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Changes in pollen autofluorescence induced by ozone

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Abstract

The changes in the autofluorescence spectra of intact *Philadelphus grandiflorus*, *Epiphyllum hybridum*, and *Plantago major* pollen have been observed with microspectrofluorometric method after 100-h exposure to ozone (3 h per day for 5 d per week in total dose 5.0 cm^3 m^{-3}). The fluorescence maximum at 530 - 550 nm disappeared in carotenoid-containing pollen of *Philadelphus grandiflorus* and *Epiphyllum hybridum*, and new maximum at 475 - 480 nm arose that correlated with lipofuscin-like substances observed in the extracts from the pollen grains. The carotenoid-less pollen of *Plantago major* showed only the increase of the maximum at 470 nm, and no lipofuscin in the extracts. It is supposed that main indicator of the pollen damage by ozone in carotenoid-containing microspores are the lipofuscin-like compounds.

Additional key words: *Epiphyllum hybridum*, exine, lipofuscin-like compounds, microspectrofluorometry, *Philadelphus grandiflorus*, *Plantago major*, sporopollenin components.

Introduction

Ozone depresses the pollen germination of various species to a different degree (Feder 1968, Mumford *et al.* 1972, Nakada *et al.* 1976). The primary target appears to be components of exine, although there are no experimental proves. Ozone is known to induce the formation of fluorescent lipofuscin-like compounds in seeds which have a characteristic emission in blue or blue-yellow spectral region (Brooks and Csallany 1978). Pollen autofluorescence excited by ultra-violet irradiation (360 - 380 nm) changes during development and ageing, and depends on the composition of the exine (Roshchina and Melnikova 1996, Roshchina *et al.* 1996, 1997, 1998, Melnikova *et al.* 1997). The aim of this paper is to show how ozone acts on the pollen cover autofluorescence.

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Materials and methods

Objects of the study were mature pollen grains of *Philadelphus grandiflorus* Willd. with high carotenoid content of 0.72 μg kg^{-1}(f.m.), *Epiphyllum hybridum* with low carotenoid content of 0.3 - 0.5 μg kg^{-1}(f.m.) and carotenoid-less *Plantago major* L. (Roshchina *et al.* 1996, 1997, 1998) collected in natural habitats or grown in greenhouse (*E. hybridum*). The choice of the species is related to the possible participation of carotenoids in lipofuscinogenesis in animals (Karnaukhov 1988). A viability estimated as pollen germination of *P. grandiflorus* and *P. major* (well-grown in control unlike *E. hybridum*) *in vitro* (in 10 % sucrose) was determined as described earlier (Roshchina *et al.* 1997). Chronic ozonation of pollen was carried out by electric charge in the ozonator chamber (0.15 cm^3 m^{-3} for 3 h per day during 5 d per week). The fluorescent products were also eluted from pollen by 20 cm^3 of chloroform:methanol or chloroform:ethanol (2:1, v/v) (Brooks and Csallany 1978), then the extracts were separated by thin layer chromatography (TLC) on *Chemapol silicagel-TLC* plates (Prague, Czech Republic) in mixture of chloroform: methanol:acetic acid:water or chloroform:ethanol:acetic acid:water (90:5:1:0.3, v/v/v/v) (Shimasaki *et al.* 1995). Protein occurrence in fluorescent spots, was determined by staining with Coomassie Brilliant blue - R 250 (*Serva*, Heidelberg, Germany) in 7 % acetic acid. Proteins were also estimated in ethanol extracts from the fluorescent spots according to Bradford (1976). Fluorescence of intact cellular surfaces induced by ultra-violet irradiation (360 - 380 nm) was observed under fluorescent microscope *Fluoval* (*Carl Zeiss*, Jena, Germany), and the fluorescence spectra of the intact cells were registered with microspectrofluorimeter of original construction (Karnaukhov 1978). The diameters of optical probe: 100 μm, 20 μm, 2 μm, the objective lens: ×10, the ocular lens: ×7. The fluorescence spectra of extracts from pollen by chloroform:methanol (2:1, v/v) or chloroform: ethanol (2:1, v/v) and the ethanol extracts (by 1 cm^3 of the solvent) from spots on TLC plates with R_f = 0.2 were done with *Perkin-Elmer* spectrofluorimeter *Coleman 575* (Norwalk, England). The fluorescence is measured in relative units. The registration time of 400 - 700 nm fluorescence spectra was 22 s. The error of the fluorescence measurement for the same sample was 1 - 2 relative units.

Results and discussion

Irreversible shifts in the fluorescence spectra of intact pollen are seen when pollen lost its viability - a capability to form pollen tubes (Table 1). Pollen of *P. grandiflorus* was not so sensitive to ozonation as pollen of *P. major*. For the first species irreversible changes in a fluorescence were seen after total dose of ozone 3.5 - 5.0 cm^3 m^{-3}, whereas in the second after 0.90 cm^3 m^{-3}. Perhaps, it deals with the protective role of carotenoids in exine of *P. grandiflorus* (Karnaukhov 1988).

After 100-h treatment with ozone (total dose 5.0 cm^3 m^{-3}) the fluorescence spectra of mature pollen of *P. grandiflorus* and *E. hybridum* differed from the control (Fig. 1). In the first case a new maximum at 465 - 480 nm arose, whereas in the

second one, in addition to this maximum, a shoulder in yellow region of the spectrum was seen. The analogous autofluorescence spectra were found in ageing pollen

Table 1. The influence of ozone dose on occurrence of irreversible shifts in the fluorescence spectra and pollen germination *in vitro* [% of control]. SE was 2 - 3 %. The germination index (ratio of germinated pollen grains to total pollen grains) in control of *P. grandiflorus* was 0.71 and of *P. major* was 0.52.

Dose of ozone [$cm^3 m^{-3}$]	*Philadelphus grandiflorus* fluorescence shifts	pollen germination	*Plantago major* fluorescence shifts	pollen germination
0	no	100	no	100
0.15	no	98	no	84
0.90	no	98	yes	0
1.65	no	97	yes	0
2.40	no	18	yes	0
3.50	yes	0	yes	0
5.00	yes	0	yes	0

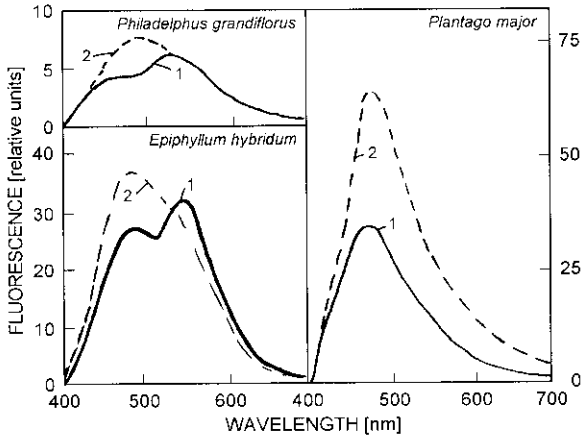

Fig. 1. The autofluorescence spectra of intact pollen from *Philadelphus grandiflorus*, *Epiphyllum hybridum* and *Plantago major*. *1* - control, *2* - treated with ozone (total dose 5.0 $cm^3 m^{-3}$ after 100-h ozonation).

of *P. grandiflorus* and *E. hybridum* stored for 1 year. In contrast, the fluorescence spectra of *P. major* pollen was not changed, although the intensity of the emission increased. The differences in the fluorescence of studied species could be associated with different qualitative and quantitative composition of their exine or with different humidity of pollen grains. *P. grandiflorus* pollen grains with water content of 30 % showed weaker fluorescence than *E. hybridum* pollen grains with water content 5 % (Roshchina and Melnikova 1996, Roshchina *et al.* 1997). Long-term action of oxidants may induce lipid peroxidation in membranes which, in dependence on the amount of unsaturated fatty acids, leads to the formation of ageing-induced

fluorescent pigments (Yun 1995). Oxidizing carotenoids can also participate on this process (Karnaukhov 1988).

According to Brooks and Csallany (1978), after 100 h of ozonation lipofuscin-like compounds arose in plant tissues. Based on the data for autofluorescence of intact pollen from *P. grandiflorus* and *E. hybridum,* we thought that similar substances could be present in the exine of pollen grains, and a search for the compounds has been done in extracts of our samples. The maximum of fluorescence at 420 - 440 nm, perhaps, belonging to lipofuscin (Brooks and Csallany 1978, Shimasaki *et al.* 1995), was found in the spectra of the chloroform:ethanol extracts from the pollen of *P. grandiflorus* and *E. hybridum*, as well as from blue-fluorescent spots ($R_f = 0.2$) of the TLC-plates after chromatography (Fig. 2). The maximum at 420 - 430 nm in ozone-treated samples was higher than maximum at 410 nm in ageing pollen stored for 1 year (Fig. 2), and was absent in fresh pollen (non-shown). The fluorescent

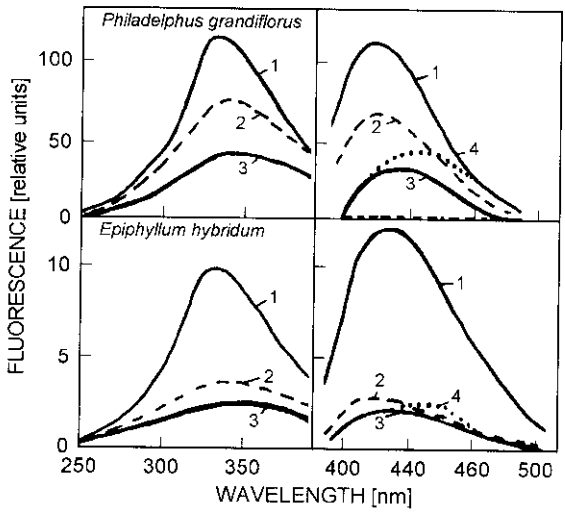

Fig. 2. The excitation spectra (*on the left*, λ_{em} = 420 nm) and emission spectra (*on the right*, λ_{exc} = 360 nm) of fluorescence of the pollen chloroform:ethanol extracts before TLC (*1*, *2*) and the ethanol extracts from fluorescent spots with $R_f = 0.2$ after TLC (*3*, *4*) from *Philadelphus grandiflorus* and *Epiphyllum hybridum*. Extracts from fresh pollen had no significant fluorescence. *1*, *3* and *4* - pollen treated with ozone; *2* - ageing pollen (1 year after collection). *1*, *2*, *3* - solutions, *4* - the film of air-dried solution *3*, which shows the spectral shift of the fluorescence maximum to more long wavelength region in comparison with the liquid.

spots of TLC-plates demonstrated maximum at 430 nm for ozone-treated pollen (Fig. 2). The spots were stained for the protein presence by Coomassie Brilliant Blue-250 because lipofuscins are lipoprotein compounds with molecular mass 3 - 5 kDa (Palmer *et al.* 1986), forming via condensation of malondialdehyde or other aldehydes link with amino groups of amino acids, proteins or amines (Brooks and Csallany 1978, Yin 1995). The reactions of the stain with the spots (R_f 0.2), relating to pollen of *P. grandiflorus,* were positive (more deep colour than in unfluorescent spots). The eluates from the spots with R_f 0.2 of *P. grandiflorus* pollen showed the

enhance of several proteins according to Bradford (1976) method. In extracts from *P. major* pollen there were no traces of lipofuscin (non-shown). Such lipofuscin-like products have been identified in soybean seeds (Brooks and Csallany 1978) and pea leaves (Merzlyak 1988). In soybean seeds they were found after 100-h ozonation in total dose 1.5 cm^3 m^{-3} like in our experiments with pollen of *P. grandiflorus* and *E. hybridum*. The fluorescence of pollen extracts in *P. grandiflorus* is approximately ten fold higher than in *E. hybridum* (Fig. 2). The difference may be due to various composition of their exines, in particular to different carotenoid contents. Moreover, short wavelength fluorescence maximum at 420 - 430 nm, beeing seen in solution may shift to more long wavelength region when it becomes dry film (Fig. 2).

The changes in the autofluorescence spectra of pollen and the fluorescence spectra of extracts from the pollen of *P. grandiflorus* and *E. hybridum* in response to ozone treatment could relate to the participation of the carotenoids in lipofuscinogenesis as reported for animal cells (Karnaukhov 1973, 1988, 1990). At earlier stages, when pollen are viable, the process of lipofuscinogenesis itself can be also useful as protection against ozone damage because it breaks a spreading of free radical chain of lipid peroxidation.

Therefore, ozone may induce the irreversible changes in pollen autofluorescence after pollen grains have just lost their viability. Perhaps, one of the causes is the formation of lipofuscin-like compounds, fluorescing in liquid state at 420 - 430 nm, and the oxidation of exine carotenoids. We suppose that shifts in the fluorescence spectra appear to be an indicator of damage induced by ozone in intact pollen grains.

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