Review Article

Vital Autofluorescence: Application to the Study of Plant Living Cells

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The application of various microscopy methods such as luminescence microscopy, microspectrofluorimetry and laser-scanning confocal microscopy has been considered as an approach to study the autofluorescence of plant living cells—from cell diagnostics up to modelling the cell-cell contacts and cell interactions with fluorescent biologically active substances. It bases on the direct observations of secretions released from allelopathic and medicinal species and the cell-donor interactions with cell-acceptors as biosensors (unicellular plant generative and vegetative microspores). Special attention was paid to the interactions with pigmented and fluorescing components of the secretions released by the cells-donors from plant species. Colored components of secretions are considered as histochemical dyes for the analysis of cellular mechanisms at the cell-cell contacts and modelling of cell-cell interactions. The fluorescence of plant biosensors was also recommended for the testing of natural plant excretions as medical drugs.

1. Introduction

The visible (400–700 nm) fluorescence of intact living cells (usually called autofluorescence) excited by ultraviolet, violet, or blue light is often observed under luminescence microscope [1, 2]. Due to special microspectrofluorimeters or confocal microscope the fluorescence spectra of objects studied and the emission intensity can be also measured. Autofluorescence is the natural emission of cellular components due to a contribution of fluorescent compounds located in different cellular compartments. In general blue and green emission reflects the participation of universal cellular fluorophores NAD(P)H (max. 460 nm), pterins (max. 450 nm), and flavins (max. 520 nm) [3], although in dry state weak blue fluorescence (420–450 nm) is also peculiar to isolated nucleotides, RNA, and DNA as well as some proteins, and seen in the films under luminescence microscope [4, 5], but water quenches the emission [4]. Besides, there are characteristic fluorescent compounds in various organisms [6]. In animal cells chitin (max. 450–460 and 520 nm),

collagen and elastin (max. 400–430, 465, 495 and 520 nm) or cellulose (max. 420–430 nm) in plants, and fungi can also contribute in blue fluorescence [6]. Orange-red fluorescence depends on organism. In plant cells main known fluorophore used for environmental monitoring is chlorophyll with maximum 675–680 nm [7] and in photosynthesizing bacteria: bacteriochlorophyll [6]. Uroporphyrinogen and haemoglobin contribute in red lightening of mammalians, while green-red range emission may reflect a contribution of sea animal fluorescent proteins used for the genetic experiments as vector for the study of cellular components [6–8]. Terpenes, some alkaloids (colchicine) and flavonoids in plants, emit in blue or in blue-green (470–525 nm) region of the visible spectrum whereas polyacetylenes, isoquinoline, and acridone alkaloids emit in yellow and orange [8]. Red fluorescence of plant cells may be also due to anthocyanins and azulenes (600–630 nm) [8]. At ageing or, if the object being undergone ozone and relative reactive oxygen species, fluorescent pigments lipofuscins are formed that may emit in wider spectral range—from blue (450–480 nm) to red

(>600 nm) in a dependence on the nature of the organism and duration of the exposure under unfavourable factor [9, 10].

Autofluorescence of the cellular compounds may serve (1) as markers for cytodiagnostics and (2) considerations of as natural indicator of cellular state because the emission often shows changes in cellular metabolism and responses to the external and internal signals [8]. This is true for the plant secretory structures analyzed under luminescent microscope (the structures are not seen in usual microscope without special histochemical staining) or laser-scanning confocal microscope (LSCM images) [8, 10, 20–22, 37–39]. The intensity and spectral composition of the emission differ in a dependence on, namely, (1) nature of the organism or (2) cell analyzed, (3) a taxonomic position of the organism tested, and (4) environmental and experimental conditions (actinic light, temperature, humidity, physiological state of a cell as a whole and phase of development, influence of neighbour cells or organisms, including parasites). Autofluorescence could be used (i) in express-microanalysis of the accumulation of the secondary metabolites in secretory cells without long biochemical procedures; (ii) in diagnostics of cellular damage; (iii) in analysis of cell-cell interactions [8, 10, 20, 21, 39].

In any case the phenomenon of autofluorescence could be used as a resource for the nondestructive vital microscopic analysis of natural organisms [41]. It has already practical application in various diagnostic procedures for cell biology analysis, ecology, medicine, and pharmacology. This paper considers the application of various types of luminescence microscopy to the study of plant cells, mainly such as secretory systems.

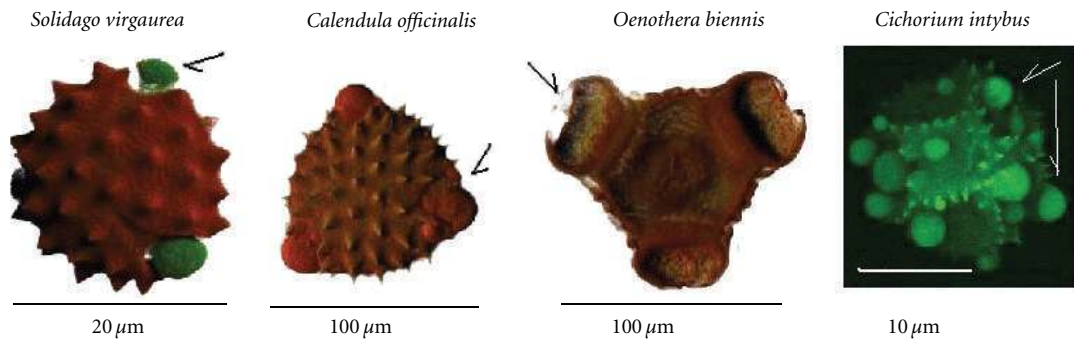## 2. Images and the Fluorescence Spectra of Fluorescing Cells in Cytodiagnostics

Today our knowledge in the problem of autofluorescence, peculiar to living cells, mainly include results of experiments made on big plant cells that are suitable for observation both *in vivo* (such as in nature) and *in vitro* (in cultivated systems). Among similar objects are generative cells of flowers: pollen (male gametophyte) and pistil (female gametophyte) and nongenerative cells, such as plant vegetative microspores and special secretory cells. Some examples dealt with the application of luminescence microscopy in various modifications to analysis of the cells are shown on Figure 1. In particular there are the images seen under usual luminescence or confocal microscopes and the fluorescence spectra measured by microspectrofluorimeter or some types of confocal microscopes. We shall also comment about Figure 1 in sections below.

The research of fluorescence maxima in secretory cells showed the correlations and differences in the emission spectra excited by UV-light among the plant species studied and their specialized structures [8]. For example, nectaries mainly fluoresce, in blue region, while sporangia of fungi, mosses, horsetails, and ferns are in green-yellow (500–550 nm). Except fungi, the structures have also maximum
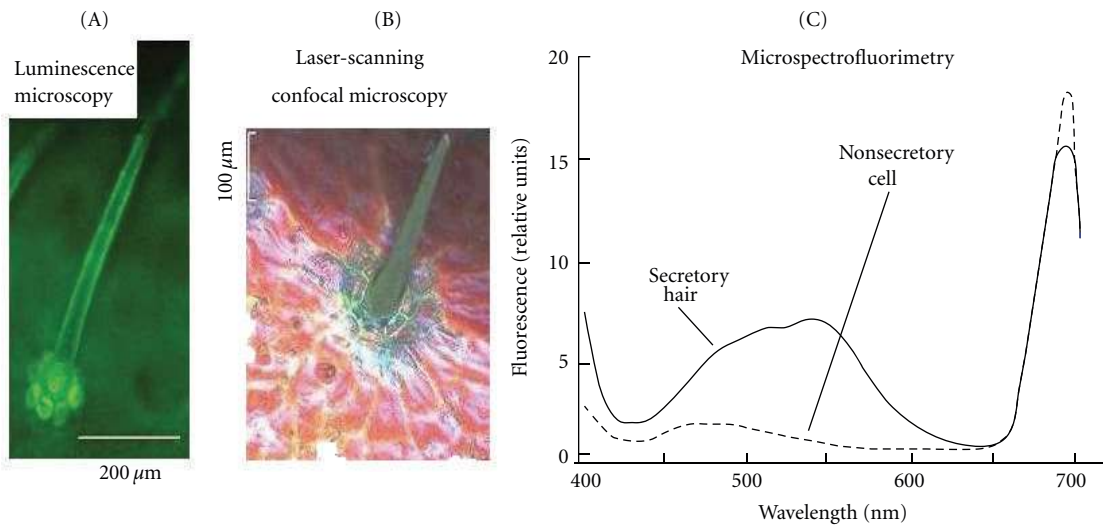
680 nm in red, peculiar to chlorophyll located in chloroplasts. Crystals of secretions emit more brightness than liquids. Today the secretion peaks were compared with the maxima of individual components of the same secretions in solutions or films [5, 8, 10]. Interference of various fluorophores in total emission may take place, but diagnostics of prevailing component of the secretion is real. Changes in the fluorescing compounds depend on stage of plant development, the influence of various factors—ozonation, redox agents and excretions of other organisms in the environment.

2.1. Pollen. Pollen is the unicellular structure (served for plant breeding of seed-bearing plants) that germinates on pistil stigma of flower forming pollen tube that grows within pistil style and carries out two spermia—male cells. As soon as the pollen tube achieves ovule (seed bag), here one sperm interacts with egg cell, and the fertilization is over by the embryo arising. Pollen often show visible autofluorescence excited by ultra violet or violet light. Luminescence microscope or laser-scanning confocal microscope was used for both the receiving fluorescing cell images and (in some cases) fluorescence spectra. Some examples of the emission are on Figure 1(a) and Table 1. Full information about the earlier studies of pollen autofluorescence as well as apparatuses for the observation and measurement has been summarized in reviews [18, 19] and monograph [8]. Here there are the references related to pioneers who first have seen autofluorescence of pollen under a luminescence microscope [43–45] and later measured first fluorescence spectra of pollen from various species [46, 47] with a microspectrofluorimeter.
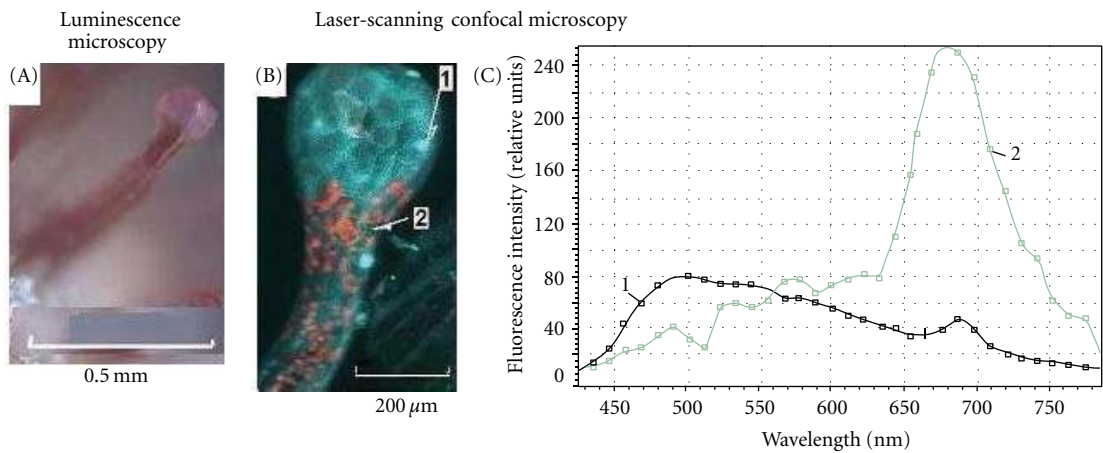
Figure 1 shows the LSCM images of pollens (from medicinal plants) released fluorescing secretions from apertures, through which pollen tube enters at pollen germination. We see different colors of autofluorescence from pollen surface and drops of secretions (marked with arrows) released from apertures (green emission of the secretions from *Solidago virgaurea*, orange-red lightening from one of *Calendula officinalis*, and colourless from *Oenothera biennis*). Today the phenomenon of autofluorescence is applied to the various diagnostics seen from Table 1. First papers in this line dealt with analysis of the exine autofluorescence by luminescence microscope. Exine, the outer layer of pollen cover, consists of sporopollenin and in various species may include various fluorescent pigments from phenols and carotenoids up to azulenes and proazulenes [8, 15]. Structure of exine was also analyzed with confocal microscopy [25], permitting to receive optical slices [8, 20, 21, 38, 39], and the fluorescence spectra were also recorded [8, 15–19]. Based on the autofluorescence the various steps in pollen development were studied [8, 13]. Moreover, the phenomenon is applied to pollen classification [30, 31], including the search pollen grains of fruitful varieties from certain plant species [33]. For understanding of sporopollenin synthesis and exine assembly a simple effective genetic screen based on their autofluorescence characteristics was represented for the *Arabidopsis thaliana* pollen (the recovery of mutants with a variety of defects in exine structure) to identify genes involved in pollen exine production [34].

Solidago virgaurea *Calendula officinalis* *Oenothera biennis* *Cichorium intybus*

20 µm   100 µm   100 µm   10 µm

(a) Laser-scanning confocal microscopy of pollen

(A) Luminescence microscopy

200 µm

(B) Laser-scanning confocal microscopy

100 µm

(C) Microspectrofluorimetry

Nonsecretory cell

Secretory hair

(b) Leaf secretory hair of *Symphytum officinale*

(A) Luminescence microscopy

0.5 mm

(B) Laser-scanning confocal microscopy

200 µm

(C)

(c) Secretory hair on flower calyx from *Rubus odoratus*

FIGURE 1: Images and the fluorescence spectra of some intact fluorescing plant secreting cells. Source: adapted from [21, 22] and unpublished data of the review' author. (a) LSCM images of pollen grains from various species under confocal microscope LSM 510 NLO "Carl Zeiss" (Laser 488 nm). Drops of secretions from apertures are seen. (b) and (c) Views of secretory hairs on leaf of comfrey *Symphytum officinale* and on the flower calyx of flowering raspberry *Rubus odoratus* under (A) luminescence microscope Leica 6000 (Excitation 360–380 nm) and (B) laser-scanning confocal microscope Leica TSC SP 5 (laser 405 nm) and the (C) fluorescence spectra measured by registered microspectrofluorimeter MSF-1 (Excitation 360–380 nm) or laser-scanning confocal microscope Leica TSC SP 5 from secretory head with released secretion (1) and stalk (2) of secretory hair.

TABLE 1: Autofluorescence in pollen diagnostics (LM—Luminescence microscopy, MS—Microspectrofluorimetry, LSCM—Laser-scanning confocal microscope).

Diagnostics	Microscopic method	Fluorescence characteristics
Vital pollen characteristics. Changes in the autofluorescence of the pollen wall during microsporogenesis and chemical treatments [11, 12]	LM, MS	Color and fluorescence spectra
Wall development of sterile and fertile pollen [13]	LM	Color
Assessment of pollen retention using exine autofluorescence [14]	LM	Images
Matured and non-matured pollen [15–19]	MS	Fluorescence spectra (pigment composition)
Self-incompatibility and self-compatibility of pollen [17]	MS	Fluorescence spectra (pigment composition)
Pollen loads analysis as well as pollen-derived products of bees [8, 17]	MS	Fluorescence spectra (pigment composition—azulenes, carotenoids)
Pollen-pollen interactions in the mixtures [8, 20–23]	LSCM, MS	Changes in the fluorescence spectra of pollen from different species reflect changes in pigments' state
Pollen viability [8, 17]	MS	Fluorescence intensity after moistening
Pollen-pistil interaction at fertilization [8, 17, 24]	MS	Changes in the fluorescence spectra reflect the receptor state
Exine structure [25]	LSCM	Images
Quantifying dynamics of pollen shed [26]	LM	Images
Ozone damages [27–29]	MS	Changes in the fluorescence spectra reflect pigments' state and forming pigment of aging lipofuscin
Damages by reactive oxygen species [28, 29]	MS	Changes in the fluorescence spectra
Classification of pollen grain species using autofluorescence image analysis [30, 31]	LM	Images and blue/red autofluorescence ratio
Testing in nanotechnology: Commercially produced fullerene fine particles [32]	LM	Adhesion of nanoparticles estimated on the autofluorescence intensity changes
The search of fruitful varieties from certain plant species [33]	LSCM	Images
Genetic screen to identify genes involved in pollen exine production [34]	LSCM	Wild-type exine fluoresces with two peaks 460 nm (higher) and 505 nm (lower)
A novel screen identifies multiple pollen mutants that have defective exine [34]	LSCM	Defective mutants differ in the emission intensity in the above-mentioned peaks
Taxonomic classification of pollen [35]	LSCM	Images
Pollen concentration in air [6, 36]	LM, LSCM	Images, emission intensity

The fluorescence spectra of pollen from 20 plant species, both wind—and insect-pollinated, were analyzed [8]. Pollen grains from wind-pollinated species (birch, pine and others) have mainly blue fluorescence whereas insect-pollinated ones belonged to different species—in blue, yellow, green, or blue-green. Nonmatured pollen demonstrated red fluorescence due to the presence of chlorophyll, while matured grains changed the color of the emission. The autofluorescence spectra of many pigmented and colorless pollens treated with ozone in various concentrations was also studied, and some characteristic damages were shown [27–29]. The fluorescence maximum at 530–550 nm disappeared in carotenoid-containing pollen grains of *Philadelphus grandiflorus* and *Epiphyllum hybridum*, and a new maximum at 475–480 nm

arose (unlike carotenoid-less pollen of *Plantago major*) that correlated with lipofuscin-like substances observed in the extracts from the pollen grains [27–29].

In pollen mixtures changes in the fluorescence of pollens from different species may reflect their chemical (allelopathic) relations [8, 21]. LSCM images directly demonstrated the alterations in the emission and the state of pollen grains from Field Scabious *Knautia arvensis* (L.) Coult. (Dipsacaceae) contacting with pollen from wormwood *Artemisia absinthium* L. (Linn.) (Asteraceae) [20].

The autofluorescence imaging alone or in a combination with other morphological parameters [33] can be considered as nondisruptive method for pollen classification since it does not require any treatment of fixing or staining of the specimens. Therefore, this simple and fast method can be used for pollen counting and taxonomical classification in multiple applications that concern disciplines like aerobiology for identification of pollens in bioaerosols [6, 36, 48, 49]. Today the pollen study is very important, because air-occurring pollen from certain plant species may induce allergy, although on the other side pollen grains of medicinal plants are used for drug preparation. Knowledge of pollen state is necessary for analysis of plant fertility in genetics and biotechnology or of the meteorological situation in ecological monitoring as well as in pharmacy.

2.2. Pistil. Pistil of flower is female gametophyte which stigma receives chemosignal from pollen as male gametophyte and recognizes pollen grains from the same plant species (as well as self-compatible or self-incompatible pollen of the species) or from foreign species. Autofluorescence reflects the pistil state before pollen application and after the pollen grains addition on pistil stigma [8, 17, 24]. If self-pollination occurs one can see changes in the peaks positions and the emission intensity in a comparison with the pistil fluorescence reply on pollen that originates from another plant of the same species like was seen on the example with *Hippeastrum hybridum* [16, 17]. When foreign pollen was applied to the pistil stigma often there are no changes in the pistil fluorescence [8]. Moreover pistils from self-compatible and self-incompatible lines of the one and same plant species, in particular as in *Petunia hybrida* clones, have different maxima in the fluorescence spectra [8].

2.3. Secretory Cells—Donors of Biologically Active Fluorescing Compounds. Any living cells may form and release secretions out, and it is better seen in specialized secretory plant cells with fluorescent products [8, 10]. Images of the secreting cell fluorescence excited with ultraviolet or violet light are especially valuable in allelopathic and medicinal plants which contain compounds named allelochemicals (simultaneously being valuable pharmaceuticals) in secretory cells [50]. Many of the chemicals excreted act on the other organisms in biocenosis. The secretory cells often show bright fluorescence at the excitation of ultraviolet (360–390 nm) or violet (400–430 nm) light [8, 10, 51, 52] due to the presence of phenols, alkaloids, terpenoids, etc.). The fluorescing images of similar cells—glandular hairs of leaf and flower—are shown under

usual luminescence and confocal microscopes as seen on Figures 1(b) and 1(c). Leaf secretory hair of comfrey *Symphytum officinale* from family Boraginaceae under the violet or blue-green excitation (the structures weakly lighten at the ultraviolet) has the green-yellow emission maximum 550 nm and shoulder 460–500 nm in the fluorescence spectrum. Another plant species—purple flowering raspberry *Rubus odoratus*—shows the emission of calycinal secretory hair on flower in blue-green with maximum 475 nm unlike nonsecretory stalk cells that have maximum 675–680 nm, peculiar to chlorophyll.

The images and the emission intensity of secretory cells included fluorescent drugs is important for medicinal plant species because this may characterise the state of pharmaceutically valuable material [40] as shown in Table 2 for fresh and dried leaves of warmwood *Artemisia* species (in pharmacy used as tonic, stomachic, febrifuge, and antihelmintic and a good remedy for enfeebled digestion and debility). The species contained fluorescent terpenes, sesquiterpenes and phenols [8]. The green emission of secretory cells on fresh leaves of wormwood *A. absinthium* L. was about 4 times higher, than in non-secretory cells. In dried material the difference increased significantly up to 20 times. This demonstrates a difference in the fluorescence intensity between secretory cells of fresh leaves than in dried leaves. In mugwort *A. vulgaris* L. dried leaf secretory cells emitted 2 times brightly in a comparison with secretory cells of fresh material. The visible difference in the fluorescence intensity between two species reflects the difference in an accumulation of fluorescent pharmaceuticals (azulenes and flavonoids) [40]. Flavonoids and coumarins as fluorophores may be also included in the leaf fluorescence of *A. vulgaris*.

3. Images of Fluorescent Plant Secretory Cells in Cell-Cell Interactions

Cytodiagnostics of cell-cell interactions has perspectives also in the analysis of autofluorescence in the studies of chemical relations (allelopathy) between species in biocenosis relations). The examples may be a visualization of the insect attack [53] or fungal invasion [54–57] and relationships between pollen grains of different species [20, 21]. Fungi or insects such as ticks, spiders, or human skin fluoresce, mainly, in blue (460–470 nm) or blue-green (<520–530 nm) whereas muscle—in blue with maxima 410 and 480 nm. Insects fluoresce by all parts of their body, and it may observe separately on the plant cell surface. The blue emission intensity of fungal hypha and mycelium are much higher than in fluorescing secretory plant cells that makes possible to recognize fungal infection.

3.1. The Parasitive Invasion. Figure 2 demonstrates the example for leaf of medical plant mint *Mentha piperita* used by aphid for nutrition. On the leaf colorless microscopic larvae are plant-louse not seen well up to the excitation by actinic light of luminescence microscope. Blue-fluorescing Mentha gland (if excited by UV-light) with maxima 475, 540, 675–680 nm in the fluorescence spectrum is covered by

TABLE 2: The fluorescence intensity (I) of leaf secretory cells of medicinal plants *Artemisia absinthium* and *Artemisia vulgaris* measured by double-beam microspectrofluorimeter MSF -2 at 520–540 nm or at 640–680 nm. Source: [40]. Excitation 420 nm.

| Cell	Plant material	$I_{520-540}$	$I_{640-680}$
Artemisia absinthium			
Glandular cell and hair	Fresh leaves	0.84 ± 0.07	5.76 ± 0.9
Nonsecretory cells	Fresh leaves	0.21 ± 0.01	7.99 ± 1.0
Glandular cell and hair	Dried leaves	0.28 ± 0.01	3.15 ± 0.08
Nonsecretory cells	Dried leaves	0.01	2.28 ± 0.03
Artemisia vulgaris			
Glandular cell and hair	Fresh leaves	0.12 ± 0.01	2.26 ± 0.09
Nonsecretory cells	Fresh leaves	0.04 ± 0.01	4.89 ± 0.20
Glandular cell and hair	Dried leaves	0.26 ± 0.02	1.83 ± 0.08
Nonsecretory cells	Dried leaves	0.14 ± 0.01	2.40 ± 0.5
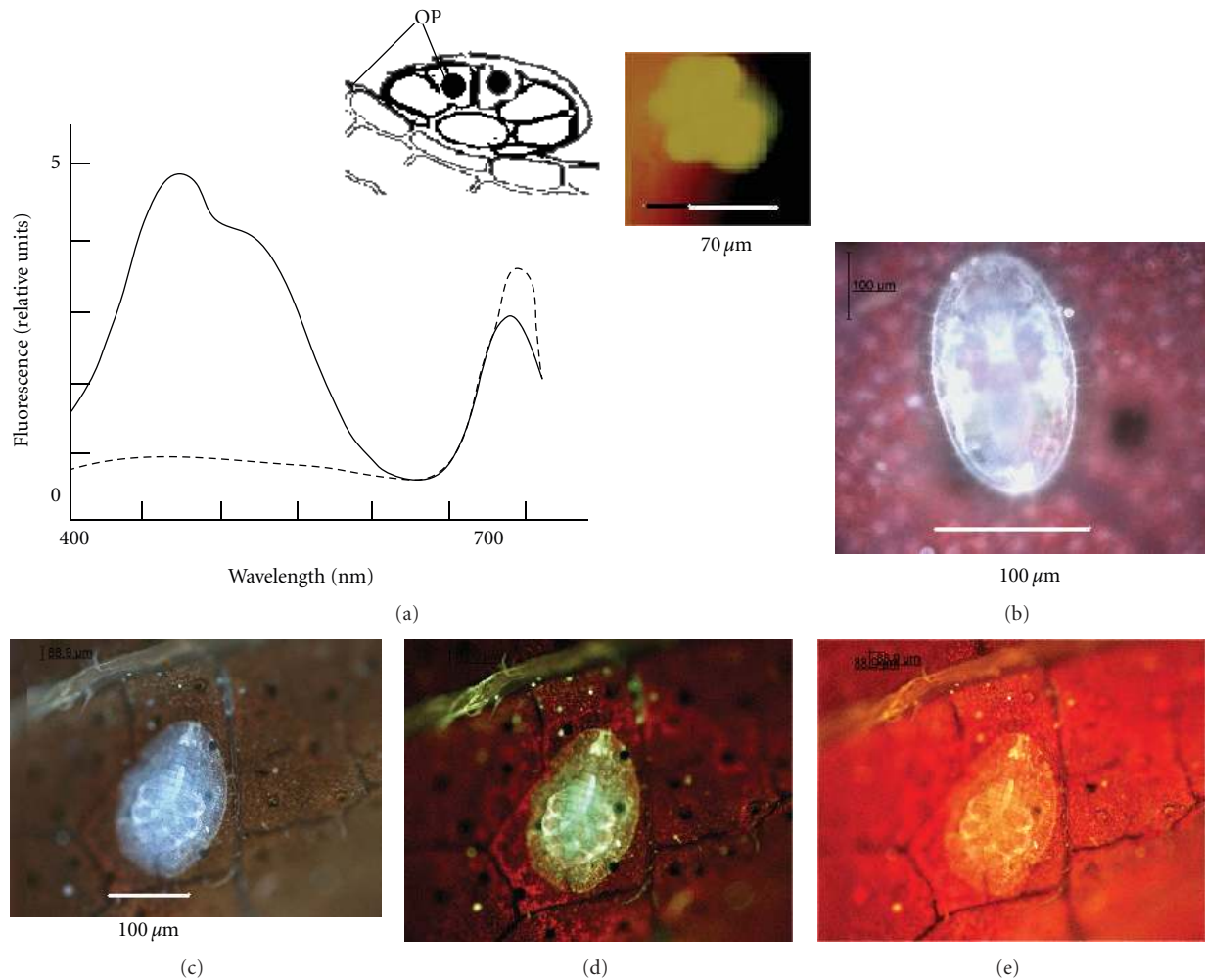
FIGURE 2: Fluorescent larva of aphis interacts with cellular complex of leaf gland of mint *Mentha piperita*. (a) Left: the fluorescence spectra of secretory cells measured by microspectrofluorimeter MSF-1. Excitation: 360–380 nm. Unbroken line—secretory cells; broken line non-secretory cells. The position of optical probe (OP) is shown. Right: the common view of gland under luminescence microscope "Carl Zeiss CD-28". ((b)–(e)) Images under luminescence microscope *Leica* DM 6000. (b) The common view of aphis larva out of the gland, ((c)–(e)) views of larva on the mint gland. Excitation by light 360–410 nm (b), 420–450 nm (c), 480 nm (d), and 560 nm (e).

weak blue-fluorescing aphis larva (chitin emission, mainly at 450 nm). At excitation by violet or green-yellow light yellow-fluoresced mint gland (surrounded by red fluorescing non-secretory cells of leaf mesophyll due to chlorophyll presence) mainly was seen through nonvisible (transparent) larva body.

Many pathogenic and saprophytic fungi fluoresce in blue green (in 390–490 nm spectral region) under actinic ultraviolet light [54–57]. Moreover the phenomenon was better seen under stress (desiccation, heat, chemicals) [56]. Recently blue-green autofluorescence of fungi of *Ustilago maydis* on maize and cell wall of this plant is an indicator of the invasion [57]. At the fungal infection the value of autofluorescence as a screening method for detecting fungi in tissues was compared with conventional histochemical stains. Autofluorescence can be used as a rapid screening method for identification of fungi in tissue sections as it does not require any other specialized staining procedure. Confocal microscopy appears to be more suitable for plant-pathogen interaction in details [58]. Fungal fluorophores may depend on species and are not well studied yet. Today fungal infection is better registered observing the enhanced blue-green autofluorescence of plant cell walls related to their phenols and sometimes auxin exchange [59].

3.2. Pollen-Pollen Interactions. Special interest is the visualization of the interaction between pollen grains of various species that named as pollen allelopathy. At the contact, excretion from pollen-donor acts as chemosignal on pollen-acceptor, inducing the changes in the fluorescence of the microspores and their germination. The interaction of big pollen from *Knautia arvensis* (fluorescing in green) and small pollen grains from *Artemisia absinthium* or from *Solidago virgaurea*, which have released drops of red-fluorescing secretion as seen under laser-scanning confocal microscope [8, 20, 21]. This secretion was seen on the surface of non-germinated pollen from *Knautia arvensis* and also on the surface of pollen tubes. The interaction of green fluorescent pollen of *K. arvensis* and red-fluorescing secretion of pollen from *Artemisia absinthium* decreased the germination in both contacting types of microspores. In this case, perhaps, the enhancement in fluorescence is related to the energy loss peculiar to nonviable pollens. On LSCM images of fluorescent pollens in their mixtures red-fluorescing secretion for large pollen of *Oenothera biennis* contacted with small pollens of *Matricaria chamomilla* emitted in green and liberated green-fluorescing secretions were seen [21]. Optical slices showed the interior of contacted pollen grains, green fluorescence was seen only on the surface of *M. chamolilla* microspore, while red emission was also seen inside the pollen grain of *O. biennis*. LSCM images of fluorescent pollen made it possible to observe directly (i) the contact of intact cell, (ii) changes in their emission, and (iii) location of contacting secretions.

Intensity of autofluorescence from interacting cells in pollen mixtures also reflects the mutual influence of contacted secretions as shown for microspores from *Hippeastrum hybridum*, *Philadelphus grandiflorus*, *Knautia arvensis*,

Plantago major, and *Oenothera biennis* used as pollen-acceptors, while other pollen grains from 42 plant species—as donor pollens [21]. Change of the light emission (excited by UV 360–380 nm or violet 430 nm light) is a visual chemical response to the pollen excretion from foreign pollens. To develop models for pollen-pollen interactions, the effects of allelochemicals secreted by foreign pollen (i.e., donor pollen) were studied *in vitro* or *in vivo* on autofluorescence concerned with the germination of acceptor-pollen [21]. Autofluorescence of acceptor and donor pollens in mixtures was changed to blue-green and red spectral regions that may serve as the indicator reply on foreign microspores. Generally increased blue-green and occasionally red emission indicated the decrease in the rate of pollen germination. However, red fluorescence was not related to germination and depended, mainly, on species used. It may be explained by redox changes in pollen components (often cover constituents) or by the synthesis of new fluorescing metabolite. Perhaps, the enhancement in blue-green emission of pollen could be related to the formation of fluoresced Schiff bases or to the formation of NAD(P)H emitted with maxima 460 nm. Red fluorescence of matured pollen is often correlated with the presence of anthocyanins or azulenes like occurs in overgrown parts of plants [8, 21].

4. Autofluorescence as Biosignal or Marker to Study Localization of Secondary Metabolites

Even 50 years ago Frey-Wyssling and Agthe have discovered fluorescence of flower nectar [8], as possible signal for insects-pollinators [60], and today the phenomenon has been demonstrated for various floral and extrafloral nectaries [8]. Moreover, some secondary metabolites served as the defense agents against parasitive invasion, for example, phenols emit under the excitation of UV-light [3], and their autofluorescence should mark their location in cells and tissues [61–63].

4.1. Biosignal for Insects. New look on autofluorescence appears with several works dealt with the search of signal for pollinators which prefer flowers of the certain plant species [64, 65]. A quantitative evaluation of the light emerging from intact petals of *Rhododendron indicum* flowers of different colors was performed based on the measurement of reflectance and fluorescence emission. Emitted photons as fluorescence were compared with reflected photons. The fluorescence quantum yield values (ϕ_f) varied from 7.6×10^{-5} to 6.3×10^{-4} for the emission in the blue region of the electromagnetic spectrum and from 2.4×10^{-5} to 1.9×10^{-4} for the emission in the red one [64]. (ϕ_f) was calculated for flowers of *Bellis perennis* (white, yellow, pink, and purple), *Ornithogalum thyrsoides* (petals and ovaries), *Limonium sinuatum* (white and yellow), *Lampranthus productus* (yellow), *Petunia nyctaginiflora* (white), *Bougainvillea spectabilis* (white and yellow), *Antirrhinum majus* (white and yellow), *Eustoma grandiflorum* (white and blue), *Citrus aurantium* (petals and stigma), and *Portulaca grandiflora* (yellow) [65]. The highest values were obtained for the ovaries of

O. thyrsoides (ϕ_f = 0.030) and for *Citrus aurantium* petals (ϕ_f = 0.014) and stigma (ϕ_f = 0.013). The fluorescence emission as an optical signal in biocommunication was negligible if to compare to the light reflected by the petals. Nevertheless, the calculation of quantum catches for each studied flower species described the visual sensitization of insect eye photoreceptors.

4.2. Localization of Secondary Metabolites in Cells and Tissues. Location of secondary metabolites fluoresced under UV or violet excitation is a new approach to visualize secondary metabolites in plants [61–63, 66–70]. Among the compounds are fluorescent phenol compounds that play a major role in the interaction of organisms in biocenosis attracting insects and functioning as signals between plants (allelopathy), as signals between plants and phenols in cell walls, vacuoles, and associated with N_2-fixing bacteria or pathogenic organisms [61–63]. The distribution of flavonoids such as anthocyanins in grape (*Vitis vinifera* L.) was measured nondestructively by chlorophyll fluorescence imaging using two excitation light bands at 550 and 650 nm [62]. Strong blue autofluorescence of living young leaf of *Coffea canephora*, species which contains very high level of chlorogenic acid, relates to vesicles filled with the compound and where the similar emission was also observed [63]. The phenol localization in the vacuole of epidermis cells, in vesicles and chloroplasts of mesophyll cells strengthen the hypothesis of its involvement in leaf protection against UV damages or pathogens. Besides phenols, yellow pigments betaxanthins also fluoresce in flowers in green region [66] and according the emission it is possible to determine dopamine-pigment complex in living *Portulaca oleracea* cells [67].

Especial attention was paid to localization in cells and tissues such flavonoids as anthocyanins in grape and apples [68, 69]. Practical tendency in the investigations is the use of multiparametric fluorescence sensor in apple quality [69]. The pixel intensity in the derived logarithm of the fluorescence excitation ratio image was directly related to the anthocyanin concentration of berry extracts. The method will be useful for the assessment of the heterogeneity of anthocyanin accumulation in berries depending on physiologic and climatic factors. It can also represent a new, rapid and noninvasive technique for the assessment of fruit ripening and the appropriate time of harvest. Moreover, well-seen autofluorescence of the anthocyanins is recommended for their traffic within cell [70]. The detection of pigments and colorless flavonoids can provide a useful indication of fruit quality [68, 69] or to localize some phenols such as chlorogenic acid in leaf tissues, demonstrating the great potential of multiphoton microscopy combined with spectral deconvolution [63].

5. Autofluorescence of Biosensors

Due to their autofluorescence plant intact cells may serve as biosensors and bioindicators because they have natural structure that determinates their high activity and stability

[71]. Criteria in the screening of the plant cells as biosensors for biologically active compounds should be as follows: (1) the object use is easy in laboratory and in the field and (2) autofluorescence should be one of the physiological responses on the external chemicals because reflects the fast biochemical changes. They could serve as the biosensors of some biologically active excretions (having drugs' characteristics) from plants known as weeds, medicinal, or poisonous plant species [37, 42, 71]. Plant biosensors are more suitable for similar testing, than animals, which use needs procedures of vivisection [40, 71].

5.1. Characteristics of Biosensors. As unicellular biosensors vegetative microspores of spore-bearing plants and pollens of seed-bearing plants [8, 42, 71] may be used as well as multicellular biosensor—pistil stigma in flowers [8, 24].

Figure 3 shows examples of fluorescent analysis of vegetative microspores of horsetail *Equisetum arvense* using various technique. Under luminescence microscope, green fluorescence of cell wall and elaters (shell employees to attach microspore to the substrate) is visible. Through the rather transparent cover bright red fluorescence of chloroplasts, located inside the cell among nonlightening cytoplasm, is also observed. The total spectra of fluorescence from cells measured by microspectrofluorimeter depended on the presence of appropriate pigments and had the three peaks—in blue with maximum 450–460 nm (azulenes, phenols), in green with maximum 540–550 nm (carotenoids), as well as the most intense red emission with maximum 675–680 nm (chlorophyll). Cell cover liberated during the microspore development does not have a maximum in red spectral region [72]. Average fluorescence intensity of 100 cells measured on the one subject slide by dual-beam microspectrofluorimeter in green (520 nm) and red (640–680 nm) was differed in many times as seen from numerical data of summary emission shown in the histogram area (3.15 relative units in red against 0.13 relative units in green region). Red emission prevailed. Its intensity increases during the microspore development in comparison with dry rest (nondeveloped) cells [72, 73].

If the division of the spore cell occurs (and duplication of chloroplasts takes place) the 2 times increase in red fluorescence is the indicator of cellular division because amounts of chloroplasts and fluorescing chlorophyll are doubled. Unlike the microspectrofluorimetry laser-scanning confocal microscopy permits to see images of optical slices of intact microspore cells at different depths and register the spectra of fluorescence from individual parts (marked as numerals on Figure 3), namely, cell wall, including its outer layer exine (1), its inner layer intine with plasmalemma (2), and chloroplasts (3).

The natural fluorescence of pollen and pistils as biosensors is served to study of various processes [10, 21, 22, 24, 27–29] from the damages by ozone and other reactive oxygen species to chemosignaling. Indicators of the emission (Table 3) based on color and intensity of fluorescence altered under ozone and reactive oxygen species or the addition

TABLE 3: Pollen biosensors.

| Plant species	Colour of pollen autofluorescence	Prevailing fluorophore	Indicator reaction
Hippeastrum hybridum L. (Herb), knight's star (Amaryllidaceae)	Blue-green	Flavins, phenols	Shifts in the fluorescence maxima and intensity at pollen-pistil or pollen-pollen interactions and neurotransmitters additions [8, 16, 17, 19, 21, 23]
Philadelphus grandiflorus, wild mock-orange (Hydrangeaceae)	Yellow-orange	Carotenoids, azulenes, anthocyanins	Shifts in the fluorescence maxima and intensity after ozone or other reactive oxygen species treatment [26–28]
Knautia arvensis (L.) Coult., field scabious (Dipsacaceae)	Green	Anthocyanins	Shifts in the fluorescence intensity at pollen-pollen interactions [20, 21]
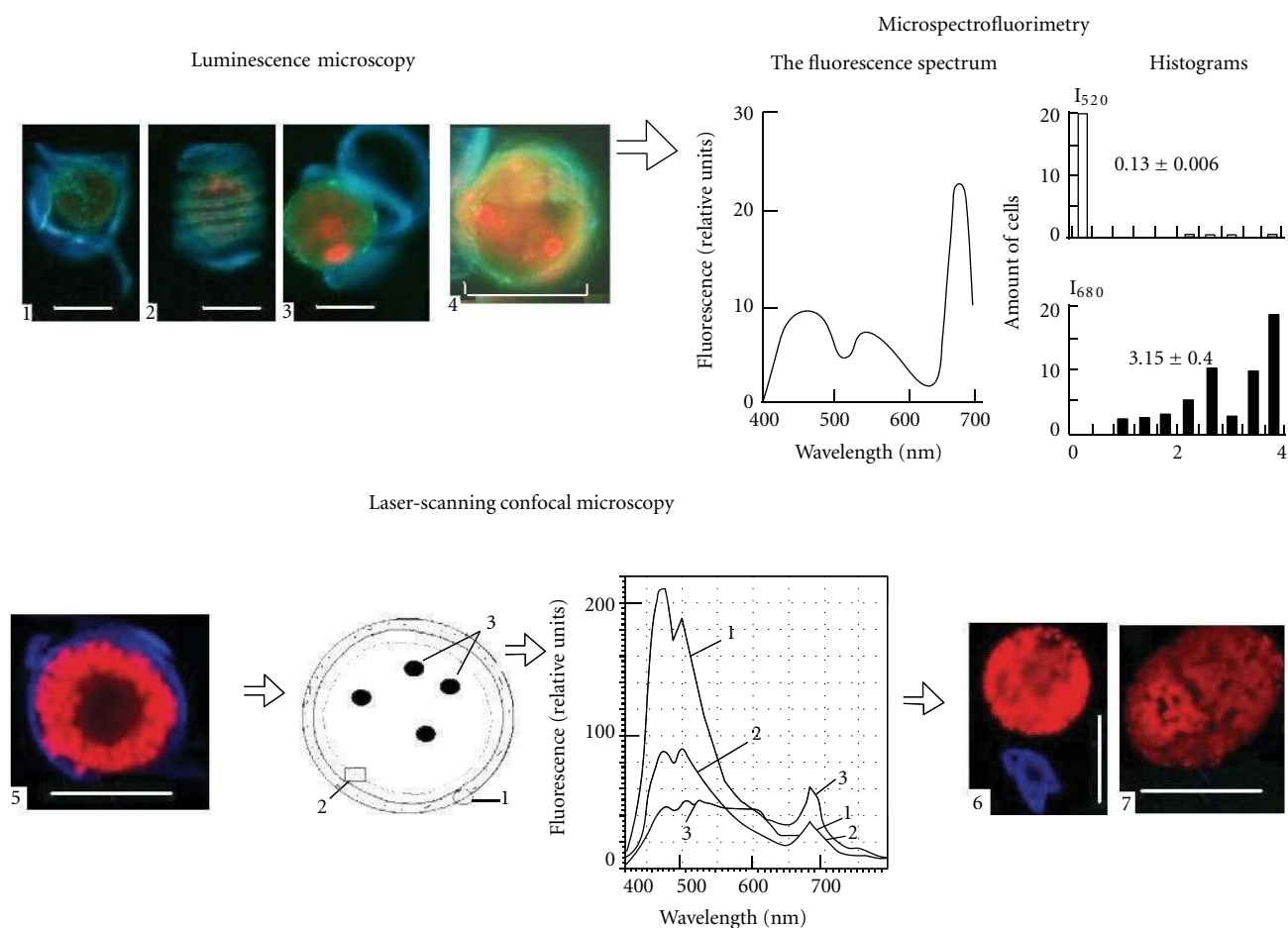
FIGURE 3: The images and fluorescence spectra of the vegetative microspores of horsetail *Equisetum arvense*. Sources: [8, 42] and non-published data of author. Upper side: Luminescence microscopy (left). Views (1–4) under the luminescence microscope Leica 6000. Microspectrofluorimetry. The fluorescence spectrum (middle) and the emission intensity distribution in form of numerical data or histogram (right). The fluorescence intensity (I) on histograms measured by dual-wavelength microspectrofluorimeter MSF-2 shows as I_{520} and I_{680}, respectively, the emission intensity in the green region with maximum 520 nm and red region with a maximum 680 nm. Lower side: Laser-scanning confocal microscopy. Images 5–7 under laser-scanning confocal microscope LSM 510 NLO (5–moistured spore) and Carl Zeiss (6 and 7 developing spore that put off the blue-fluoresced rigid cover-envelope and then divided, resp.). On the scheme of optical image slice under laser-scanning confocal microscope (at the middle of the picture numbers mean cellular parts, from which the appropriate fluorescence spectra have been received with laser-scanning confocal microscope *Leica* TCS SP-5. By numerals exine (1), its inner layer intine with plasmic membrane (2) and chloroplast (3) are marked. Excitation for the luminescence microscope or microspectrofluorimeter was 420 nm and for laser-scanning confocal microscopy—405 nm laser.

TABLE 4: Fluorescence of biologically active compounds (medical drugs) out and within cells of microspores—biosensors. Source: [38, 40].

Component of plant secretions with pharmaceutical features	Fluorescence (color/maxima in nm)			Cellular component—target of a fluorescent staining
	Pure compound	At the interaction with microspores		
		E. arvense	*H. hybridum*	
Azulene	Blue/425	Blue/425, 550	Blue/425, 550	Nucleus, chloroplasts
Atropine	Blue/415	Blue/465–470	Blue/465–470	ChR in Plm
d-Tubocurarine	Blue/415	Blue/425	Blue/465–475	ChR, Plm
Yohimbine	Blue/455	Blue/4305	Blue/475	AdR, Plm

*Plm: plasmalemma, CW: cell wall, ChE: cholinesterase, ChR: cholinoreceptor, AdR: adrenoreceptor, and Glc: glucosidase.

of some natural compounds (including complex secretions) and artificial chemicals. Pollen grains of *Hippeastrum hybridum* were often used as biosensors [37, 42, 71] as well as a pistil stigma of the same species [8, 24]. Multicolor pictures received looked as fantastic art photo that makes them fine illustrations [20, 21]. Besides fluorescent pollens, weak-emitted pollen such as from plantain *Plantago major* is also used for the analysis of interactions with fluorescent plant secretions, in particular from colored pollen grains) and the action mechanisms of drugs [8, 20–22, 40].

5.2. Biosensors in the Study of Chemosignal Processes. Chemosignaling is a first process occurred at contacts of sexual and somatic cells. For the studies it is necessary to distinguish two groups of fluorescing objects—cells which serve as donors of fluorescing compounds excreted and cells-acceptors of the chemical signal that are biosensors. Analyzing both cell types the researcher can observe, namely: (1) the changes in natural fluorescence of contacting cells, (2) the penetration of emitted fluorophore into the cell acceptor, (3) binding of the lightening compounds within cells, and (4) quenching or strengthening the total cell-acceptor fluorescence.

Chemosignaling at direct cell-cell contacts is possible to observe, using some sensitive pollen as biosensor (like to study of pollen allelopathy in Section 2.1). Application of LSCM technique permits to observe direct interaction of the contacted pollens and the alterations in their state, basing on the fluorescence changes. In example with LSCM images the secretion released from red-fluorescing pollen of *Hypericum perforatum* may stain the surface of the color-less pollen from *Plantago major* [8, 20]. The fluorescence of slices showed that the red-emitting pigment hypericin concentrated only on the surface without penetration into a cell.

Besides the direct observation of chemosignaling at cellular communications on LSCM-images, there is modelling of the interaction in the system biosensor–contacting chemical. Use of cellular autofluorescence as a response to the chemosignal can be one of the approaches to study mechanisms of action of biologically active substances on sensitive cells [37, 59]. Cellular mechanisms of the effects of many natural compounds as chemosignals often are not suitable to observe on the tissue slices of animals after vivisection or special conditions of cell storing and cultivation; plant cells have advantages as biosensors. For example, neurotransmitters acetylcholine, catecholamines, serotonin and histamine

that also found in excretions of plant and microorganisms [74, 75] changed the position of maxima in the emission spectra and intensity of pollen and pistil autofluorescence in *Hippeastrum hybridum* [8, 24] or vegetative microspores of horsetail *Equisetum arvense* [42]. The neurotransmitters in the presence of other components of secretions—lipids and proteins or membrane-active agents of fungal infection—decreased or enhanced the fluorescence intensity at 680 nm of biosensors-vegetative microspores from horsetail *Equisetum arvense* [42]. Some of the contacted secretory products also fluoresce that allows observing how they interact with unicellular biosensors [8, 21, 22, 24]. Fluorescent components of plant secretions are used to study of chemosignal processes at cellular level, stain living cells and provide valuable information about the level of their entering into a cell and binding sites [21, 22] as well as the mechanisms of chemoreception and action of some drugs [40, 42, 71]. Blue-fluorescent antagonists (blockers of appropriate receptors), in particular antagonist of acetylcholine atropine or d-Tubocurarine and antagonist of catecholamines yohimbine, were binding with plasmatic membrane while histaminic antagonist azulene penetrated into a cell that was seen on LSCM images [42]. Table 4 demonstrates the targets of the compounds' bindings. Antagonists of neurotransmitters are included in secretions of medicinal (allelopathically active) plants as pharmaceuticals with own fluorescence. All compounds studied decreased number of the cells of *E. arvense* fluorescing in red (Figure 4). d-Tubocurarine and yohimbine did not penetrate the cells binding on the cellular surface, but azulene was linked at both the cell surface and inside the cell, demonstrating blue or blue-green emission. Tubocurarine bound with cholinoreceptor in plasmatic membrane stimulated blue emission and decreased red chlorophyll fluorescence masking plastidal emission. The evidence that tubocurarine and acetylcholine are linked in one and the same site of the membrane is the absence or small blue fluorescence and enhanced red fluorescence in the variant with preliminary treatment of the biosensor cells with acetylcholine.

Besides unicellular microspores, the long-lengthed pistil of *Hippeastrum hybridum* which the stigma autofluorescence (maxima in the fluorescence spectra and the emission intensity) is sensitive to acetylcholine and histamine as well as their antagonists d-Tubocurarine and azulene [8, 24] may be suitable biosensor for the study of chemosignalling.
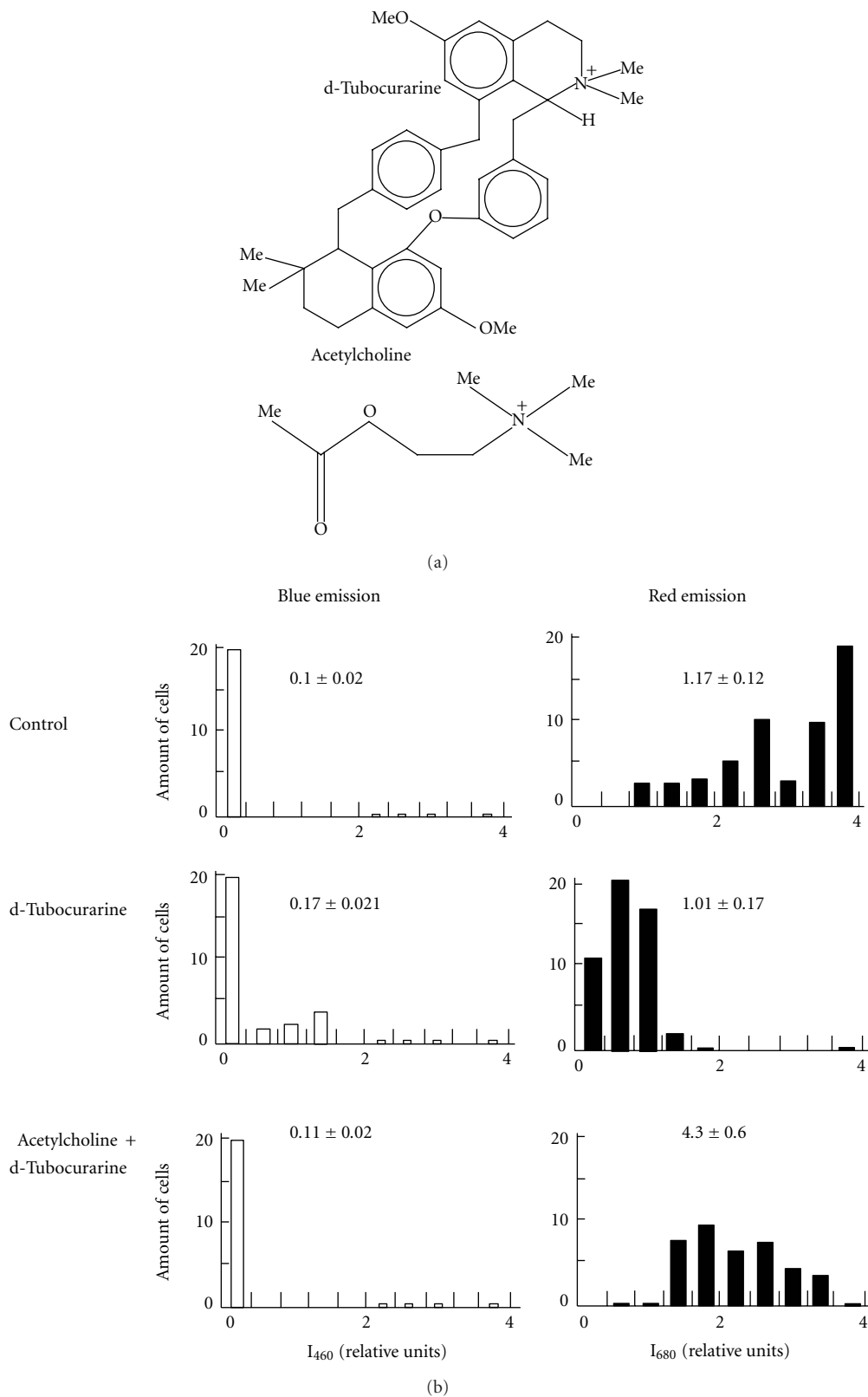
(a)

(b)

FIGURE 4: Summed distribution histograms of blue (maximum 460 nm) fluorescence intensity of vegetative microspores of horsetail *Equisetum arvense* before and after the treatment with components of plant secretions during 24 h. Excitation 360–380 nm. Total amount of studied cells in a variant = 100. Summed values for 100 analyzed cells are also given.

5.3. Potentials for a Search of New Biosensors. Among potential biosensors may be multicellular systems having autofluorescence. Similar biosensor based on petal fluorescence in flowers such as *Mirabilis jalapa* may be also applied to analysis of chemosignalling dealt with certain pigments-fluorophores [60]. The visible fluorescence emitted by one pigment, a yellow betaxanthin, is absorbed by another, a violet betacyanin, to create a contrasting fluorescent pattern on the flower's petals for pollinators. The analysis of free radical processes with the participation of the pigments [76] opened new horizons to search biosensors among pigmented plant cells.

Perspective biosensors today appears to be mycorrhizal roots of plants sensitive to a colonization of arbuscular mycorrhizal fungal structures (such as hyphae, vesicles and spores) that brightly autofluorescence among root tissues under ultraviolet, blue or green light excitation [76–78]. Whole roots of lucerne *Medicago sativa* may be suitable for the same aim.
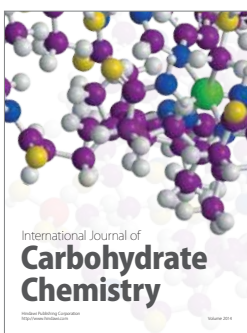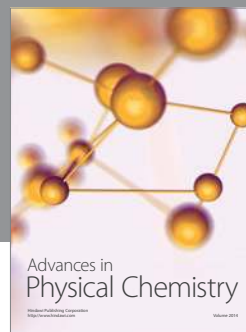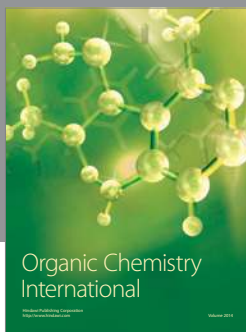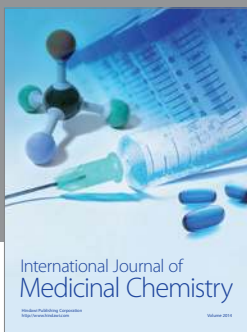
6. Conclusion

Autofluorescence of living cells may serve as biosensor and bioindicator reaction for various studies of plants–from taxonomic position of plant or pollen viability to the chemosignalling processes. This reaction is analyzed by following ways, namely: (1) on undamaged structures, if their surface composed of fluorescent components or interior of the structures filled of fluorescent secretion; (2) modeling with individual cellular components showing the picture of the fluorescing cellular structure observed. The autofluorescent structure images are studied in a dependence on a purpose either for anatomic characteristics applying to taxonomic sign in plant systematics or for a comparison of samples in the control variants and those that exposed under someone factor. Moreover, optical slices made with laser-scanning confocal microscopy permit to show a location of fluorescing secondary metabolite naturally synthesized within the cell or exometabolite interacted with the cell at cell-cell communications or at experimental addition of the chemical agent. Besides images, the fluorescence spectra of emitted structures may be recorded with microspectrofluorimetry or certain laser-scanning confocal microscopes. Measuring the absorbance and fluorescence spectra there is a possibility to estimate quantitative yield of fluorescence not only for individual fluorophore, but also for certain cells, such as flower petals.

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