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Spectral Analysis for the Search of Azulenes in Plant Microobjects on Different Evolution Steps

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Abstract

The spectral study of the microobjects such as a surface of single cells and isolated organelles from azulene-containing plants of Russia was carried out. The presence of azulenes in diatom *Ulnaria ulna* Ulnara, lichen *Parmelia sulcata Taylor.,* vegetative microspores of horsetail *Equisetum arvense* L. and microspores of fern *Asplenium scolopendrium* L. as pollens - male gametophytes from pine *Pinus sylvestris* L. (Family Pinaceae) and common juniper *Juniperus communis* L. (Family Cupressaceae) as well as *Clivia miniata* (Lindl.) Regel. (Family Amaryllidaceae) and *Tulipa sylvestris L*. hybrid form (Family Liliaceae) was detected by microspectrophotometry. This was assessed by the appearance of blue color and characteristic maxima in the region of 580 - 640 nm in the absorption spectra and 405 - 430 nm in the fluorescence spectra. These data were confirmed in experiments on usual spectrophotometry with extracts of these pigments with acetone or ethanol solvents, respectively, from the surface of microobjects and from the inside (after infusion of microobjects for 10 minutes or 24h). The results obtained may be of interest for cellular monitoring of azulene-containing plants species useful in the future for pharmacology.

***Keywords**: Absorbance; Azulenes; Cell Wall; Fluorescence; Microspectrophotometry; Spectrophotometry*

Introduction

Recently blue pigments azulenes have been found in plant leaves with blue or silver colour [1-6]. These compounds are used in cosmetics and for medical purposes [6-8], and therefore the search for azulene-containing plant objects and the study of their cells is of great interest to the pharmaceutical industry. The chemical nature of azulenes, which have numerous double bonds, gives them their antioxidant properties that explain their therapeutic [6-8] and preventive effects, such as increasing cell viability and protective role against the adverse effects of ultraviolet radiation and ground-level ozone [10,11]. They could convert external chemical signals and perform a protective function against the damaging effect of active oxygen forms. The appearance of azulenes in plants are connected with the geography and ecological peculiarities [4-6,12].

Earlier, main pharmacological method to search azulenes based on the distillation of essential oils from raw plant material [7-9]. Among suitable methods, new application of the microspectrophotometry and other spectral methods, unlike pharmacological approach, permit to do the compounds' discovery in intact plant leaves via the receiving of their absorbance spectra with characteristic maxima 580 - 640 nm from the surface of the photosynthesing objects [4-6,12]. The method data were supported by the extraction of the blue pigments and the analysis of the spectral absorption of their extracts. In most cases maxima peculiar to azulenes were found in many plant species analyzed

[1,2,4-6,12]. In some cases, the azulene fraction was purified from chlorophyll by thin-layer and column chromatography based on silica gel [12,13]. The above-mentioned approach to search azulenes was applied to higher plant multicellular structures, concentrating on the leaf azulenes in Gymnosperms and Angiosperms. It was not considered yet for microscopic objects such as single photosynthesizing cells of microorganisms and plant microspores. Earlier there were first several publications where the presence of azulenes in pollen [13] and spores [14], using fluorescent methods were studied. The functions of blue pigments azulenes for the plants themselves have begun to study at2020 year [1,15]. It has been shown that they can react with reactive oxygen species and biogenic amines [16] and act as electron donors in the electron transport chain of photosynthesis in model systems [15,16]. This last feature is particularly interesting because azulenes have been found in isolated chloroplasts of legume plants such as *Pisum sativum* L., *Trifolium repens* L. [17,18], *T. pratense* L., and *T. medium* L. [18].

Basing on the important data, the question arises: are azulenes in unicellular plant organisms or not, and when they may appear in evolution.

Aim of the Study

The aim of this work is the use of spectral methods (microspectrophotometry and usual spectrophotometry) to study the presence of azulenes in various photosynthesing single cells of species standing on the different stage of evolution - from diatoms, lichens, microspores of horsetails and ferns up to pollen of Gymnosperms and Angiosperms.

Purpose of the Study

The purpose of the paper is an application of the spectral analysis, including microspectrophotometry and usual standard spectrophotometry, for the search of blue pigments azulenes in intact microscopic cells and extracts from them among various plant species - diatom algae, lichen, horsetail, ferns and pollen of Gymnosperms and Angiosperms.

Materials and Methods

Objects: The list of analyzed species included: 1. diatoms *Ulnaria ulna* (Nitzsch.) Compere (Diatomeae or Bacillariophyceae) received originally from Karadag biostation of the Kovalevsky Institute of south seas (Feodosia, Crimea) and cultivation in our laboratory of intracellular signaling of the Institute of Cell Biophysics RAS in Pushchino, Moscow Region as described earlier [19,20]; 2. blue-color thallus of lichen *Parmelia sulcata* Taylor. (Family Parmeliaceae) from the forest near Pushchino, Moscow region; 3. blue-green colored vegetative microspores of horsetail *Equisetum arvense* L. (Family Equisetaceae) were collected in Pushchino coast of Oka river in Spring 2024-2025 years; 4. matured plant leaves of fern spleenwort *Asplenium scolopendrium* L. (Family Polypodiaceae) with soruses and microspores were given in Lazarevskaya coast, Sochi region, Caucasus, Russia by our Specialist Alexander Kunyev; 5-6. Pollen of Gymnosperms common juniper *Juniperus communis* L. and pine *Pinus sylvestris* L. (Family Cupressaceae) with a noticeable blue leaf color and were selected as research objects for cellular analysis in Pushchino state reserve; 7. Pollen of Angiosperms *Clivia miniata* (Lindl.) Regel (Family Amaryllidaceae) and *Tulipa sylvestris L*. hybrid form (Family Liliaceae) were grown at room conditions.

Spectral methods: The absorbance spectra or the fluorescence spectra of intact cells and isolated organelles were measured directly on glass slides using microspectrophotometer/microfluorimeter MSF-15 (LOMO, Sankt-Peterburg, Russia) [5,11,18,21]. The position of the maxima in the absorption spectra of intact cell surfaces, recorded using the MSF-15 microspectrophotometer, was determined according to the option of differentiating the reflection spectra according to Zolotaryov method for low-intensity bands, which is built into the instrument's software [22]. As described earlier and presented as the first derivative of the external reflection spectrum in our previous work [12,16], the upper part of the first derivative graph with positive values allows for the direct determination of the position of the absorption band maximum (Figure 1). The absorption and fluorescence spectra of the extracts from intact objects were recorded

in 1-0.5 cm cuvettes, using the Specord M-40 spectrophotometer (K. Zeiss, Germany) and Perkin-Elmer 350 MPF-44B spectrofluorimeter (UK), respectively. Fluorescence excitation was 360-380 or 360 nm. Azulenes in the ethanol and acetone extracts of the samples were determined spectrophotometrically at 580 nm [1,2,12,16], considering their content in 10 minutes and 24h. Images of cells were observed and photographied under above-mentioned MCF-15 microspectrophotometer/microspectrofluorometer or laser-scanning confocal microscope Leica-TCS SP-5 (Wetzlar, Germany).

Extracts from plants: Extracts from the surface of intact plants with 100% acetone or 95% ethanol from cells (1 : 10 wt/vol) were obtained within 5 - 10 minutes, when the chlorophyll release was still absent or minimal [1]. After a longer extraction period of 24h, the samples contained azulenes not only on the surface but also inside the cells. Fluorescence with maxima in the region 400 - 430 nm was used for extracts as an additional confirmation of the presence of azulenes in the extracts [1,12]. The violation of the Stokes law (short wavelength emission instead long wavelength peak as usual case), which is characteristic of all azulenes, was observed for conformation of azulene parameter.

Chromatography*:* To identify azulene bands, the extracts were chromatographed on Silufol thin-layer plates, as described earlier for pollen from various seed plants and microspores from horsetail [17,18]. After separation from chlorophyll, for the blue bands of azulenes, the retention factor Rf of the individual substance, which characterizes its position on the chromatogram, was calculated. Three to four repetitions of chromatography were performed for each plant species, and the average Rf value was compared with the position of azulene synthesized by Fluka as a quality control. In all cases, the Rf value was from 0.92-0.94 or to 0.80-0.82. Then, azulene bands separated from chlorophyll were cut out, extracted in acetone or ethanol, and the absorption and fluorescence spectra of the extracts were recorded.

Results and Discussion

Analysis of the microobject surface for the search of the azulenes' presence was beginning after the visual estimation cells by usual and fluorescent microscopy included the following steps: (1) Record of the absorbance spectra of intact cells (single cells, microspores, leaves by microspectrophotometer); (2) Extraction of the blue pigments with 95% ethanol or acetone from cells for 10 minutes (from the surface according to [1,16]) or 24h (all content of compounds); (3) Record the absorbance and fluorescence spectra of the obtained extracts; (4) The determination of the azulene concentrations in the extracts according to maxima 580 in the absorption spectra [1]. The way of the experiments includes two directions for analysis with microspectrophotometry of intact cells and usual spectrophotometry of the extracts from the objects. Further, the azulene fraction may be purified from chlorophyll like it was described earlier for leaves of some plants [2,6,16]. But here the task was not for the direction of the present paper.

Diatom: Unicellular algae *Ulnaria ulna* characterized by the presence of frustule, rigid silicified cell wall (included silicium) that is a pillbox-like shell composed of overlapping halves (epitheca and hypotheca) perforated by intricate and delicate patterns. The microspectrophotometry technique at the first stage of research allows one to quickly identify various azulene peaks in the absorption spectra (first derivative of the reflection spectra) of intact cell (Figure 1a). This spectrum is first derivative of the reflection spectra according to Zolotarev method for small maximum 617 - 618 nm in the spectrum is related to the region of the absorbance of azulenes. Following stage of the experiment includes the extraction of the azulenes from cells. For this aim the two probes, each equal 2 ml of culture, containing silica gel that necessary to the frustule [19,20], were air-dried in the 2 glass vessels - one for 10 minutes extraction and another - for 24h extraction with ethanol or acetone. The silica gel adsorbs cells of the diatoms (approximately 200 cells in probes controlled as red cell fluorescence under luminescence regime of microscope in figure 1c) and permits to treat by solvent for the receiving of extract (By ethanol in figure 1b) from the air- sample. One could see weak maximum in the region 620 nm in 10 minutes extract (black curve) and the absence of chlorophyll maximum 666 nm that may characterized the surface azulenes. More visible maximum 605 nm was in 24h extract (red curve) and with chlorophyll peak here. In the fluorescence spectra of 24h extract was the emission region of 405 - 430 nm, non-illustrated), characteristic for azulenes [1,12] that is anti-stocks effect. Peculiar to azulenes.

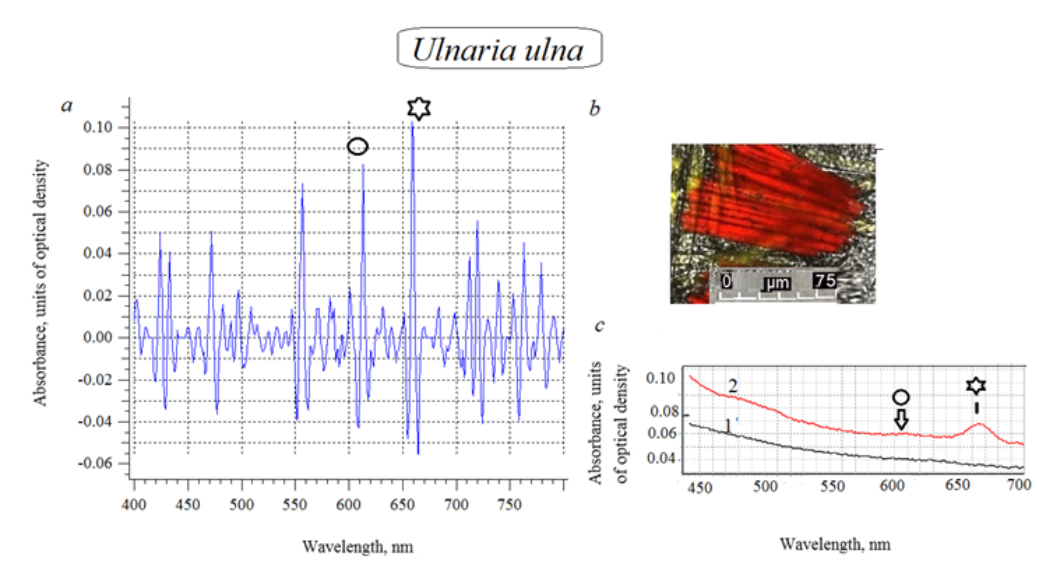

Figure 1: *Spectral analysis of diatom Ulnaria ulna. a- Differential absorbance spectra (First derivative of the reflection spectra) of whole cells recorded by microspectrophotometer. The circles indicate the azulene maxima, and the asterisks - the chlorophyll maxima; b- The image of the fluorescing diatoms population under luminescence microscope of microspectrofluorometer at the excitation 430 nm (Red-fluorescing photosynthesizing cells were seen); c- The absorbance spectra of the 1 ml ethanolic extract from the probe with 500 cells, where (1- Black line for 10 minutes extraction, while 2- Red line for 24h, the arrow shows characteristic peak of azulenes).*

Lichen: An example of the azulene search for lichen is shown in figure 2 for *Parmelia sulcata*. The absorption region of the intact thallus peculiar to azulenes included three maxima 590, 595 and longest 610 nm (Figure 2a). This correlated with blue color of thallus (Figure 2b). In 10 minutes and 24h extracts with ethanol were visual blue color of thallus and its maximum 610 nm (Figure 2b). In 10 minutes and 24h extracts with ethanol were visual maxima 610 nm, smaller in first case as seen on Y-scale. It may be estimation of the azulenes both on the cell surface and within as it has been shown earlier for blue leaves from species of Gymnosperms and Angiosperms [4-6]. Blue color was correlated with the presence of azulenes.

Horsetail: Spectral analysis of horsetail *Equisetum arvense* vegetative microspores was carried out according to above-described scheme (Figure 3). There were seen main two maxima 580 and 610 nm on the surface absorbance spectra of intact microspore served for breeding of the spore -bearing species. But in dependence on the probe given in various spring days (during one-two weeks), one can see more peaks, related to azulenes, in the addition. It may be also due to phase of the non-developed or developing microspores (Figure 3b, 1 and 2). In the 10 minutes extracts by ethanol (non-illustrated), we could not observe the azulene-linked maxima. Main pigments with

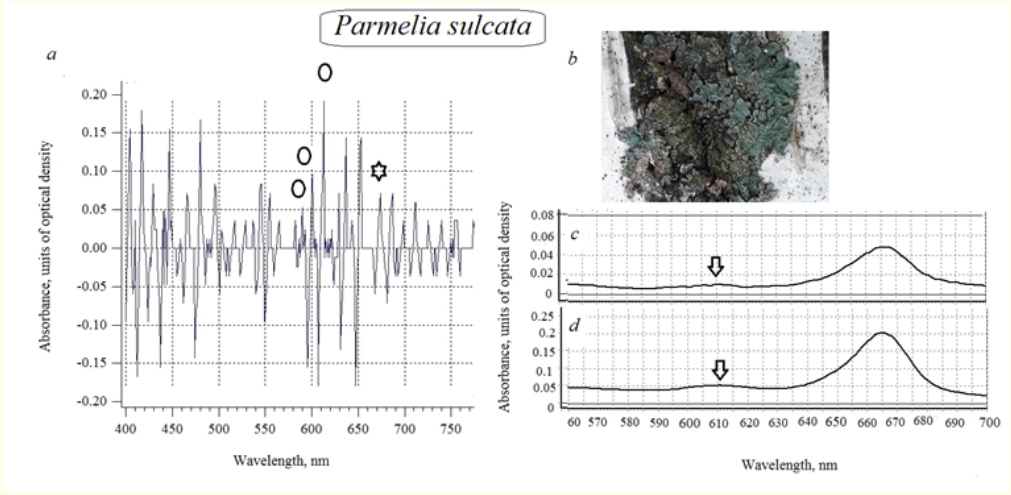

Figure 2: *Spectral analysis of lichen Parmelia sulcata. a- Differential absorbance spectra (First derivative of the reflection spectra) of whole thallus cells recorded by microspectrophotometer; b- Image of thallus; c- The absorbance spectra of the 10 minutes ethanolic extract from the probe for 10 minutes extraction, d- For 24h extraction). The circles indicate the azulene maxima, and the asterisks indicate the chlorophyll maxima, while the arrows show the maxima of azulenes.*

maximum 610 - 615 nm in the absorption spectrum and 410 nm in the fluorescence spectrum, characteristic for azulenes [4-6], were seen. 24h-extracts (Figure 3c) showed maximum 610 nm, peculiar to phenyl azulenes [23]. Anti-Stocks effect was seen in the fluorescence spectra of the extract (Figure 3d) maximum 405 - 410 nm instead maxima after fluorescence 620 - 630 nm.

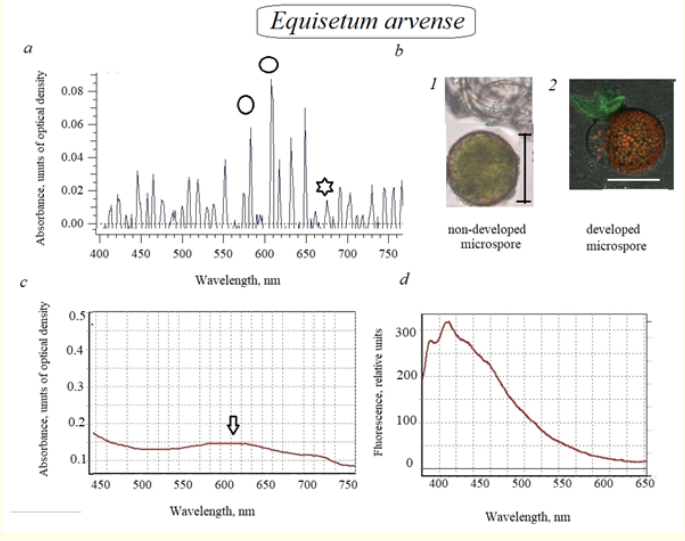

Figure 3: *Spectral analysis of microspores from horsetail Equisetum arvense. a- Differential absorbance spectrum (first derivative of the reflection spectra) of whole cells recorded by microspectrophotometer (the circles indicate the azulene maxima, and the asterisks indicate the chlorophyll maximum); b- Images of microspore in transmitted cell of usual microscope with elaters (1) and at excitation 430 nm laser of confocal microscope (2) seen liberated green-lightening cover and red fluorescing chloroplasts; c- The absorbance spectra of the 1 ml ethanol extract for 10 min extraction; d- The fluorescence spectra of the extract.*

Fern: Microspectrophotometry was applied to record the absorbance spectra from of the soruses - yellow sporangium's with microspores (Figure 4a-4c) on the leaf surface of the fern *Asplenium scolopendrium* L. and individual microspores served for the fern breeding. Soruses (a) Filled by microspores (Well-seen under luminescence microscope as b and c images) served for non-sexual stage of the fern development. For the soruses in the spectra there is maximum 593 nm (surface of thick external cover of the sporangium-Figure 4d), while for individual microspore - 610 nm (Figure 4e), peculiar to phenyl azulenes [23]. Last maximum was seen both for 10 minutes and 24h ethanolic extracts (Figure 4f and 4g). Anti-Stocks effect also was in the fluorescence spectra of the extract maximum 405 - 410 nm (non-illustrated).

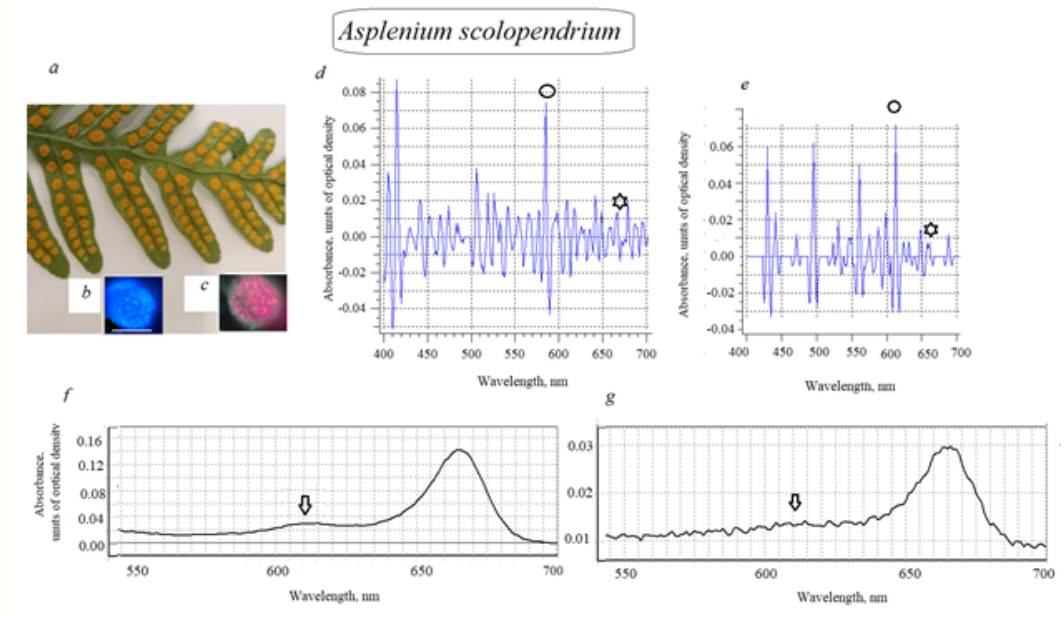

Figure 4: *Spectral analysis of fern Asplenium scolopendrium. a- Leaf with yellow sores filled by microspores; b and c - Fluorescing sorus with spores within at excitation with light 360 and 430 nm, respectively; d and e - The absorbance spectra of the surfaces of sorus and microspore, relatively; f and g- 10 minutes and 24h- extracts from microspores.*

The absorption spectra of the part of intact leaf out soruses have no the azulenes' maxima (non-illustrated).

Pollen as microspores of Gymnosperms and Angiosperms. Pollens are male microspores (male gametophyte) playing important role in the sexual breading. Seed-bearing plants have stored the elements of microsporia in the form of pollen. In this paper we pay attention to pollen as a following step of microsporia in evolution. Twenty years ago, using chromatography, azulenes were found in pollens [13]. This fact was linked with the phase of the maturation of pollen when it is ready to fertilize [24]. Our last investigations of azulenes were linked with the phenomenon of blue color in leaves of Gymnosperms and Angiosperms [1-6,21]. In the firsts, blue color may be met here constantly as in some species related to families Pinaceae and Cupressaceae [1,5,21] or may be dependent on the season [4-6]. In most cases "blue phenomenon" of leaves was linked with the presence of azulenes.

In the present work we have chosen pollen of Gymnosperms such as *Juniperus communis* and *Pinus sylvestris*, while pollens of Angiosperms were represented by yellow pollen of *Clivia miniata* (Lindl.) Regel and black pollen of *Tulipa sylvestris* L. (hybrid form). In both types of species there were azulenes in leaves.

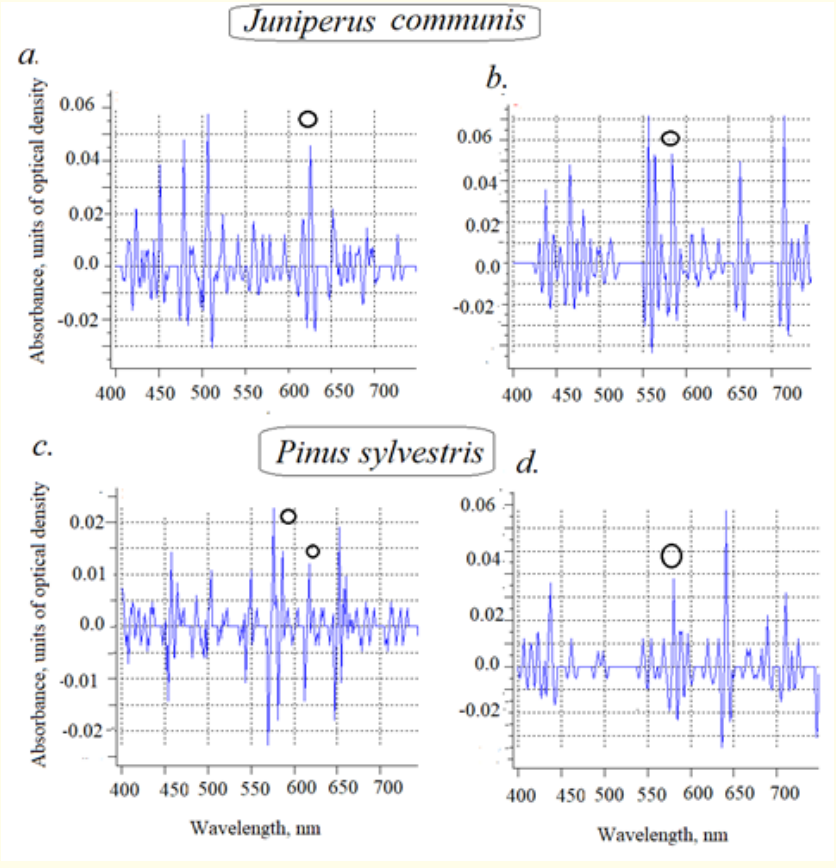

Figure 5: *Spectral analysis of pollen (a, c) and needle - leaf (b, d) of Gymnosperms Juniperus communis and Pinus sylvestris. a- Differential absorbance spectrum (first derivative of the reflection spectra) of whole cells recorded by microspectrophotometer (the circles indicate the azulene maxima).*

In figure 5 one can see spectral analysis of intact pollen and needles with microspectrophotometer and may to compere the absorption spectra a- leaf with yellow sores filled by microspores; pollen from Gymnosperm plant species *Juniperus communis* and *Pinus sylvestris.* Both species had maxima, peculiar to azulenes. The maxima of azulenes were also observed in the extracts by ethanol. They differed in the pollen and needle. The clear maxima were summarized in following section in table 1.

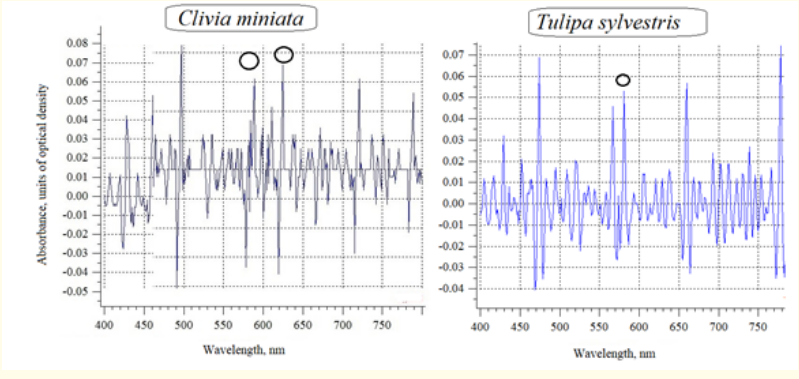

Figure 6: *Spectral analysis of pollen from Angiosperm plant species Clivia miniata Fa- differential absorbance spectrum (First derivative of the reflection spectra) of whole cells recorded by microspectrophotometer*

Figure 6 demonstrated the search of azulene maximum in intact pollens of Angiosperm species *Clivia miniata* and *Tulipa sylvestris*. *Clivia* had two maxima in the absorption spectra the 580 and 610 nm, characteristic for azulenes, while *Tulipa*-one maximum 580 nm. We also confirmed the presence of azulenes by the receiving of the ethanolic extracts with similar maxima in the absorbance spectra and calculated the concentrations of the pigments (see table 1 in following section).

In all studied species there were the azulene fractions, after thin-layer chromatography on silica gel we have their blue bands with Rf 0.90-0.94 from with peaks 580 nm. Among the species only in *Tulipa sylvestris* one can see not only one blue band, but one rose band with Rf 0.82 is belonging to the pigment anthocyanin. It shows that azulenes may be synthesized simultaneously with anthocyanins, but unlike them, do not change their blue color in acid medium [23].

The role of azulenes in pollen was related to possible defence from reactive oxygen species and linked with the maturity of the male gametophyte and capability to fertilize [24]. For example, in some species like *Matricaria chamomilla* and self-compatible clone of *Petunia hybrida* azulenes were found in matured pollen and were absent in non-matured and self-incompatible clone, relatively [24]. The synthetic azulene and proazulenes added to pollen *Hippeastrum hybridum* prevented the damages induced by ionizing radiation [25]. Analogous cause may be for more ancient plant cells as microspores of horsetails and ferns that needs a special study like experiments with microspores 'vitality [26].

Comparison of the azulenes maxima and concentration of azulenes in studied objects

We did the estimation of the spectral characteristics and concentrations of azulenic fractions in the species studied after the analysis of individual objects and methodical demonstration of similar calculations of the azulene concentrations for species studied [1,4-6]. Table 1 shows the maxima of the azulenes in intact objects and ratios between the surface and internal concentrations of azulenes. At the beginning of the evolution single organism such as diatom *Ulnaria ulna* already had maximum in the absorption spectra related to azulenes in range 580 - 630 nm. The presence of the pigments has been supported by the data with ethanolic extracts from the objects' studies. Moreover, it was able to compere the concentrations of the compounds on the surface (10 minutes extracts) and within the cells (Concentration in 24h extract minus the amount in 10 minutes extract). In *Ulnaria ulna,* the pigment and concentration within was smaller, than on the surface. In lichen *Parmelia sulcata* the picture changed. In the cell interior it became 2 - 5 times higher. Moreover, lichen as organism contained the fungus and algae already is seen as thallus of blue color. Similar tendency to have more blue pigment within a cell was observed for horsetail *Equisetum arvense.* It should mark that concentration of azulenes on the cellular surface for some species may be connected with their participation in sensory reactions of allelopathic and medicinal species [27]. In the all three types of the organisms the intact cell demonstrated in the absorption spectra maxima in the region with optical density at 615 - 619 nm.

Plant species	Wavelength, nm	Optical density	Concentration of azulenes, mg /g fresh mass		
			10 minutes	24 minutes	24h - 10 minutes amount
Ulnaria ulna	617	0.0546	0.0167 ± 0.004	0.0269 ± 0.006	0.0100 ± 0.002 © and ®.
Parmelia sulcata thallus	588 590 610 615	0.0598 0.0598 0.0747 0.1911	48.7 ± 4.0	243 ± 8.0	195 ± 3.7 © < ®.
Equisetum arvense microspore	583 590 598 619	0.0508 0.0516 0.0579 0.0824	80 ± 2	256 ± 10	176 ± 4.0 © < ®).

Asplenium scolopendrium L., dried spore sporangium	606 613 593 616	0.0568 0.0717 0.0679 0.597	0.406 ± 0.035 11.7 ± .1.3	0.233 ± 0.061 2.2 ± 0.09	-0.123© > ® -9.5© > ®
Juniperus communis pollen	589-590 584	0.0627 0.0520	2.1 ± 0.2	44.1 ± 5	42 ± 1.2 © < ®
Pinus sylvestris pollen	580	0.0598	30.6 ± 3.2	70 ± 1.9	40 ± 2.9© < ®
Clivia miniata pollen	583 589-590	0.0512 0.1554	81 ± 6.1	131 ± 7.3	50 ± 4© > ®
Tulipa sylvestris pollen	610 620	0.05 0.08	142 ± 7	286 ± 11	144 ± 9© =®

Table 1: *Presence of azulenes in cells of plant organisms on various steps of evolution. Vertical values for maxima recorded for intact cell is equal or higher 0.0500 units of optical density as a limit for our microspectrophotometer sensitivity. Main blue pigment is concentrated -on the surface© or within cell ® or is present in both parts © and ® (© > ® or © < ®).*

Following evolution step in fern supported similar peaks both to for the sporangium and microspores, while the surface of microspores concentrated almost all azulenes unlike the cell interior, which lack it. This situation is similar for the sporangium surface, though it should mark that the azulene concentration mainly in cellular surface in much smaller concentrations at all.

In the plant kingdom hierarchy, the microsporia transformed to pollens (Male microspores or male gametophyte) both in Gymnosperms and Angiosperms. In the first ones, are *Juniperus communis* known as one of most species *Juniperus* azulene- containing plant in genus with blue needles [21], and pine *Pinus sylvestris* (Family Cupressaceae). Second group of species were represented with yellow pollen of *Clivia miniata* (Family Amaryllidaceae) and dark-blue pollen *Tulipa sylvestris* (Family Liliaceae). All pollens had maxima in the absorption spectra in the region 580 - 640 nm. Azulenes were founded in all pollen of 4 species. Ratio between the surface and internal concentrations varied, but often pigments presented in both 10 minutes and 24h extracts.

The data presented in table 1 are consistent with the earlier publications concerning seasonal concentrations of azulenes [4-6] and it is seen that in the surface layer of cells of the studied plant species azulenes are present in different concentrations. In most cases azulenes are also found inside the cells. But there are also exceptions, for example, ivy *Hedera helix* and rock rose *Cistus tauricus* [5]. In these species there are practically no azulenes inside the cells, the main part of the blue pigments is concentrated on the surface. Azulene concentrations on the surface and inside cells are approximately equal in temperate climate zone plants such as *Anthriscus sylvestris, Centaurea phrygia*, and *Saponaria officinalis*. At the same time, the olive *Olea europea* growing in the Crimea and the *Hylotelephium maximum* plant of Moscow region had a significantly higher amount of azulenes inside the cells, than on the surface [4,5]. As seen from the data received, the application of the spectral methods have perspectives for the testing of the azulenes' presence in Nature conditions without pharmacological technique of oil distillation.

Conclusion

The use of spectral methods - microspectrophotometry and standard spectrophotometry permitted to apply them for the analysis of the presence of blue pigment azulenes in microspores of the organisms on the evolution tree - from unicellular diatom to multicellular organisms, able to bread with various type of exomicrosporia. Basing on the work data, it has been supposed azulenes to be antioxidants, necessary for many organisms for a defense from reactive oxygen forms.

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Ethic Approval and Consent to Participate

This work does not describe any studies involving humans or animals as subjects.

Conflict of Interest

The author of this work declare that she have no conflict of interest.

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