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The Autofluorescence Response of Flower Cells from Saintpaulia Ionantha as the Biosensor Reaction to Ozone

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Abstract: Anthocyanin-containing cells from blue petals of flowers of Saintpaulia ionantha Wendl have been considered as models for the fluorescent analysis (by luminescence microscopy including confocal microscopy technique) in the indication damages arisen under the action of various ozone concentrations. Laser-scanning confocal microscopy permitted to observe the changes in images of autofluorescence and the fluorescence spectra of individual cells of petal and multicellular secretory hairs. First alterations were fixed in the emission spectra of some petal cells just after 2.5 h -exposure in O_3 (total dose 0.005 µl/l) – missing of maxima 620-630, 640-650 and 665 nm, peculiar to anthocyanins. Although at the ozone concentrations 0.005 µl/l under luminescence microscope any changes in the fluorescence images were not seen yet. Acute experiments during 25 h-50 h-exposures with O_3 (doses 0.05 -0.1 µl/l) led to changes both in common fluorescence images (quenching of the emission in main cells of the petal surface and stalks of the secretory hair) and in the spectral position of maxima at region 620 - 660 nm, peculiar to anthocyanins, that disappeared in main petal cells. The chlorophyll maximum 675-680 nm has seen up to 25 h of the exposure, and disappeared to 50 h of the ozonation. In the secretory hairs, similar picture was registered in some cells of stalk, whereas head of the trichome differencing from main cells fluoresced with maximum 480-500 nm, and here additional emission in yellow-orange region have also seen. Experiments with individual phenols and chlorophyll treated with ozone showed that only anthocyanins in vacuole and chlorophyll in chloroplasts are targets of O_3 even in smallest concentrations. The sensitive cells of flowers have been recommended as possible ozone bioindicators both outdoors and indoors.

Keywords: Aromatic Acids**,** Anthocyanins, Chlorophyll, Coumarins, Laser-Scanning Confocal Microscopy, Luminescence Microscopy, Phenols, Secretory Hairs

1. Introduction

Although ozone may be hazardous for every living organism in large doses, including plants [1, 2] there is a few information about effects of O_3 as exogenous oxidative chemosignal on plant cells in the acute experiments using concentrations lower that dangerous [3]. Its influence both outdoors and indoors (where electric and ultra-violet light systems such as the copy or computer technique, fluorimeters and other apparatuses worked all day) may establish by finding of model plants-biosensors. The response of 473 wild plants species to O_3 has known, and some of them characterized as “ozonophobic” even at O_3 levels lower than the hazardous threshold [4]. In nature whole plants such as forest species [5], Bel-W3 tobacco in agriculture [6], etc, are known as indicators, while cellular models that include, mainly, pollen and vegetative microspores of spore-bearing plants [3, 7] are rarely used yet. Fluorescence (autofluorescence and visible emission after staining with dyes) as a parameter for the study of ozone effects on the cellular models was also considered in some cases [3, 8]. At doses of O_3 0.5-5 µl/l emission of leaf secretory hairs from *Raphanus sativus, Lycopersicon esculentum* and *Fragaria vesca* significantly changed [3]. However, a reaction to ozone, as common exogenous signal, on plant fluorescing secretory cells in low concentrations is unknown yet as well as possible targets of the gas. In this case, fluorescence of plants and possible concrete sensitive fluorescing targets for ozone is of special interest for bioindication. For these experiments, one could choose suitable object as model.

Among new suitable models for similar studies perspectives belong to pigmented parts of plants, for example those, containing phenols [9]. Degradation of flavonoids, mainly anthocyanins, in fruit juice under the high doses of ozone has shown earlier [10], although fluorescence of the extracts has not analyzed. Emission of anthocyanin-containing flower petals with secretory cells has not studied yet. Earlier the attention paid to the nature-lived and house-cultivated species which anthocyanin-containing cells are used for the analysis of the membranous damages under the action of various natural compounds, in particular blue petals from flowers of *Saintpaulia ionantha* Wendl [11, 12]. This object is wider-spread as room-living plant, and a sensory ability of the flowers may be relate to the ozone effects too. Blue or violet-blue color of the plant flower petals depends on the presence of phenolic pigments – anthocyanins. Anthocyanin-containing cells from *Saintpaulia* contain acylated anthocyanidin 3-rutinoside-5-glucosides [13]. The anthocyanins were identified as 3-*O*-[6-*O*-(4-*O*-(acetyl)-α-rhamnopyranosyl)-β-glucopyranoside]-5-*O*-(β-glucopyranoside) of malvidin, peonidin, and pelargonidin. On the visible absorption spectral curves of fresh violet-blue petals and in their crude extracts in pH 5.0 buffer solution, there are two characteristic absorbance maxima at 547 and 577 nm, with a shoulder near 620nm [11]. In the anthocyanin extracts by water one can see only one maximum 620 nm, and after the acid addition - at 525-530 nm. In the fluorescence spectra peaks at 430-450 nm and 675-680 nm have observed that peculiar to anthocyanins (also seen in water extracts) and chlorophyll, relatively. Our paper was devoted to the fluorescence of model system such as *Saintpaulia ionantha* undergone doses of ozone lower than threshold (smell of the gas is at total dose 0.1 μl/l).

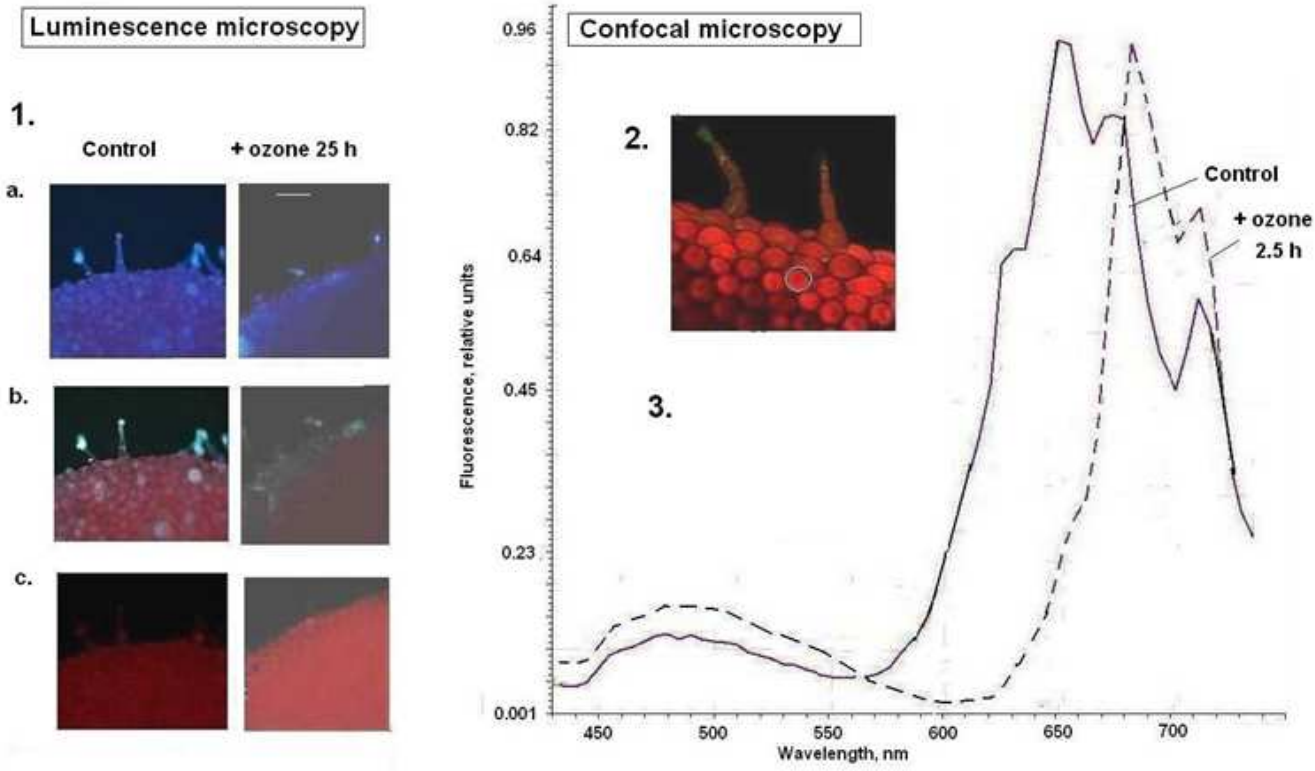

Figure 1. *The images of petal surface of Saintpaulia ionantha under luminescence (1) or confocal microscope (2) and fluorescence spectra recorded by confocal microscope (3). 1. fluorescent images of surface before the treatment (control) and after 25 h - exposure in ozone (total dose 0.05 μl/l). Bar = 50μm, a, b, c, - light excitation 340-380, 355-425, 515-560 nm, relatively. 2. the fluorescing image of surface, analyzed ROI is shown by ring. 3. the fluorescence spectra from this ROI in control and after 2.5 h - exposure in ozone (total dose 0.005 μl/l). Laser excitation 405 nm.*

2. Materials and Methods

Objects of the investigations were pigmented petals from flowers violet line of *Saintpaulia ionantha* Wendl grown in room conditions at 22°C. Extracts from intact petal cells obtained by 60 min exposure with an aqueous or 96% ethanol solvents (3 ml solution on 20 mg of petal).

Autofluorescence of living cells was observed and photographed on the glass slides at room temperature 20-22° as described previously [8, 11] using *Leica* apparatuses (Germany) -luminescence microscope Leica DM 6000 B (natural emission colors) and laser-scanning confocal microscope Leica TCS SP-5 (pictures were seen in pseudocolors). As the source of the exciting light in the confocal microscope a mercury lamp - for visual observation and laser Argon/2 (wavelengths 405, 458, 477, 488, 514 nm), He Ne1 (543 nm wavelength) and HeNe2 (633 nm wavelength) were used. Registration carried out three channels.

The fluorescence spectra of cells have recorded with the

above-mentioned laser-scanning confocal microscope Leica TCS SP-5 (Germany). Individual cells or parts of the secretory structures have been analyzed first as a region of interest (shortly ROI) marked by rings.

Fluorescence spectra of the water and ethanol extracts from the 1 g of studied material have recorded by spectrofluorimeter Perkin Elmer 350 MPF-44B (UK) in 1 or 0.5 cm-cuvettes.

Impact of-level ozone has studied in pleksiglass cell volume 439 cm^{-3} with ozone generator KPMZ (Russia). Duration of exposures in O_3 was continuous 2.5, 4, 6, 8, 25, 50, 100 h hours. There were low doses of ozone 0.005 µl/l (2.5 h exposure) - 0.05 µl/l (25 h exposure), higher - 0.1 µl/l (after 50 hours-exposure) and highest 0.2 µl/l, danger for human (100 h exposure).

Following reagents were used in the work: pelargonidin, phenolic acids such as chlorogenic, ferulic, cinnamic, coumaric acid and coumarin esculetin from "Sigma" (USA). Chlorophyll a+b and anthocyanins were extracted with 50% ethanol from the *Urtica urens* leaves and petals of *S. ionantha*, relatively, and then purified on Silicagel plates as shown earlier [11].

3. Results and Discussion

The noticeable changes in the intensity of the cells' emission have seen visually under luminescence microscope after 25 h of the ozone (total dose 0.05 µl/l) treatment (Fig. 1, images 1). Common fluorescence was quenched in different degree at the excitation by light 340-380 nm (image 1a - ultra-violet light), 355-425 nm (image 1b - UV/violet light), and 515-560 nm (image 1c - green light). First and second light may excite the emission of phenols, while third one – mainly, chlorophyll only. All cells, including cells of the hairs, had positive staining with $FeCl_3$, reagent for phenols. Perhaps, the emission drop has related to disappearance of anthocyanins. Laser-scanning microscopy has given more clear information for main petal cells (Fig. 1, image 2). On the fluorescence spectra (Fig. 1, image 3), received from surface of the petal (out hairs) changes in the maxima position were observed just after 2.5 h exposure in ozone (dose 0.005 µl/l). Instead maximum 650 nm, peculiar to anthocyanins, there was only peak 680 nm related to chlorophyll. Similar picture was stored up to 25 h of ozonation (total dose 0.05 µl/l). After 25 h of the ozone exposure, when dose achieved 0.05µl/l, most cells have lost in anthocyanins, because the maximum 640-650 nm disappeared. Only maximum 680 nm related to chlorophyll has observed, but after 50h - ozonation (total dose 0.1 µl/l) the pigment disappeared too. This dose is threshold sensitivity for human nose.

Unlike main massive petal cells, cells of multicellular cells

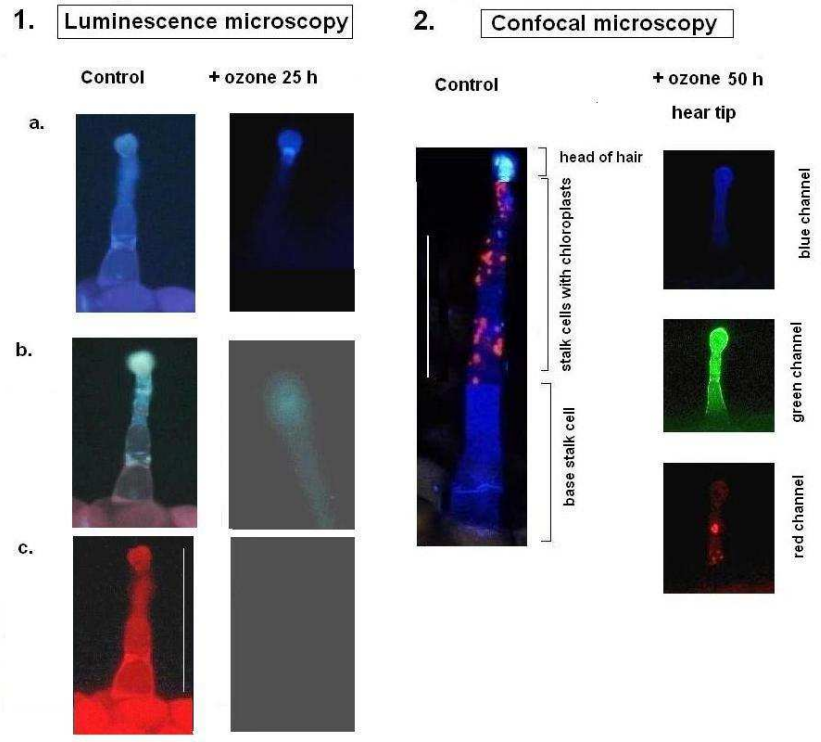

Figure 2. *The images of multicellular secretory hair on flower petal from Saintpaulia ionantha before any treatment (control) and exposure in ozone received with luminescence microscopy (1a-c) or laser-scanning confocal microscopy (2). Bar = 50µm. 1. a,b, c, - light excitation 340-380, 355-425, 515-560 nm, relatively. 25 h - exposure in ozone (total dose 0.05 µl/l). 2 After 50 h-exposure in ozone (total dose 0.1 µl/l) fluorescence was seen only in the hear tip that shown in 3 channels. Laser excitation 405 nm.*

Unlike main massive petal cells, cells of multicellular secretory hair demonstrated different emission in tip cells of head, stalk cells and base stalk cell (Figs. 2 and 3). The head of secretory hair that lack of chloroplasts brightly fluoresced in blue (Fig. 2, image 1a) or blue-green (image 1b) as seen under luminescence microscope and in confocal microscope

(Fig. 2, image 2 control). In the head fluorescence spectra (Fig. 3 a, spectrum 1) we saw maximum 500 nm (and no maximum 680 nm belongs to chlorophyll). Stalk cells, lied just below the head, fluoresce in blue and red because have chloroplasts (Fig. 2, image 2 control). Unlike the top of the hair, first and especially second cells of stalk (Fig. 3b-d, spectra 1) have maximum 680 nm peculiar to chlorophyll in plastids. Maximum 490 nm in blue spectral region has also seen. Stalk cells also have maximum 650 nm related to anthocyanins. Shoulder 620 nm in stalk cells has often observed too. Third and fourth cells of the stalk (Fig. 3 , images c ,d, spectra 1) as well as base stalk cell(image e, spectrum 1) have minimal amount of chlorophyll or practically lack at all, unlike upper stalk cells, and have clearer maximum at 460-490 nm.

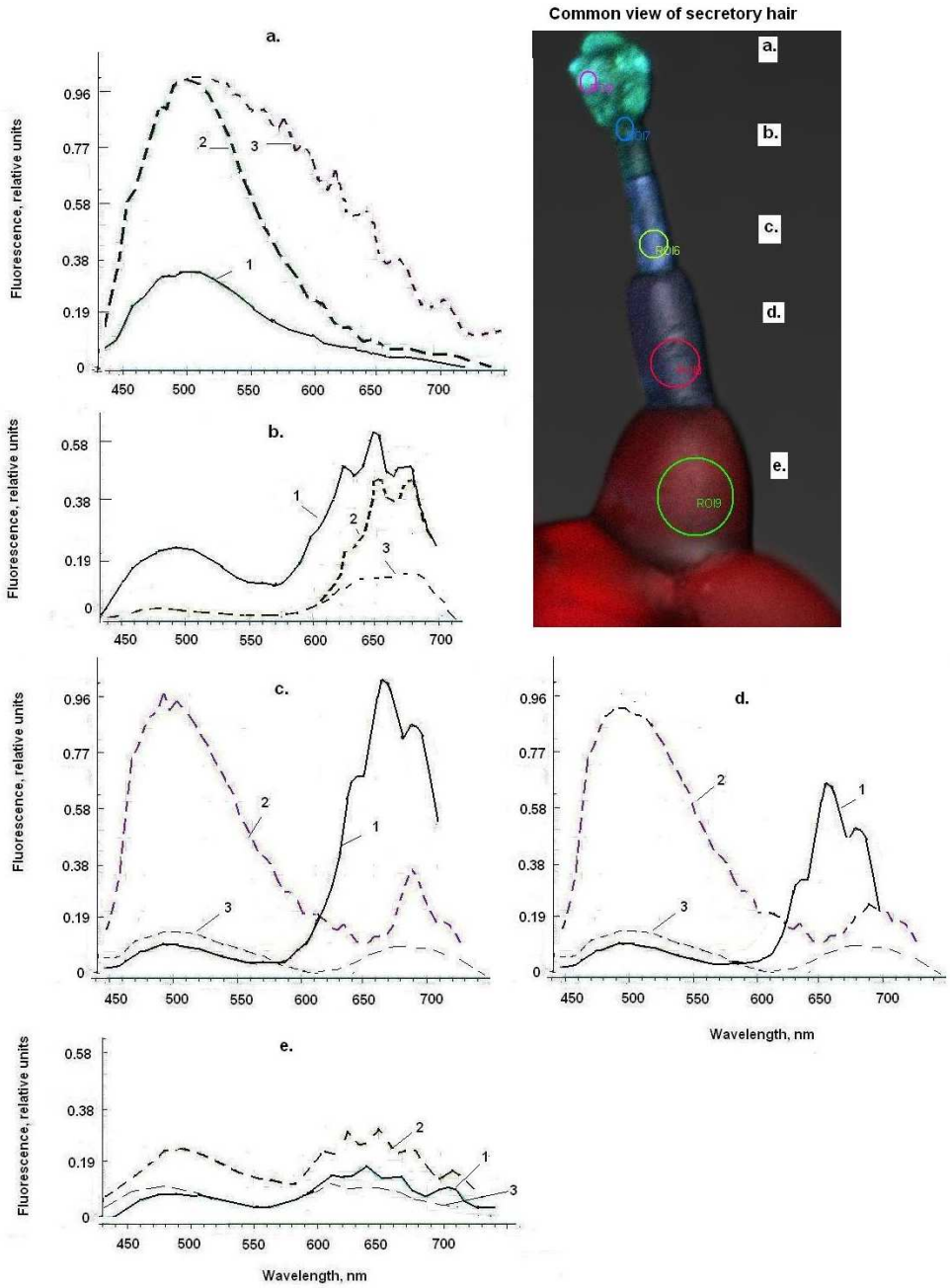

Figure 3. *The fluorescence spectra and common image of the secretory hair on petal of Saintpaulia ionantha received by the confocal microscope before (spectrum 1) and after the treatment with ozone during 25 h -and 50 h-exposure (spectra 2 and 3, relatively). Bar = 50 µm. Total doses of ozone for spectra 2 and 3 were 0.05 and 0.01 µl/l relatively. The position of optical probe (ROI) in control hair is shown with color rings.*

Visual experiments with luminescence microscopy showed significant influence of ozone and sensitivity of the especially secretory hairs of petal to the gas concentration. After the 25 h of ozone exposure (dose 0.05 µl/l), the fluorescence of secretory hairs has not been observed at all in the cells of stalk excited by UV/violet light (Fig. 2, image 1). Only head of hair demonstrated the significant blue emission in ultra-violet. After 50h-exposure in ozone we could see

under confocal microscope the weak blue emission of the head of secretory hair and brighter lightening in green spectral region. In red channel, head of the hair had no emission. Fluorescing hair looks as a microscopic antenna with tip cells as detectors of ozone.

Analysis of the hairs' fluorescence spectra also has carried out (Fig. 3). After the 25 h-ozone treatment in dose 0.05 μl/l the fluorescence of the trichome head (Fig. 3 a) in maximum 500 nm increased more than twice (spectrum 2) in comparison with the control (spectrum 1). It shows the liberation of energy from the structures in form of the emission. At 50 h - (dose 0.1 μl/l) ozone exposure the width of the spectrum shifted from green to orange-red region (spectrum 3). Unlike the head, cells of stalk had another picture in their fluorescence. Stalk cell near head (Fig. 3 b) and stalk cell lying abreast (Fig. 3 c) included anthocyanins (maxima 630 and 650 nm) and chloroplasts with chlorophyll (maximum 680 nm) that seen in the emission spectra of control (spectra 1). Third stalk cell (Fig. 3 d) has smallest amount of chlorophyll (spectrum 1). Under 25 h -ozone exposure in stalk cell 1 (Fig 3b) the maximum 620-630 nm was lost (spectrum 2), and after 50 h of ozonation peaks 650 and 680 nm decreased and smoothed (spectrum 3). In stalk cell 2 (Fig. 3 c) 25 h in O_3 led to appearance of high emission intensity at 500 nm (spectrum 2), and only smaller maximum 680 nm was seen. Similar picture with smallest peak at 675-680 nm (spectrum 2) was shown for stalk cell 3 (Fig. 3 d) too. Later, after 50 h ozonation (total dose 0.1 μl/), all maxima decreased and smoothed in all three stalk cells (spectra 3). Unlike cells of head and above-mentioned stalk cells, in base stalk cell (Fig. 3 e) having weak emission even without ozonation (spectrum 1) small changes were seen at the ozone exposures. Only after 25 h- ozone treatment the fluorescence intensity both in blue and red increased, approximately two fold (spectrum 2) in the comparison with the control, but it decreased at 50 h- exposure in O_3 (spectrum 3).

We can try to consider possible targets for ozone in the cells studied. It appears to be formation on some polyphenols [14] seen as specific spots on leaves. According to investigations of 7 the phenols in plant tissues by confocal microscopy at normal natural conditions their main fluorescence was in blue- green 460-520 nm, but the existence of more peaks in orange and red is not clear yet [15]. Many flavonoids fluoresce in green spectral region if pH is higher, than 8, but the emission disappeared or decreased at lower pH [16]. The question is what fluoresced compounds undergone the ozone influence in the first turn. Among visible targets of ozone in the structures of petal are native pigments such as chlorophyll and anthocyanins, which are possible to analyze measuring the fluorescence characteristics. In our model, it should consider mainly flavonoids that include anthocyanins seen in the emission spectra of intact cells with maxima 480, 615, 630, and 650 nm (Fig. 1 and Fig. 3 b-d, spectra 1 control). Three last maxima disappeared in first turn, after 2.5 – 25 h of the ozone exposure. Later, up to 50 h of the O_3 influence, chlorophyll was decomposed. However, the colorless substances such as phenols (phenolic acids and coumarins) could be also the tolerant contributors in the observed fluorescence in the head of secretory hair (Figs. 2 and 3). In many cases in chronic ozone exposure polyphenols may be formed [3]. In Brazil woody plants, for example, *Astronium graveolens,* the leaves' fluorescence spectra recorded by confocal microscopy showed maxima between 400 and 650 nm peculiar to polyphenols after prolonged exposures (90 days), and ozone stimulated the fluorescence intensity in this spectral region [9]. This object was enriched in quercetin- and miricetin-hexosides as well in tannins gallic and ellagic acids which can transformed to polyphenols under the ozone influence. All the substances appear to be indicators of the damage. In the fluorescence spectra received by FRET apparatus [17] polyphenols have emission with maxima 440 and 585 nm at the excitation 380 nm. In our experiments top of the hair emitted in yellow-red when the cells undergone 50 h -ozone exposure (dose 0.1 μl/l), although without definite maximum in this spectral region (Fig. 3 a, spectrum 3). Stalks and base stalk cells fluoresced in enhanced intensity, mainly with maxima 480-500 nm, at 0.05 μl/l ozone dose (Fig. 3 b-e), but the emission decreased at higher O_3-concentrations. Not excluding the possible formation of polyphenols under acute action of ozone, we also carried out experiments dealt with a behavior of individual compounds (which are contained in our object) undergone ozone.

In table 1, the effect of ozone on the maxima position of dry individual compounds contained in our object is shown. Among individual substances treated with ozone at room temperature (22°C) anthocyanins are more sensitive

Table 1. *The effects of ozone on the fluorescence maxima of individual compounds solved in 96 % ethanol. Excitation 360 nm.*

Individual compound	**Maximum, nm/ Fluorescence intensity changes**			
	+ dose of ozone (μl/l)			
	Control	**0.05 (25 h)**	**0.1 (50 h)**	**0.2 (100 h)**
Sum of anthocyanins from *Saintpaulia* petals (pH 7.8).	430, 630-650	430 / decrease	0	0
Anthocyanin pelargonidin (pH 5.5.)	450-460, 665	450-460	410-420/ decrease	0
Chlorophyll	675-680	675-680/*	675-680/increase	675-680/decrease
Caffeic acid	450-460	450-460*	450-460*	450-460*
Ferulic acid	450-460	450-460*	450-460*	450-460*
Cinnamic acid	450-460	450-460*	450-460*	450-460*
Esculetin (Aesculetin)	500-520	450*	450/decrease	450/decrease
Coumaric acid	450	450*	450*	450*

* - no changes in the emission intensity

(blue color cyanidins at pH > 7 for sum of *Saintpaulia* anthocyanins or at pH< 7 for red anthocyanin pelargonidin), and their emission disappeared after the threshold 0.1 μl/l ozone at all. Cyanidins, form of anthocyanins at pH> 7, in water solutions have maxima at 610-630 nm at the excitation 220-230 or 270-280 nm [18]. In literature the fluorescence of the compounds, such as malvidin, and its glycosides have maxima in blue-green up to 520 nm and in red 610-630 nm in acid/water solutions [19-21]. Anthocyanins may fluoresce not only in blue, some of them emitted with maxima at orange – red 580-620 nm too [17]**.** According to investigations with secretory cells [22], in longer spectral region, besides green fluorescence (at 510 and 515 nm), yellow- orange and even red (peaks 555, 570-585, 610 nm) emission has seen. For example, under ultra-violet light of the microscope the yellow fluorescence of anthocyanin pelargonin (aglycon) and its glycoside pelargonidin has observed. Pelargonidin in 96 % ethanol (pH <7) has the emission maxima in blue, and the anthocyanin fluorescence depends on the formation of complexes or conjugates with metals or other cellular components. When the compounds applied to Silicagel plates or Avicell ST microcrystallic cellulose, delphinidin, petunidin and malvidin fluoresce in blue, green and red, while cyanidin and peonidin– in red, and pelargonidin – in orange –red spectral region ([16]. In rigid, crystalline state flavonoids, although most of them also fluoresce in blue 450-470 nm, flavones also may have maximum 580 nm, but after the fumigation with ozone (dose 5 μl/l) the shifts observed from blue to orange spectral region [22, 23].

Chlorophyll is not too sensitive in a comparison with anthocyanins – maximum 675-680 nm decreased only to 100 h (dose 0.2μl) ozone action (Table 1). Unlike chlorophylls and anthocyanins, chlorogenic, ferulic and caffeic acids as well as coumaric acid solved in ethanol may fluoresce in blue with maxima at 450-460 nm, and the emission intensity did not change during our acute exposures (Table 1). Thus, strong yellow emission in the head of the secretory hair on the *Saintpaulia* petals to 50 h-ozonation may belong to above-mentioned phenolic acids and coumaric acid. Emission of coumarin esculetin at 450 nm under higher doses of O_3 decreased. 7-Alkoxy coumarins generally have a purple fluorescence, whereas 7-hydroxy and 5.7-dihydroxycoumarins tend to fluoresce in blue [16]. In dry state o-coumaric acid emits in blue with maxima at 440-450 nm and esculetin - at 460 nm [22, 23]. It does not exclude that new phenols may formed under acute ozone doses too.

4. Conclusion

Changes in plant autofluorescence excited in ultra-violet, violet or green may be useful in analysis of ozone effects on living systems. Chemical mechanisms of the action are in a center of the attention dealt with the biggest danger for humanity of ground level of O_3. Search of the ozone indicators (organisms sensitive to low doses ozone - lower than 0.1 μl/l, in which there is a characteristic odor) is actual today. In this case simple model systems such as blossoming *Saintpaulia ionantha*, which the flower cell responses based on their fluorescence, seems to be suitable for biomonitoring and showing the earlier stress state induced by ozone. Anthocyanins and in smaller degree chlorophyll are considered as primary targets for O_3 in this object. More tolerant are aromatic acids and coumarins.

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