

ENVIRONMENTAL POLLUTION CHANGES IN MEMBRANE LIPIDS, ANTIOXIDANTS AND VITALITY OF SCOTS PINE (*PINUS SYLVESTRIS* L.) POLLEN

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ABSTRACT

Investigations were carried out on pollen grains of Scots pine (*Pinus sylvestris* L.) collected from trees at 1.5, 3, 4 km and control, 20 km from the Luboń factory producing mineral fertilisers. The percentage of germination of pollen formed close to the pollution source was ca 20% lower compared to the control pollen. Lowered vitality of the pollen was effected in changes of the structure of cytoplasmic membranes. Pollen from the polluted area contained ca 15% less total phospholipids, mainly phosphatidylcholine and phosphatidylinositol and had a lower content of soluble proteins and less of low molecular antioxidants, such as thiols and ascorbic acid. Composition of total fatty acid in phospholipids fractions showed a significant reduction in the degree of unsaturation of fatty acids. Pollen originating from the polluted area and stored at -30°C showed considerably stronger degradation of cytoplasmic membranes than control.

KEY WORDS: antioxidants, environmental pollution, freezing stress, fatty acids, membranes, phospholipids, *Pinus*, pollen grains.

INTRODUCTION

Pollen of Scots pine (*Pinus sylvestris* L.) is sensitive to unfavourable environmental conditions (Cox 1992; Chałupka 1998). Numerous stress factors such as low and high temperature, desiccation or imbibition (Hoekstra et al. 1991 and 1992; Sowa et al. 1991), and industrial pollution, (Mulcahy and Mulcahy 1987), cause serious changes in the cytoplasmic membranes which affect vitality of the pollen grains. Most sensitive to injuries are water transportation and enzymes as well as the lipid-protein structure of membranes which carry the receptors of light, transmitters of signals, and channels of ions. They are intensively investigated to identify the molecular basis for sensitivity of pollen to abiotic stress factors (acidity, heavy metals, sulphur dioxide, ozone, aluminium and fluoride, detergent which strongly affect pollen in laboratory conditions (Cox 1992; Paoletti 1992). Scots pine pollen formed under air-soil pollution also had a significantly higher concentration of S, Al, Cu, Ni and Cd and lower content of Zn (Oleksyn et al.

1999) than the control. Enhanced accumulation of nutrients and heavy metals by pollen may affect its function and the molecular studies may prove helpful in explaining both the decrease in pollen viability and the physiological behaviour of forest trees affected by industrial pollution (Smith 1990). It is known that pollen grains are more tolerant to abiotic stresses owing to: (1) greater accumulation of unsaturated fatty acids which maintain fluidity of membranes on the physiological level, (2) accumulation of greater amounts of phospholipids and water soluble proteins responsible for transmutation of the isotropic membrane structure into a hexagonal H_{II} one, and (3) maintenance of an adequately high level of low molecular antioxidants functioning as free radical scavengers preventing oxidation of phospholipid fatty acids than other plant tissues i.e. the needles (Pukacka and Pukacki 2000a).

Undisturbed intracellular control of these processes prevents damages and decelerates the ageing process of pollen (Hoekstra 1986) as well as of seeds during long-term storage (Pukacka and Kuiper 1988). This is especially important in breeding work when maintenance of high viability of pollen is needed for several years (Towill 1985; Charne et al. 1988). Plant material for our investigations came from the areas located at different distance from phosphate fertiliser factory Luboń. The Luboń factory was established in 1917 and until 80-ties continuously emitted more

Abbreviations

FA – fatty acid; 16:0 – palmitic acid; 18:0 – stearic acid; 18:1 – oleic acid; 18:2 – linoleic acid; 18:3 – linolenic acid, PL – phospholipids; PA – phosphatidic acid; PC – phosphatidylcholine; PE – phosphatidylethanolamine; PG – phosphatidylglycerol; PI – phosphatidylinositol

or less increasing amounts of various pollutants to the environment (Oleksyn and Bialobok 1986). The Luboń area influenced for many years by high pollution emission: SO₂, from 2608 t in 1980 to 80 t in 1996, and fluorine compounds from 169.1 t to 1.15 t in 1996. Soil at the polluted site is 10-fold more acidic, has higher AL and P, and lower K, Ca, Mg, than the soil from the control site (Kórnik). The full characteristic of experimental area are described elsewhere (Oleksyn and Bialobok 1986; Reich et al. 1994; Pukacki 2000; Pukacki and Kamińska-Rożek 2002). In the late 80-ties air pollution substantially declined owing to the use of more modern technology and the polluted soil became the main factor influencing the physiological processes and development of Scots pine trees (for more details on the soil and plant organs characteristics see; Reich et al. 1994; Giertych et al. 1997; Oleksyn et al. 1999). In addition Fober (1999) on the same polluted area observed ca 30% decline in height growth of Scots pine trees. The purpose of the present study was to test whether the Scots pine pollen formed under pollution shows visible and destabilising changes in the structure of cytoplasmic membranes and in the level of low molecular antioxidant and soluble proteins.

MATERIAL AND METHODS

Plant material

Mature male strobili of Scots pine were collected from 16-30 year-old trees growing in zones of different levels of air and soil pollution at 1.5, 3, 4 km and control 20 km, (Kórnik) of southeast the Luboń phosphate fertiliser factory. Each site was represented by three trees. Pollen was cleaned by sieving through a fine nylon mesh allowed to dry in dry air, under incandescent lamp at 32°C until the water content reached 8% moisture (a H₂O/g fresh wt.) and stored at 4°C before use.

Cold storage treatment

After collection, two 15 g samples of pollen were cleaned and dried to 8% moisture content and placed in a plastic tube. Samples of pollen were transferred to a cooling chamber at -30°C ± 2°C and stored for 16 months. The cooling and thawing rate of samples was 6°C h⁻¹.

Estimation of pollen vitality

Fresh pollen grains as well as those from 4°C and grains stored for 16 months at -30°C, were placed on petri dishes (d = 6.0 cm), containing sterile medium with 7% sucrose, 1% of agar and 0.01% boric acid. The pollen was incubated at 23°C for 7 days. Percentage of germinating grains and length of their pollen tubes were examined under a microscope count of 250 grains. The experiment was repeated three times.

Membrane composition

Pollen samples of 250 mg weight both, fresh and after storage at -30°C were used for the extraction of total phospholipids. Before extraction, pollen was washed in 1.5 ml hexane and then ground in a mortar with 2 ml chloroform:methanol (2:1, v/v) and 0.005% butylated hydroxytoluene (BHT) as an antioxidant. The sample was centrifuged at 10 000 g to remove the debris; and supernatant wa-

shed with 0.3 volumes of 0.7% NaCl. After phase separation, the CHCl₃ layer was collected, dried under N₂ and used as a total lipid extract.

Separation of polar and non-polar lipids was performed using SEP-PAK Silica cartridge (Waters As, No. 51900) according to Juaneda and Rocquelin (1985). Total and individual phospholipids were determined in aliquots of the lipid extract, separated by TLC (Merck, No 1.05721, Silica gel 60), developed in chloroform:methanol:acetic acid: water (85:15:10:3.5, v/v), (Nichols et al. 1965), as described by Pukacki and Kamińska-Rożek (2002). The bands were scraped off for phosphorus analysis. Quantities of particular phospholipids were expressed as µg P mg⁻¹ of total lipids, according to Ames (1966), as described by Pukacka (1989) and Pukacki (1995).

Fatty acids were determined in aliquots of the phospholipid fraction according to Kendall and McKersie (1989), and Pukacki et al. (1991). Fatty acids were saponified, methylated for 50 min at 90°C in the presence of 14% boron trifluoride (BF₃) in methanol (Sigma) in a N₂ atmosphere and separated using a gas chromatograph (Model 5890, Hewlett Packard).

Analyses of methyl esters of fatty acids from phospholipids were performed with temperature increase programmed between 160° and 280°C at 5°C min⁻¹. Fatty acids were quantified and identified by comparison of retention times using methylheptadecanoate (C17) (Sigma) as internal standard.

Assay of antioxidant

For the assay of ascorbate (AA) and non-protein thiols (-SH), 1 g and 500 mg respectively, samples of pollen were ground to a fine powder under liquid nitrogen in a mortar with pestle. The grinding was continued after adding 5 ml 5% trichloroacetic acid (TCA) containing 10 mM EDTA (ethylenediaminetetraacetate). The homogenate was filtered through two layers of cheese cloth, centrifuged at 12 000 g for 30 min at 4°C and the supernatant was used for the assays (Pukacka and Pukacki 2000a).

Ascorbate content

Ascorbate acid (AA) content was assayed according to Kampfenkel et al. (1995). The assay is based on a reduction of Fe³⁺ to Fe²⁺ by ascorbic acid in an acidic solution. Fe²⁺ forms complexes with bipyridyl, giving a pink colour with a maximum absorbance at 525 nm.

The concentration was determined by comparison with a standard curve.

Thiols content

Nonprotein thiol content was determined at 412 nm according to Sedlack and Lindsay (1968) using Ellman's reagent 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). The standard curve was made with glutathione (GSH), (Sigma).

Protein content

For analysis of total soluble protein, 500 mg of pollen were ground in 10 ml of 10 mM Hepes buffer pH 7.4 The homogenate was centrifuged at 10 000 g for 20 min (4°C) and the supernatant filtered through a blotting-paper. Protein was determined in an aliquot of each sample using bovine serum albumin (Sigma) as a standards, according to Bradford (1976). The absorbance was read using a UV-

-2401PC spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD).

Statistical analysis

All data were subjected to analysis of variance (ANOVA). Different letters in the figures indicates significant differences according to the, Duncan's test. Linear regression analysis was used to test the correlation between germination percentage of pollen grains and amount; total phospholipids, thiol group and soluble proteins.

RESULTS

Tests of vitality *in vitro* showed a distinct influence of the polluted soil and air at Luboń on the germination percentage of Scots pine pollen grains. Pollen collected from trees growing in the most strongly contaminated zone (1.5 km from the chemical factory) showed germination ability of pollen tubes lower by ca 20% in comparison to the control, pollen from the relatively non-polluted site of Kórnik which germinated in more than 90 % (Fig. 1). The significantly negative effect of pollution on pollen vitality was observed after storing pollen for 16 months in -30°C (Fig. 2). Ability to germinate decreased by ca 8-10%, for pollen

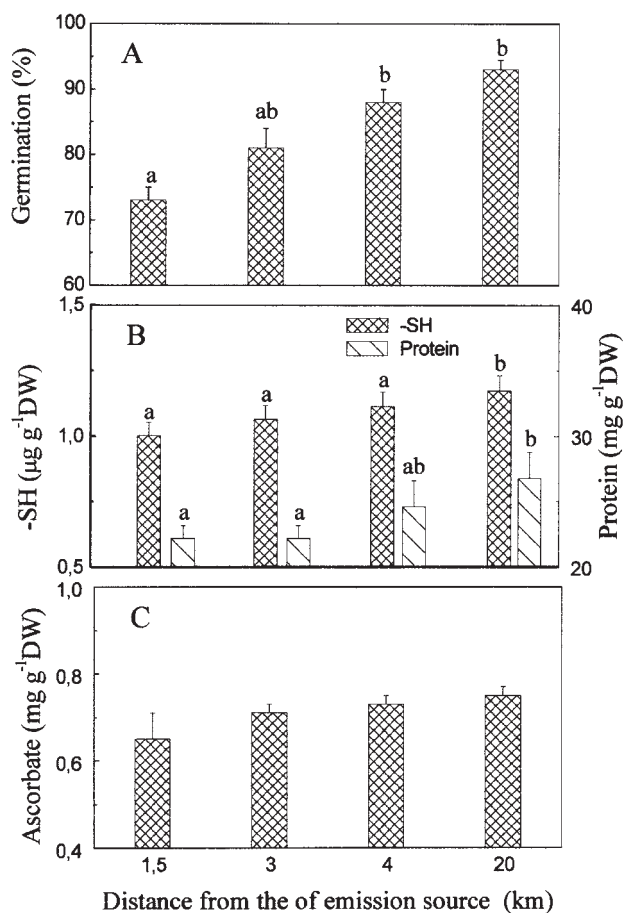


Fig. 1. Germination (a), thiols and soluble protein content (b), ascorbic acid (AA) content (c) in fresh pollen grains of Scots pine growing in at different distances from the Luboń factory (source of emission). Each value represents a mean from three trees ($n = 6$) \pm SD. Different letters on the top of bars in same analysed component indicate significant difference at $p < 0.05$.

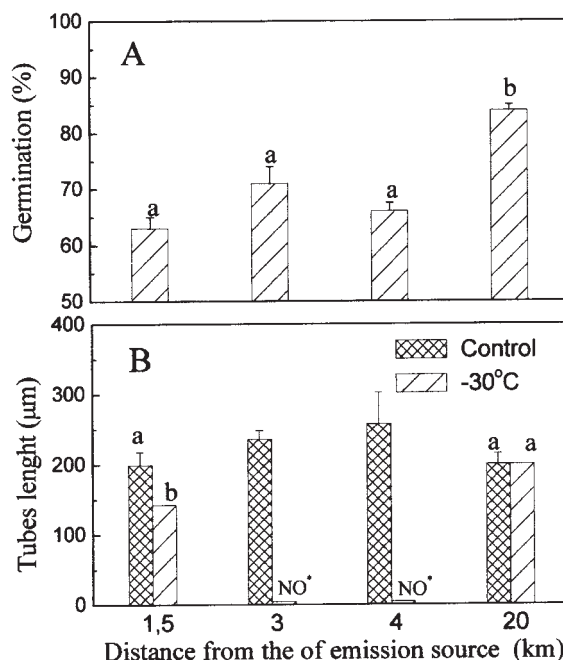


Fig. 2. Effect of long-term (16 months) cold storage (-30°C) on germination (a) pollen tubes length (b) of Scots pine pollen versus different distances of trees from the source of emission. Each value represents a mean from three trees ($n = 6$) \pm SD. Different letters on the top of bars indicate significant difference at $p < 0.05$, NO*, not analyse.

from the polluted and the control site. The mean length of pollen tubes decrease by ca 30%, only in samples from trees near Luboń factory. Storage of pollen at low temperature had no effect on the length of the germinating pollen tubes from the control site in Kórnik. A highly significant correlation was observed between germination percentage of pollen grains and the content of soluble proteins ($r = 0.916$, $p < 0.05$), total phospholipids (PL), ($r = 0.966$, $p < 0.05$) and thiols content ($r = 0.989$, $p < 0.01$). Analysis of soluble proteins, low molecular antioxidant -SH groups showed a significant decrease of their contents in pollen from the polluted site as compared to the control area (Fig. 1). A strong positive correlation was detected between the content of soluble proteins and -SH groups in pollen and the distance from emitter to the site of pollen sampling ($r = 0.896$ and $r = 0.867$ at $p < 0.05$, respectively).

These results show that pollen from the most heavily polluted area (1.5 km nr Luboń) has a lower ability to detoxify free radical scavengers, because in cells of their pollen there is less low molecular antioxidants. The content of phospholipids, was lower in pollen collected from pines growing near the polluting factory in comparison to the pollen collected from the control area (Fig. 1). Stability of membranes in pollen grains increased with increase in distance from the source of pollutant emission. Similarly distinct decrease was observed in the content of phospholipids in pollen samples stored at -30°C . The greatest differences among pollen samples collected at different distances from the pollution source were found for among phospholipids, phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatidylethanolamine (PE) (Table 1). In the pollen from the polluted site, a ca 50% decrease in PC content after storage was in comparison to pollen from the control site. The total fatty acid content in the fraction of

TABLE 1. Composition of membrane phospholipids in *Pinus sylvestris* pollen grains collected at different distances from the source of pollution emitted by the Luboń factory before (control), and after 16 months cold storage at -30°C . Data are presented as means from three trees ($n = 6$) \pm SD.

Distance from pollution source		Phospholipid $\mu\text{g mg}^{-1}$ lipids					
km	Treatment	PG	PI	PC	PE	PA	PC/PA
1.5	Control	0.29 (0.07)	0.80 (0.08)	3.2 (0.5)	0.33 (0.1)	0.96 (0.1)	9.6
	-30	0.17 (0.04)	0.44 (0.1)	1.65 (0.4)	0.17 (0.04)	0.46 (0.1)	9.7
3	Control	0.30 (0.1)	0.80 (0.1)	3.50 (0.4)	0.30 (0.1)	0.94 (0.05)	11.7
	-30	0.22 (0.01)	0.45 (0.1)	1.76 (0.02)	0.35 (0.2)	0.51 (0.1)	5.0
4	Control	0.36 (0.1)	0.92 (0.2)	3.70 (1.2)	0.30 (0.1)	0.77 (0.3)	12.3
	-30	0.30 (0.06)	0.65 (0.02)	2.45 (0.1)	0.39 (0.1)	0.66 (0.1)	6.3
20	Control	0.78 (0.1)	0.75 (0.1)	3.20 (1.4)	0.31 (0.1)	0.81 (0.2)	10.3
	-30	0.32 (0.01)	0.27 (0.01)	3.23 (0.01)	0.29 (0.1)	0.71 (0.06)	11.1
Mean ^a	Control	0.43*	0.81*	3.40**	0.31	0.87*	10.9*
	-30	0.25	0.45	2.27	0.30	0.58	8.0

Mean^a at; *, ** indicate significance at $p \leq 0.05$, $p \leq 0.01$, respectively.

phospholipids after storage at -30°C was lower in the pollen sampled in the polluted areas 1.5 km away emission source, than in the control one (Fig. 3). This concerned mostly unsaturated acids such as oleic acid (18:1) and linoleic acid (18:2).

DISCUSSION

Our results indicate that the decades of environmental pollution had an influence on Scots pine causing a decrease both in the percentage of pollen germination and in the length of pollen tubes during germination on agar medium. This confirms earlier results of Mejnartowicz and Lewandowski (1985), and Ostrolucka et al. (1995), who also showed an inhibitory effect of pollution on the ability of pollen to germinate. However, in these studies no explanation of the phenomenon of pollen injuries was proposed. Investigations of Hoekstra et al. (1992), Wolkers and Hoekstra (1995) and Golovina et al. (1998) showed that cytoplasmic membranes were prime targets for destructive changes in pollen grains as a result of abiotic stresses. There is little information in the literature about the structural and biophysical function of membranes in the tolerance of pollen grains to industrial pollution. It is known that concentration of several elements such as Al, S, Mn, Cu, Ni and Cd is significantly higher in Scots pine pollen formed at the polluted site and this may affect pollen functions (Oleksyn et al. 1999). The gas chromatograph analysis of membrane phospholipids can provide information on membrane changes in the pollen grains. The results concerning fatty acids and phospholipid compositions indicate damages in pollen caused by pollution; showed a lower content of these components in pollen from polluted site (Fig. 3 and Table 1). The decreased ratio of unsaturated to saturated fatty acids (18:2/18:0), indicates a process of peroxidation of membrane lipids of pollen formed at the site with contaminated soil (Fig. 3b).

In other study, lipid changes were attributed to the stress-induced degradation process (Pukacki et al. 1991). Modifications of the content of polyunsaturated fatty acids (18:1, 18:2) and some phospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in membranes, cause physical changes, which are responsible for ion permeability and transition temperature (T_m), phase change from the liquid-crystalline to the solid-gel. The changes observed in pollen show a potential possibility for a change of the phase transition temperature for membranes of pollen grains. Such a tendency of changes was found for pollen of *Rosa* and *Quercus* upon imbibition (Hoekstra et al. 1992). Many authors have also reported lateral phase separation of mem-

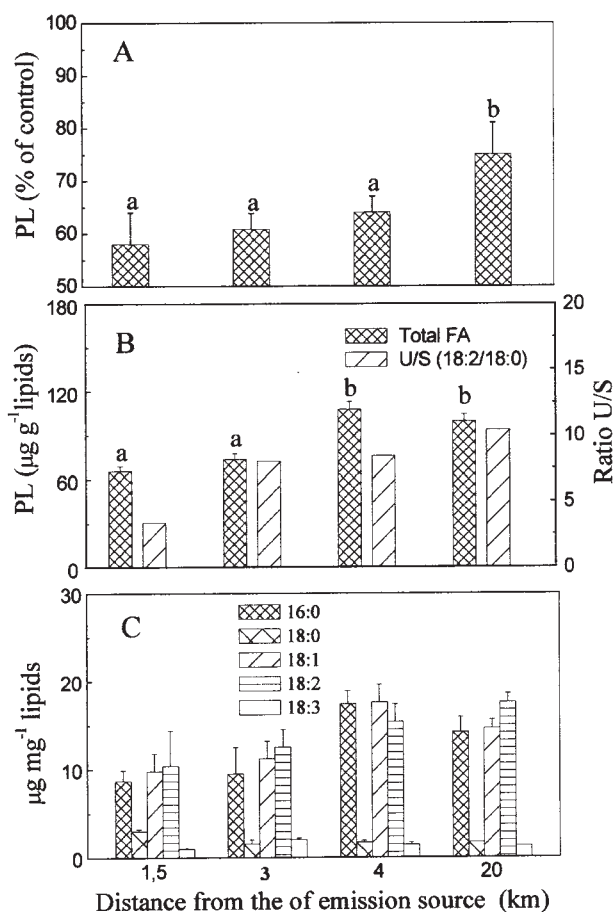


Fig. 3. Effect of pollution on total phospholipids (PL) content (a), total fatty acids (FA), unsaturated to saturated fatty acid ratio, i.e. (18:2/18:0) (b), and fatty acid composition in the phospholipid fraction (c), of pollen Scots pine versus different distance of trees from the source of emission, after 16 months storage at -30°C . Each value represents a mean from three trees ($n = 6$) \pm SD. Different letters on the top of bars indicate significant difference at $p < 0.05$.

brane constituents in plant cells that had been frozen (Bryant and Wolfe 1992). A decreased content of low molecular antioxidants, thiol groups (-SH) and of soluble protein in pollen from the polluted site (Fig. 1b), clearly indicates participation of these molecules in the process of free radicals scavenging from the cells. Increased free radical production in cells was ascribed to intensive catabolism of adenine nucleotides, injury of mitochondria and lowering of DNA repair efficiencies (Bartosz 1997). Żytkowiak et al. (1996) have reported that the free proline content was 32% lower in the Scots pine pollen formed in the polluted area (Luboń) compared to the control (Kórnik). Protein denaturation might contribute to loss of membrane integrity, since protein are integral components of biological membranes. Under stress, there is no synthesis of new proteins which could protect sensitive cellular components of pollen grains and pollen tubes (Hopf et al. 1992). Thus, it is possible that low molecular antioxidants such as glutathione might participate as one of the main factors of protein protection against environmental pollution. A similar effect was observed in needles of Scots pine in the same polluted areas (Pukacka and Pukacki 2000a, b). In the present study we found that pollen from the polluted area after storage at low temperature (-30°C) showed poorer vitality and larger changes in the cytoplasmic membranes. Combination of long-term air/soil pollutants and low temperature stress led to additive membrane injury effects. Under natural conditions polluted pollen grains and pollen tubes can be injured during winter and/or spring freezing stress. There is no direct data on the relationship between observed biochemical changes in pollen under abiotic stress and their influence on fertilisation process and seed quality but such a hypothesis is plausible. Thus the effects of air/soil pollutants are very complex and require more investigation because they may soon become a major constraint on forest productivity.

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WPLYW ZANIECZYSZCZONEGO ŚRODOWISKA
NA LIPIDY BŁON, ANTYUTLENIACZE ORAZ ŻYWOTNOŚĆ
PYŁKU SOSNY ZWYCZAJNEJ (*PINUS SYLVESTRIS* L.)

STRESZCZENIE

Badania były prowadzone na ziarnach pyłku sosny zwyczajnej (*Pinus sylvestris* L.) zebranego z drzew oddalonych od fabryki produkującej nawozy mineralne w Luboniu w odległości 1,5, 3, 4 i 20 km (powierzchnia kontrolna). Procent kiełkującego pyłku uformowanego blisko źródła emisji zanieczyszczeń był o 20% niższy w porównaniu ze zdolnością do kiełkowania pyłku kontrolnego. Niska żywotność pyłku była wynikiem powstałych zmian w strukturze błon cytoplazmatycznych. Pyłek pochodzący z zanieczyszczonego stanowiska charakteryzował się o 15% niższą zawartością ogólnych fosfolipidów, głównie fosfatydylocholiny i fosfatydyloinozytolu, białka rozpuszczalnego oraz niskocząsteczkowych antyoksydantów: grup tiolowych i kwasu askorbinowego. Kwasy tłuszczowe we frakcji fosfolipidowej wykazały istotną redukcję stopnia nienasycenia. Pyłek pochodzący z sosen rosnących na powierzchni skażonej po przechowaniu w temperaturze -30°C wykazał znaczącą degradację składników błon cytoplazmatycznych w porównaniu do pyłku kontrolnego.

SŁOWA KLUCZOWE: Antyutleniacze, skażenie środowiska, stres mrozeniowy, kwasy tłuszczowe, błony, fosfolipidy, *Pinus*, ziarna pyłku.