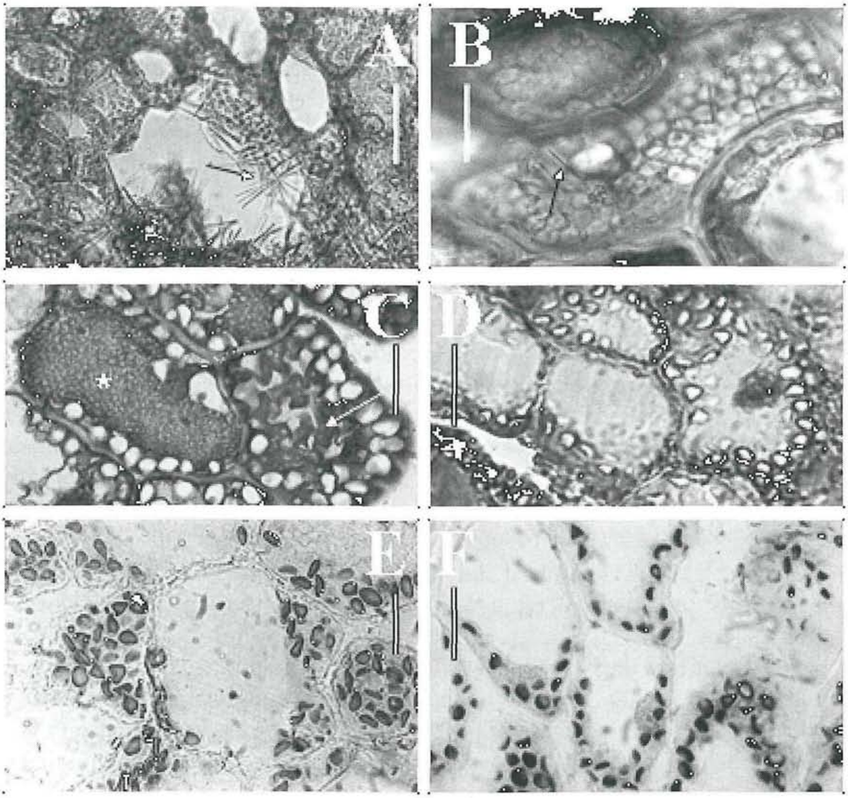

Fig. 4. A. Druses of Ca-oxalate crystals (arrow) observed in free hand-made sections treated with sulphate acid in cement dust affected needles. B. Few small Ca-oxalate crystals (arrow) were found in control needles. C. Polyphenolic substances detected with Toluidine blue O stain in cement dust affected needles mostly present as massive granulated deposits (asterisk) or thick ribbon-like forms emerged by clustering and fusing well abundant globules (arrow). D. Control needles with no polyphenolics in vacuoles. E. Regularly distributed starch grains in mesophyll cells of cement dust affected needles. Starch grains are bigger and more abundant compared to control. F. Mesophyll cells in non-affected control needles showing usual size and distribution of starch grains. Bar = 20 µm.

of tannins was detected in the mesophyll cells next to the necrotic areas, which indicates more advanced stage of the cell injury. LEPEDUŠ & al. 2003a compared the picture of polyphenolic substances in mesophyll cells of chlorotic spruce needles using the light and electron microscopy proposing the possible ways in originating those different forms. As pointed out by FINK 1999, the synthesis of polyphenols is one of the first reactions of parenchyma cells to the mechanical injury. A comprehensive investigation of the cytological injuries in conifer needles done by SOIKKELI 1981 revealed that it is possible to distinguish three different stages of cell injuries using the tannin appearance. The description of the tannin deposits in her investigation corresponded very well with those we have observed in our study (Figs. 4C, 4D). The occurrence of the grain structured tannin deposits was also reported in the needles of *Picea omorika* Pančić suffering from mineral deficiency (LEPEDUŠ & al. 2001).

The starch distribution in mesophyll cells of both dust affected and non-affected needles was regular (Figs. 4E, 4F). However, the starch grains were bigger (Fig. 3B) and more abundant in dust affected needles (Fig. 4E) compared to non-affected ones (Fig. 4F). Such homogeneous accumulation of starch was demonstrated in the Mg and K deficient spruce needles (FINK 1989, 1993). Our previous investigation (CESAR & LEPEDUŠ 2001) of the needles of spruce trees grown near the cement factory in Našice revealed the changes in the anatomy of vascular bundle characteristic for such nutritional disturbances. The declining of the phloem function that occurs in Mg and K deficient needles indicates inhibition in carbohydrate translocation process (FINK 1993, 1999, PUECH & MEHNE-JAKOBS 1997).

In conclusion, it appeared that besides the necrosis of mesophyll cells, a numerous biochemical changes took place in the tissues of Norway spruce needles affected with the cement dust pollution. The observed changes in callose deposition should be considered as the protective event, while other histochemical alterations indicated the stress situation caused by a direct deposition of the alkaline dust. Our future investigations will be concentrated on the tissues of spruce vegetative buds as well as early needles development in the atmosphere polluted with the alkaline dust.

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