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ORIGINAL PAPER

Fluorescent Analysis for Bioindication of Ozone on Unicellular Models

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Abstract Unicellular model plant systems (vegetative microspores of horsetail *Equisetum arvense* and pollen of six plant species *Corylus avellana, Dolichothele albescens Populus balsamifera, Salix caprea, Saintpaulia ionantha, Tulipa hybridum*, on which autofluorescence and fluorescence after histochemical treatment studied, have been represented as bioindicators of ozone. It has found that low doses of ozone 0.005 or 0.008 μl/l did not affect or stimulate the autofluorescence of the samples with the ability to germinate in an artificial medium. In higher ozone concentrations (0.032 μl/l) either the decrease in the intensity of the emission or changing in the position of the maxima in the fluorescence spectrum (new 515–520 nm maximum characteristic for the green-and yellow area has appeared) were observed. In dose of 0.2 μl/l, higher than above the threshold of danger to human health, autofluorescence in all samples fell down to up to zero, and there was no the ability to germinate. In this case the formation of lipofuscin-like compounds fluoresced in blue with maxima from 440 to 485 nm was observed. Stress metabolites, known as neurotransmitters biogenic amines, were found in treated cells as determined on the characteristic fluorescence at 460–480 nm in the samples after a specific histochemical reactions for catecholamines (with glyoxylic acid) or for histamine (with o-phthalic aldehyde). Increased intensity of the emission under the treatment with ozone (total doses from 0.012 to 0.032 μl/l) was associated with an increase in the concentrations of catecholamines and histamine. The fluorescent analysis on undamaged cells-possible bioindicators of ozone can be useful in ecomonitoring for earlier warning about health hazardous concentrations of this compound in the air.

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The high ground-level ozone concentrations (higher than 0.1 μl/l per hour, in which there is a characteristic odor) is an biggest danger for human health. In environmental biomonitoring of O_3 hazardous concentrations in air or chronic levels of this gas (less than 0.05 μl/l for 10 h) indication of early reactions of living organisms to O_3, or cellular damages caused is very important, and the using plant cells as bioindicators has some preferences [1]. In plants of different ozone concentrations cause various reactions - from stimulating processes (O_3 below the 0.1 μl/l) before breaking the growth and emergence of necrotic and age spots (at high concentrations in the range of 0.1–2 μl/l) [1, 2]. Earlier pollen sensitivity has also been shown to germinate in the presence of various concentrations of ozone in the maize *Zea mays* [3], mock orange *Philadelphus grandiflorus,* knight's star *Hippeastrum hybridum* and plantain *Plantago major* [2, 4–6], well as horsetail vegetative microspores *Equisetum arvense* [7]. Moreover, it is shown that high concentrations of ozone increased allergenicity by Birch pollen [8]. Formation and accumulation of stress metabolites, including biogenic amines known as neuromediators [9, 10], also occur under the influence O_3 [1].

Autofluorescence of plant microspores served for reproduction -vegetative microspores of spore-bearing plants or generative (pollen that means male gametophyte) in Gymnosperms and Angiosperms can be an indicator in early diagnosis of ozone effects on single models [3, 11–13]. On the changes in the maxima position of fluorescence spectra or in emission intensity after chronic exposure to O_3, one can judge the extent of the damage to the cells. It is especially true for the

alteration or destruction in pigments of pollen or vegetative microspores [4, 11–13]. Fluorescence microscopic analysis of individual plant cells is simple, fast, and permits to avoid a destruction of tissue [13–15]. In addition to the autofluorescence measure of reactions to O_3 the approach may be hoarding stressful metabolites -biogenic amines [9, 10], which we could define by fluorescence method [16, 17].

This paper is devoted to the possibilities to use fluorescent analysis to identify bioindicators for ozone. In their analysis of the experiments would be considered autofluorescence of objects studied and their emission after the staining with histochemical reagents to catecholamines and histamine, as compounds that are accumulated at stress.

Materials and Methods

Objects of Research Objects of research were vegetative microspores of horsetail *Equisetum arvense* l. (collected between April and May in the Oka river flood-land), pollen of hazel forest *Corylus avellana* L., goat willow *Salix caprea* L., balsamic poplar *Populus balsamifera* L., hybrid tulip *Tulipa hybrida* . (collected from April to May in the Park area of the city of Pushchino), as well as dolihotele *Dolichothele albescens* (Tiegel) Backeb., african violets *Saintpaulia ionantha* Wendl. (grown in greenhouse). The responses to ozone were their autofluorescence, the formation of biogenic amines and germination, beginning from 2 h after exposure to O_3. Selection of the objects was defined by the seasonality and the relative ease of collecting material in nature, and the possibility of artificial cultivation of plants for experiments. Cultivation techniques of microspores and pollen at slides glass in Petri dishes, as well as statistical processing described earlier [4, 5, 18].

Preparation of Samples for Histochemical Analysis Cells in subject glasses were placed in a Petri dish and poured in drops of 1 % aqueous solutions of glyoxylic acid by the method for detemination of catecholamines described for animal embryo cells [19] and applied to plant vegetative microspores [20] or the cells treated with 1 or 0.5 % o-phthalic aldehyde to determine histamine in algae [21] or in pollen and vegetative microspores [16, 17]. Samples after 10–20 min of staining were dried at 50–80° C.

Preparation of Fluorescent Products from Microspores Fluorescent products were extracted according to [22] by chloroform: ethanol (2:1 v/v) from microspores in ratio 10:1 w/v. Then the extracts were separated by thin-layer chomatography on TLC *Chemapol* silicagel plates in mixture of chloroform:ethanol:acetic acid:water (90:5:1:0.3 v/v/v/v), and the ethanol extracts (by 1 ml of the solvent) from spots with Rf 0.2 as described for the identification of lipofuscins [23] were analyzed with spectrofluorimetry [24].

Study of Fluorescence Images and spectra of fluorescent cells were recorded by laser-scanning confocal microscope *Leica* TCS SP-5 (Germany-Austria- United States) on the glass slides at room temperature 20–22 °C as published earlier [20]. The excitation by laser wavelength was 405 nm. Three photomultipliers can catch the fluorescence, separately or simultaneously by use the pseudocolor effects.

The emission intensity of cells studied on the glass slides was also measured at two different wavelengths (460–475 and 640–680 nm or 520 and 640–680 nm) by dual-wavelength microspectrofluorimeter MSF-2 (Institute of Biological Techniques (Pushchino)) with the special computer statistical program *t*-test, as described previously [14, 20]. The excitation was by actinic light 360–380 or 430 nm. In every experiment an average fluorescence intensity of 100 cells was measured on one subject slide. Results were expressed statistically with standard error of mean - mean±SEM of four replications (four subject slides) for every variant and control.

Determination of catecholamines and histamine in microspores using glyoxylic acid or 0-phthalic aldehyde respectively were made of the fluorescent methods described previously [16, 17, 20]. Fluorescence of reaction products was analyzed using laser-scanning confocal microscope *Leica* TCS SP-5 at excitation with laser 405 nm [16, 17] or measured at 460 nm or 475–485 nm at excitation by ultra-violet 360–380 nm using the microspectrofluorimeter MSF −2 [17].

The fluorescence spectra of solutions were recorded in 0.5 см-cuvette by spectrofluorimeter Perkin Elmer 350 MPF-44B (UK). The fluorescence spectra of the original chlorofom/ethanol extracts from samples and from spots after TLC-chromatography were recorded at excitation 360 nm.

The Exhibition in Ozone Impact of ground-level ozone in pleksiglass cell volume 439 $cm^{-3}$with ozone generator KPMZ (Russia). Duration of exposures in O_3 was: 1. short continuous 2.5, 4, 6, 8 h or 2. prolonged exposure 4 h ozone + 12 h break without the gas + 4 h ozone + break 12 h, etc. to a total dose. There were low doses of ozone 0.005 μl/l (two and a half hours exposure) or 0.008 μl/l (4 h exposure), higher ozone concentrations (after 16 hour-exposure in ozone, total dose of 0.032 μl/l) and highest, danger for human, concentration (about 100 h exposure in O_3, total dose 0.2 μl/l)

Reagents In the work following reagents are used: biogenic amines dopamine from “Sigma” (USA) and histamine from “Fluka” (Switzerland), reagents on biogenic amines glyoxylic acid from “Serva” (USA) and *o*-phthalic aldehyde from “ICN Pharmaceuticals” (USA).

Results and Discussion

The fluorescent image of cells (control and after exposure to the ozone concentrations) and fluorescence spectra were analyzed. Emission of cells were recorded as autofluorescence (that is the native form without any treatment by histological chemicals) or the characteristic fluorescence after processing with reagents for biogenic amines such as glyoxylic acid (defining the presence of catecholamines dopamine, norepinephrine, and epinephrine) or o-phthalic aldehyde (for determination of histamine).

Autofluorescence of Cells Figure 1 presents data for the vegetative microspores horsetail *Equisetum arvense* and pollen of plants flowering in the spring. Effects after exposition in ozone to 2.5–4 h in the total dose of 0.005 μl/l–0.008 (parts per million) were compared with controls, and noticeable change in the fluorescence of 500–550 nm were observed. Starting from 6 h- treatment with O_3 (total dose of 0.012 μl/l) in *E. arvense* the characteristic peak in the green-and-yellow 510–540 nm appeared that was absent in control. It was found earlier in secretory hairs of seedling leaves of the *Raphanus sativus* exhibied in O_3 3 h [2], but the total dose of ozone in this case was much higher (0.15 μl/l). Increased ozone concentrations led to visible changes in the cells usually fluorescent in red as in control due to presence of chlorophyll (maximum of 680 nm), gradually began to acquire more and more yellow emission (Fig. 1a), as has been shown for some objects in our publications [2, 7]. Unlike vegetative microspores of *E. arvense* in pollen, selected in this study, position of the maxima in the spectrum of autofluorescence was mainly in blue or green (500–520 nm) range and essentially un-

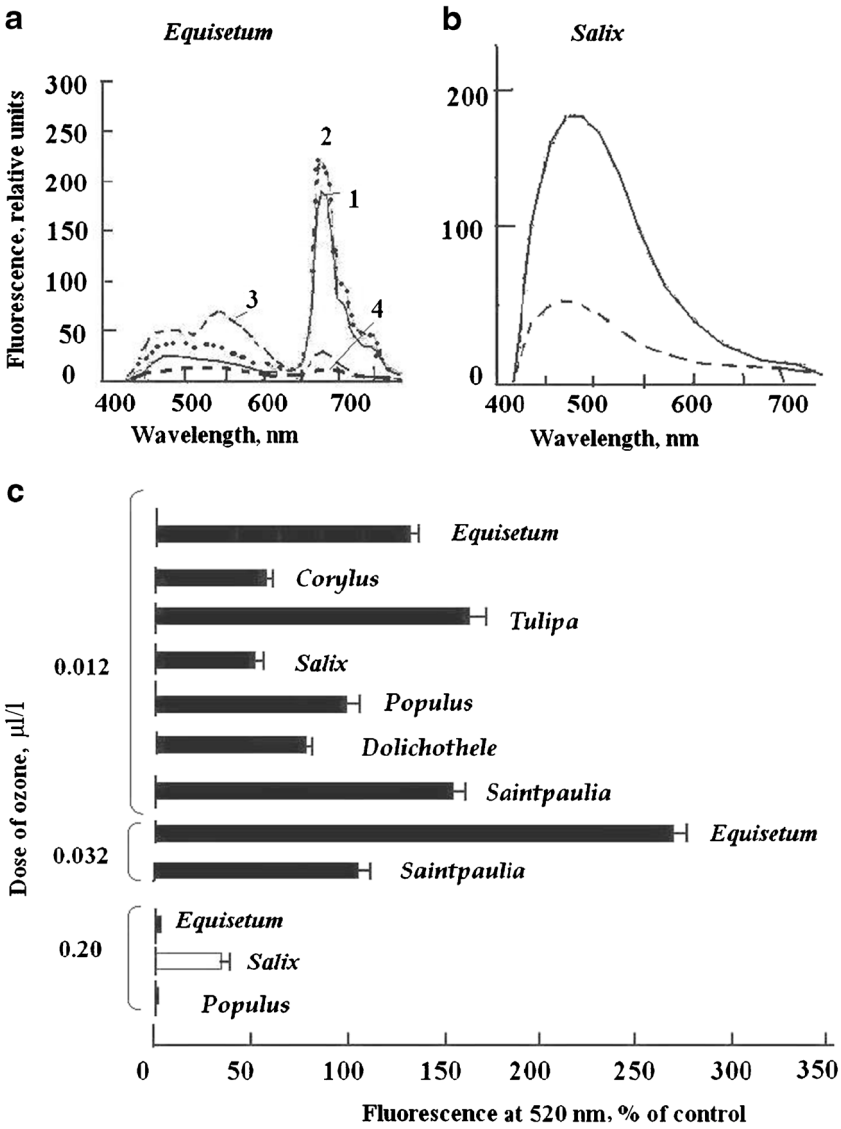

Fig. 1 Effect of ozone on the changes in the autofluorescence spectra (**a** and **b**) and the intensity of emission at 520 nm (**c**) of the plant microspores. Measurement of the spectra was by using laser-scanning confocal microscopy (405 nm laser), and of the emission intensity at 520 nm with microspectrofluorimeter with excitation light 430 nm. **a**. vegetative microspores *Equisetum arvense*: 1-control, 2–4 – total doses of ozone 0.012, 0.032, 0.2 μl/l. **b**. pollen *Salix caprea* after ozone fumigation in a total dose of 0.012 μl/l

Table 1 Influence of ozone on the pollen germination of *S.caprea* and *S.ionantha*. Index = the ratio of the number of germinating pollen grains to the total number of pollen grains

Pollen *S.caprea*	Index	Pollen *S. ionantha*	Index
Control	0.63±0.02	Control	0.64±0.01
+ ozone 0.012 μl/L	0.55±0.01	+ ozone 0.012 μl/L	0.82±0.01
+ ozone 0.032 μl/L	0.84±0.04	+ ozone 0.032 μl/L	0.86±0.06

changed. The pigments dominate in studied pollen is known as quercetin glycoside, as for example in *C. avellana* [25], that leading to a yellow color and green fluorescence. On the sample of *Salix caprea* spectrum (Fig. 1b) under the influence of ozone only the height of the peak of 495–500 nm decreased. The same picture was also for the rest of the objects, and the data to change the intensity of the samples were presented in Fig. 1c.

Under the influence of a small ozone dose 0.012 μl/l (after 6–6, 5 h of exposure) the intensity of the autofluorescence at 520 nm could decline by about 50 % compared with controls for pollen *S. caprea* and *C . avellana* or increased up to 130–150 % like for the pollen*T. hybrida* and *S . ionantha,* or vegetative microspores *E. arvense* (Fig. 1c). When the total dose of

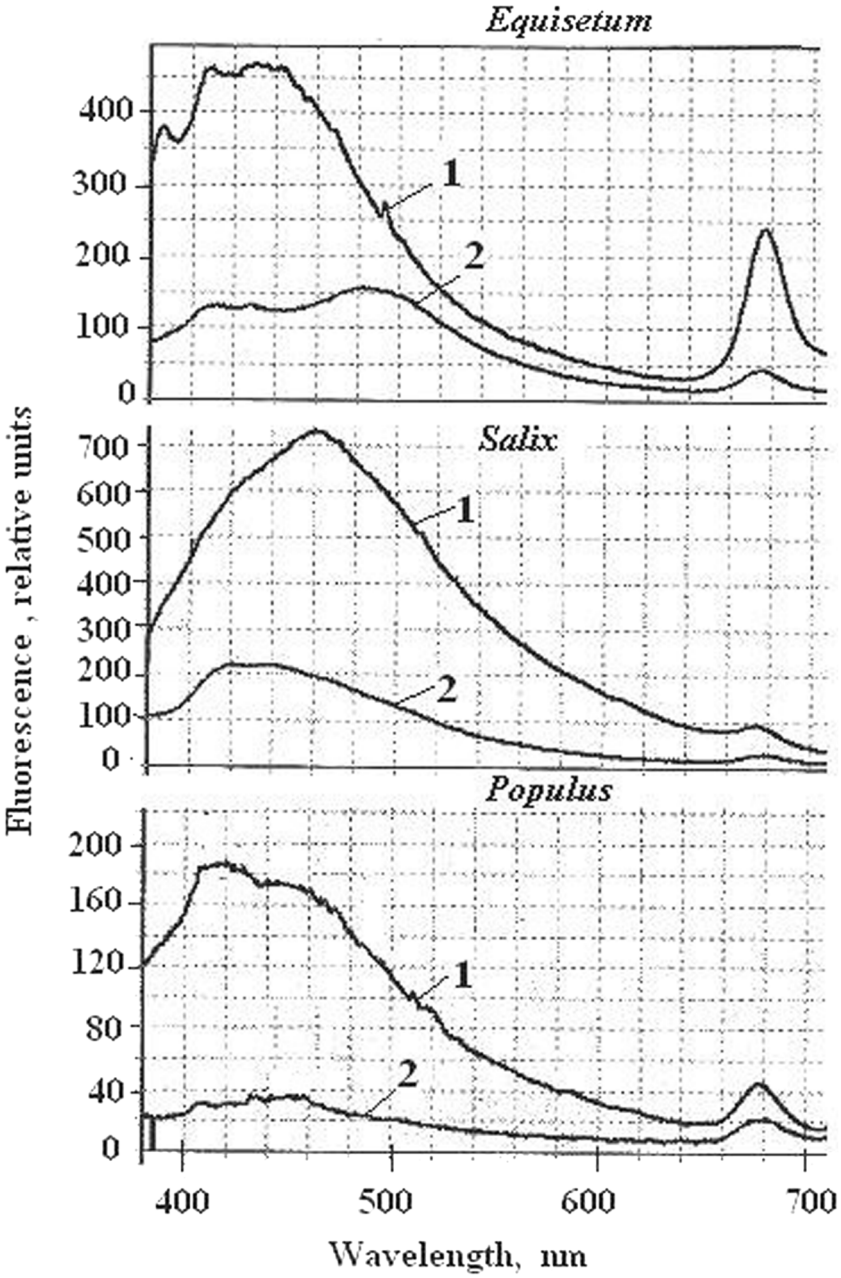

Fig. 2 The fluorescence spectra of chloroform extracts from untreated sample (1) as control and (2) spot with Rf 0.2 of thin-layer plate for component formed after the ozone exposure 100 h (total dose 0.2 μl/l)

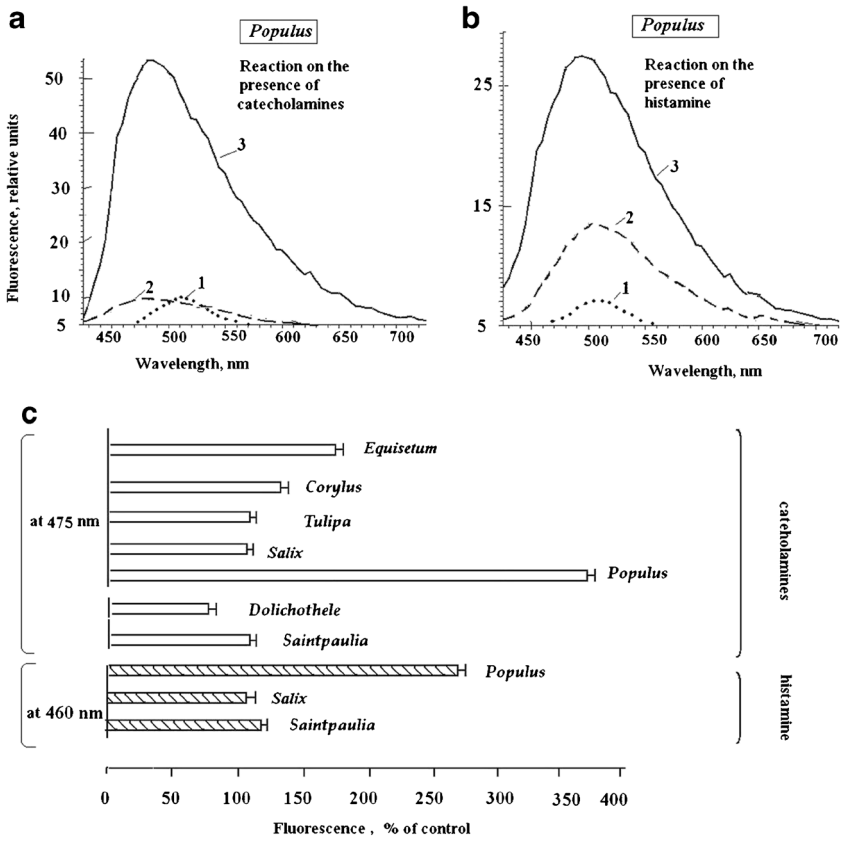

Fig. 3 Ozone effects on the fluorescence spectra and the emission intensity at 460 and 475 nm of cells after staining with reagents on biogenic amines. (**a**) and (**b**)- the emission spectra of pollen from *Populus balsamifera* recorded using laser-scanning confocal microscope (excitation 405 nm laser): 1-control (without staining with reagents), 2 – control after treatment with glyoxylic acid (*left*) or o-phthalic aldehyde (*right*), 3. the exposure to ozone 6 h in total dose of 0.012 μl/l and then staining with reagents on biogenic amines. (**c**). the intensity of the cell fluorescence at 460 or 475 nm upon exposure to 16 h ozone at total dose 0.032 μl/l recorded by microspectrofluorimeter. Excitation light 360–380 nm

O_3 increased up to 0.032 μl/l in intermittent exposure emission of microspores *E. arvense* achieved more than 250 % of the control, while the rest of the samples remained approximately at the same level. The intensity of the fluorescence in pollen of *S. ionantha* is reduced to control value. It is significant that pollen *S. caprea* and *S. ionantha,* good developed usually, even after exposure to ozone during 16 h (total dose 0.032 μl/l) retained the ability to germinate (Table 1). The index of the germination (ratio amount of germinated pollen grains/total amount of pollen grains studied) even increased, indicating a greater number of pollen grains that formed pollen tubes.

Studies of the effect of doses greater than threshold health risk (>0.1 μl/l) in intermittent O_3 regime were also conducted. After exposure to high dose (0.20 μl/l) for 100 h in all samples the quenching of autofluorescence was observed, except of the dampening weak emission from *S . caprea.* Besides, the ability to germinate disappeared. Such phenomena can be associated with a change of state of phenolic and terpenoid compounds with double bonds and a formation of lipofuscin, known as pigment of ageing [24].

Figure 2 shows the fluorescence of similar components formed in the cells after 100 h of the exposure in ozone (total dose 0.2 μl/l) and purified by thin layer chromatography (TLC) in order to compare the emission of the extracts from similar spots and the original extracts from untreated pollens. In untreated variants (controls) TLC-plates had no spots with Rf 0.2, related to lipofuscins [22–24]. Similar spots appeared in the ozone variant only. The spots had maxima in blue like lipofuscins: maximum 485 nm for microspores of E. arvense, maximum 420–440 nm in the spot component from S.caprea pollen, while in the spot from P.balsamifera pollen - small maximum 455 nm.

It should be noted that ozone in high concentrations (about 0.1 μl/l and above) affect the germination of the pollen is anemophilous species negatively, for example, so the ryegrass pasture *Lolium perenne* [26] or common ragweed *Ambrosia artemisiifolia* [6]. The latter increases pollen allergenicity due to stimulation of the enzyme NAD(P)H oxidase that generates reactive oxygen species [6].

The autofluorescence change occurs as a result of the formation of oxidized products under the influence of ozone and its derivative-free radicals and peroxides. Free radicals-superoxide anion radical and hydrogen peroxide from the pollen and vegetative microspores of horsetail upon exposure to ozone has been shown by measuring chemoluminescence [5]. Autofluorescence of pollen from seed-bearing plants and vegetative microspores from spore-bearing plants should be seen as a sensitive test for ozone.

Fluorescence with Reagents on Biogenic Amines

Stress metabolites of biogenic amines (known as neurotransmitters of animals, but also found in plants and microorganisms) in the studied samples found after specific histochemical reactions characteristic of catecholamine fluorescence at 480 nm after the staining with glyoxylic acid or 460–480 nm luminescence belonging to histamine after the staining of a sample with o-phthalic aldehyde. The example of these responses is shown in Fig. 3 for pollen *P. balsamifera* . The object had characteristic emission resulting from the presence of catecholamines (dopamine, noradrenaline, adrenaline) and histamine. On Fig. 3a, b one can see the enhancement of the pollen cells emission under the influence of ozone (total dose of 0.012 μl/l) compared to control, which means that significantly increased both the amount of catecholamines and histamine content. The stimulation of the formation of catecholamines by ozone was observed as the increase in the fluorescence intensity, not only for pollen *P. balsamifera,* but also for vegetative microspores *E . arvense* and pollen *of C . aveliana* (Fig. 3c), while verifical results had not been received for the pollen from rest plant species. The slight increase in histamine (not more than 5 %) was also observed in pollen *S. ionantha* and *S. caprea* after exposure to ozone in some variants.

Components that might cause allergies of human may be found in pollen [27, 28]. This is important for the diagnostics of allergens in medicine. Although it has not been established exactly yet that biogenic amines met in plant microspores cause allergic reactions, but the presence of catecholamines and histamine (direct agent of allergic response) in pollen [16, 17] allows us to consider such mechanism too. The fact that ozone in high concentrations increases the content of these compounds in some of our objects deserves the attention of physicians.

Fluorescent analysis as a whole is useful method for aerobiology too because the ozone enhanced 351/355-nm-excited emission of bioaerosol particles (which may include pollen grains) in visible spectral region, especially at high relative humidity that permits fast and simple monitoring [29, 30]. Histochemical staining on bioactive compounds has also perspectives in similar studies.

Conclusion

The use of fluorescent analysis on cellular models- biosensors permits quick on their autofluorescence or the fluorescence after staining of special reagents for biogenic amines diagnose an increase in concentration of O_3 and changes induced under the influence of ozone. As a model systems plant cells can be used as bioindicators in monitoring of the chronic effects of ozone on natural and urbanized habitats of organisms.

References

1. Feder WA (1978) Plants as bioassay systems for monitoring atmosphere pollutants. Environ Health Perspectives 27(1):139–147
2. Roshchina VV, Roshchina VD (2003) Ozone and plant cell. Kluwer Academic Publishers, Dordrecht, p 240
3. Mumford R, Lipke H, Laufer DA, Feder WA (1972) Ozone-induced changes in corn pollen. Environ Sci Technol 6(5):427–430
4. Roshchina VV, Melnikova EV (2001) Chemosensitivity of pollen to ozone and peroxides. Russian Plant Physiology 48:89–99
5. Roshchina VV, Miller AV, Safronova VG, Karnaukhov VN (2003) Reactive oxygen species and luminescence of intact microspore cells. Biophysics 48(2):259–264
6. Pasqualini S, Tedeschini E, Frenguelli G et al (2011) Ozone affects pollen viability and NAD(P)H oxidase release from *Ambrosia artemisiifolia* pollen. Environ Pollut 159(10):2823–2830
7. Roshchina VV, Melnikova, EV (2000) Contribution of ozone and reactive oxygen species in the development of plant cellular systems. In: Skulachev BP., Zinchenko VP (eds) Mitochondria, cells and reactive oxygen species. Proceedings of the International Conference, 6–9 June 2000, Pushchino, Biological Center of Russian Academy of Sciences. Pushchino, p 127–129
8. Beck I, Jochner S, Gilles S, McIntyre M et al (2013) High environmental ozone levels lead to enhanced allergenicity of birch pollen. PLoS ONE 8(11):e80147. doi:10.1371/journal.pone. 0080147
9. Roshchina VV (1991) Biomediators in plants. Acetylcholine and biogenic amines. Biological Center of USSR Academy of Sciences, Pushchino, p 192
10. Roshchina VV (2001) Neurotransmitters in plant life. Science PubL, Enfield, Plymouth, p 283
11. Roshchina VV (2003) Autofluorescence of plant secreting cells as a biosensor and bioindicator reaction. J Fluoresc 13:403–420
12. Roshchina VV (2006) Plant microspores as biosensors. Trends Mod Biol (Russia) 126:262–274
13. Roshchina VV (2008) Fluorescing world of plant secreting cells. Science Publishers, Enfield, Plymouth, p 338
14. Roshchina VV, Yashin VA, Kononov AV (2004) Autofluorescence of plant microspores studied by confocal microscopy and microspectrofluorimetry. J Fluorescence 14(6):745–750
15. Roshchina VV (2014) Model systems to study excretory function of higher plants. Dordrecht, Heidelberg, Springer, p 220
16. Roshchina VV, Yashin VA, Kuchin AV, Kulakov VI (2014) Fluorescent analysis of catecholamines and histamine in plant single cells. Int J Biochem, Photon 195:344–351
17. Roshchina VV, Yashin VA (2014) Neurotransmitters catecholamines and histamine in allelopathy: plant cells as models in fluorescence microscopy. Allelopathy J 34(1):1–16
18. Roshchina VV, Melnikova EV, Yashin VA, Karnaukhov VN (2002) Autofluorescence of intact spores of horsetail *Equisetum arvense* L. during their development. Biophysics (Russia) 47(2):318–324
19. Markova LN, Buznikov GA, Kovačević N et al (1985) Histochemical study of biogenic monoamines in early ("Prenervous") and late embryos of sea urchins. Int J Develop Neurosci 3(5):493–499
20. Roshchina VV, Yashin VA, Vikhlyantsev IM (2012) Fluorescence of plant microspores as biosensors. Biochemistry (Moscow), Suppl ser.A: Membrane and Cell Biology 6(1): 105–112 (Biological Membranes 28 (6): 547–556)
21. Barwell CJ (1989) Distribution of histamine in the thallus of *Furcellaria lumbricalis*. J Appl Phycol 1:341–344
22. Brooks RI, Csallany AS (1978) Effects of air, ozone, and nitrogen dioxide exposure on the oxidation of corn and soybean lipids. J Agr Food Chem 28:1203–1209
23. Shimasaki H, Maeba R, Tachibana R, Ueta N (1995) Lipid peroxidation and ceroid accumulation in macrophages cultured with oxidized low density lipoprotein. Gerontol 41(2):39–49

24. Roshchina VV, Karnaukhov VN (1999) Changes in pollen autofluorescence induced by ozone. Biol Plant 42(2):273–278
25. Strack D, Meurer B, Wray V, Grotjahn L et al (1984) Quercetin 3-glucosylgalactoside from pollen of *Corylus avellana*. Phytochemistry 23(12):2970–2971
26. Schoene K, Franz J-T, Masuch G (2004) The effect of ozone on pollen development in *Lolium perenne* L. Environ Pollut 131:347–354
27. Weber RW, Nelson HS (1985) Pollen allergens and their interrelationships. Clin Rev All 3:291–318
28. Motta AC, Marliere M, Peltre G, Sterenberg PA, Lacroix G (2006) Traffic-related air pollutants induce the release of allergen-containing cytoplasm granules from grass pollen. Int Arch Allergy Immunol 139:294–298
29. Pan YL, Santarpia JL, Ratnesar–Shumate S et al (2014) Effects of ozone and relative humidity on fluorescence spectra of octapeptide bioaerosol particles. J Quant Spectrosc Radiat Transf 133:538–550
30. Pan YL (2015) Detection and characterization of biological and other organic-carbon aerosol particles in atmosphere using fluorescence. J Quant Spectrosc Radiat Transf 150:12–35