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Possible Role of Azulenes in Plant Life: Experiments with Models

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Abstract

Possible role of blue natural azulenes in plant life has been studied on two models. First model was bluish-silver leaves of silver dollar *Eucalyptus cinerea* F. Muell. ex. Benth (fam. Myrtaceae) which serves for microscopic analysis of the surface by transmitted microscope, luminescence and confocal microscopes as well microspectrometer/ microfluorimeter. The microscanning of the surface by the technique permitted to see azulene-containing wax plates, covered leaf. The presence of azulenes also showed the absorbance spectra of the surface with characteristic maximum 580 and 607 nm, which earlier has been seen in the extracts of blue pigments by ethanol or acetone. Their emission was seen in blue in laser-scanning confocal microscope and similar with known for artificial base azulene. Second model- isolated chloroplasts from leaves of *Kalanchoe pinnata* (Lam.) Pers. (fam. Crassulaceae) with small content of chlorophyll and lack of natural azulenes– was used for experiments for effects of exogenous synthetic base azulene on photochemical activity because some blue pigments earlier were found in chloroplasts of some species of Fabaceae (Leguminosae). Photoreduction of $NADP^+$, potassium ferricyanide and dichlorophenol indophenol with or without inhibitors diuron or antimycin A in the reaction medium stimulated with azulene. According redox-potential it may be donor of electrons in electron transport chain at the level of cytochrome f-plastocyanin. Blue pigment missed color at the interaction with these isolated electron carriers. Both models have demonstrated that azulenes being antioxidants may be defensive components out cell (on the surface) and within cell (in chloroplasts).

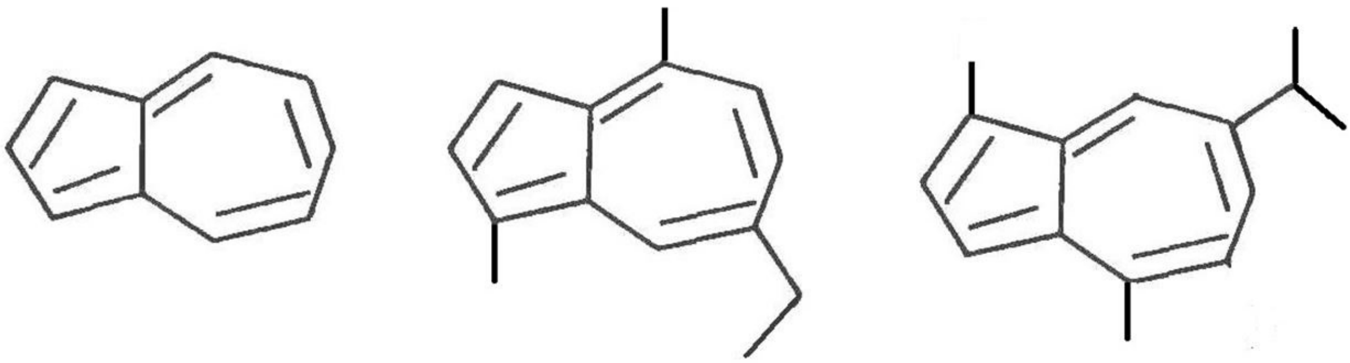
Keywords: Absorbance; Autofluorescence; Cell Surface; Chloroplasts; *Eucalyptus Cinerea; Kalanchoe Pinnata;* Laser-Scanning Confocal Microscopy; Luminescence Microscopy; Microspectrophotometry; Photochemical Activity

Introduction

If you see blue or bluish image of leaves in herbs or in many woody species, the question arose: what pigment does it? The attention to this problem connected with plant ecology was far from the study many years, although this pigment is known and applied for medicine, cosmetics and technics.

The blue pigment azulene is a natural substance that functions in many types of plants, fungi, and invertebrate organisms. Originally, azulene-containing plants have been used since ancient times for medical and cosmetic purposes. However, the presence of the pigment itself has been discovered in the 15th century in extracts of the German chamomile herb. Azulenes did not attract the attention of chemists until 1863, when the French perfumer Septimus Piesse became interested in the plant, studying the azure product of the distillation of chamomile, and called the Spanish word "azul", which means blue. By the end of the 19th century, scientists discovered azulene in other plants, including yarrow and wormwood. Later, in 1934-1937, Lavoslav Ružička established the chemical structure of this compound and studied its characteristics, and Placid Plattner synthesized it. In the post-war period, azulenes became of interest to chemists as seen in reviews [1,2]. Since about the second half of the twentieth century, azulene has already been used in skin care products and some cosmetics, as it is considered a useful ingredient due to its bright blue color and healing properties. Various medicinal plants contain azulene that is usually found during the oil distillation of plant raw materials. Familiar plants to us: chamomile, wormwood, St. John's wort, valerian, tansy, etc. may use as the origins of the blue pigment [1-4].

Blue azulene is a monoterpene and isomer of white crystalline solid naphthalene [5,6]. Main types of natural azulenes are shown on Figure 1. Their derivatives originate from this base samples.



Azulene Chamazulene Gujazulene

Figure 1: Main types of natural azulenes

Azulene consists of a fused cyclopentadiene ring and a cycloheptatryene ring and has an electronic system of 10 pi, like cyclodecapentaene. The dipolar nature of the ground state is reflected in its deep color, which is unusual for small unsaturated aromatic compounds [7]. Another notable feature of azulene is that it violates the Kashi rule by exhibiting fluorescence from the upper excited state (S_2 → So). This pigment has a high basicity, and therefore, when mineral acids or aprotonic acids act, salts are formed. On this property of azulenes, the methods of their isolation are based. Since the azulene molecule consists of two components - a five-member and a seven-member, it has special characteristics. The five-membered part of the molecule carries a small negative charge, and the seven-membered part carries a positive charge, as a result of which it has a dipole moment. The first part of the 5-member cycle has the ease of electronic substitution, dissolving in 60% sulfuric or 85% phosphoric acid H_3PO_4. Azulenes are oxidized to naphthalene in air and in the presence of K_2MnO4 is converted to CO_2.

Among the main areas for study of the azulene properties in the modern world are medical and technological. The first direction is the oldest, since azulene-containing plants have long been used by mankind to treat cosmetic diseases because the blue pigment reduces inflammation in skin tissues. A developing line in research relates to medicine, in addition to cosmetics and personal care products [8]. The technological direction is associated with the study of the physicochemical properties of azulenes and their application in the optical and chemical-analytical industries [9]. For many years, compounds have been studied by physicists and chemists due to their unique physical properties. Azulene derivatives have been synthesized for use in materials as color probes and sensors (including fluorescent ones) or ligands for metallurgy [9] as well as the base for optoelectronic systems. Azulenes are also included in polymers, oligomers and conjugates with furanes and phenols in order to create new chromophore materials [9]. Due to its physicochemical properties, azulene and its derivatives with many double bonds and their conjugated biologically active compounds have found application in technology, especially in optoelectronic devices [10].

Unlike a burst in technological use of azulenes, there is small information about biological effects of azulenes. Natural azulenes and synthesized, such as azulenic retinoids, may have antitumor activity [11] or have an antioxidant effect [12] or affect dopamine receptors [13]. Moreover, in some cases they can affect other living organisms, including negative effects [14]. As part of medicinal herbs, these substances have been widely used for hundreds of years in medical practice, and now they are used in antiallergic, antibacterial and anti-inflammatory therapy [8].

The roles of azulenes for plant itself is unknown yet. If to see where the compounds occur, we may do first steps to understanding of the problem. For many years, chemists discovered azulenes by distilling essential oils from the medicinal herbs of wormwood bitter *Artemisia absinthium* L. or common yarrow *Achillea millefolium* L, which acquired a blue color [2]. But such pigments were also found in extracts of organic solvents in mosses of the hepatic *Salypogeia azurea* and others [15,16], pollen of different flowering species and pollen collected by bees [17,18], cells of horsetail microspores *Equisetum arvense* L. [19], surface cells of the needles of the blue spruce *Picea excelsa* [20], isolated chloroplasts of pea *Pisum sativum* L. and clover *Trifolium repens* L. [20]. It should be noted that the role of azulenes for the plants themselves has not yet been considered in the literature, although there is only one interpretation of their properties as growth regulators belonging to water soluble azulene [21]. In experiments with exogenous azulene on vegetative microspores of horsetail, the properties of this sesquiterpene as a histamine antagonist are shown [22]. Recently it was shown that short time-extracts by organic solvents from leaves having blue color in some woody plants contained azulenes [23]. These species were tolerant to the high acute or chronic influence of tropospheric ozone. The fact gives us the idea that their tolerance may connected with the defensive role of azulenes. Short-time of the azulene appearance in extracts shows a possibility of the compound location on the leaf surface as a barrier or filter for active ultra-violet radiation and ozone as the origins of dangerous oxidants [23,24].

Basing on the data, we had a goal to do model experiments where may see possible significance of azulenes for plants themselves. For this task, it should need to observe the leaf surface and penetrate into the cells of model objects.

Material and Methods

Objects

Analysis of possible role of natural azulenes on the surface and within the cells studied was carried out on two model systems. First model was leaf of *Eucalyptus cinerea* F. Muell (fam. Myrtaceae), which has silver blue surface. In their extracts from whole intact leaves by acetone or ethanol (1:10 weight/v) azulenes have been found [24]. Second model represents isolated chloroplasts from leaves of *Kalanchoe pinnata* (Lam.) Pers. (fam. Crassulaceae), which contain small amount of chlorophyll and lack of azulenes, unlike chloroplasts of pea and clover, where the pigments were observed [20].

Visualization

For the analysis of the plants surface the leaves of *Eucalyptus cinerea* has been chosen as model. The surface in transmitted light was analyzed by Invitro Evos M5000 microscope (Thermo Fisher Scientific, USA and the absorbance spectra of the cells were recorded by microspectrophotometer/microspectrofluorimeter MCF-15 (LOMO, Russia). Autofluorescence of the samples was observed and photografied after excitation in various color rays by Leica DM 6000 B luminescence microscope (USA-Germany-Austria) and laser-scanning confocal microscope Leica TCS SP5 (Germany) as described earlier [25-27].

Isolation of chloroplasts and their photochemical activity

Isolation of chloroplasts from leaves of *Kalanchoe pinnata* (Lam) Per. was carried out according to Robinson with co-workers [28] in the phosphate buffer medium containing 0.3 M mannitol,0.08 M KCl, 0.066 M KH_2PO_4, .0066 M Na_2HPO_4 pH 7.25. Photosynthetic electron transport during 5 minutes was estimated as photoreduction of $NADP^+$($NADP^+$ 0.9 μmoles/ml and pea ferredoxin 0.1 mg /ml), potassium ferricynide and 2,6- Dichlorophenol indophenol (4 x 10^{-2} μmoles) without and with inhibitors of electron transport diuron known as 3-(3,4-dichlorophenyl)-1,1-dimethylurea (10^{-6} M) or antimycin A 10^{-5} M (Serva USA). Actinic light transmitted through the interference filters 550 or 670 nm. Intensity of the light flow was 38 and 53 $kerg.cm^{-2}.c^{-1}$, relatively. Used maximal concentration of azulene (Merk, Austria) – 2 μg/ml. Chlorophyll was determined according to Arnon [29]. Results were expressed as mean ± SEM. The relative standard deviation was 5-6 % (n -3-4 samples of each variant; P + 0.95).

Reagents in experiments; cytochrome f was isolated from *Chlorella* sp. cells according to [30], while ferredoxin, and ferredoxin-NADP-reductase - according to [31,32].

Results

Since in some plant species azulenes may be present on the surface and in chloroplasts within cells, it is possible to analyze them on appropriate models using microscopy and determination of photosynthetic activity, relatively.

Azulenes on the plant surface

Earlier we studied the plant responses to ozone, modelling its negative effects on the individual cells, and showed how the atmospheric gas acts on the leaves washings from woody plants [25,26]. Moreover, under O_3 –action, the color and fluorescence of the surface secretory and non-secretory systems of were changed that was suitable for the express-testing of cell damage. Among the woody species studied, some tolerant to ozone have silver or blue color of leaves and contain azulenes washed by organic solvents from their surface. We concluded that the compounds may be optic and antioxidant filters for action of unfavorable factors such as tropospheric ozone of urbanistic regions or/and ultra-violet irradiation. We did some direct experiments where surface images may be studied by various microscopic technique, keeping in mind data of washings from intact leaves of *Eucalyptus cinerea* F.Muell (fam. Myrtaceae), which has silver blue surface. Azulenes are present here as a by -products of distillated oil [3].

As shown on Figure 2, the leaf surface of *E. cinerea* was studied with various microscopy. In transmitted light of usual regime of luminescence microscope Leica DM 6000 b (Figure 2a), one could see only dark green image with included greenish-yellow multicellular oil glands. Special construction of new RGB-transmitted light of Inviter Evos M5000 microscope permitted to do optical slices (in our experiment 10 slices) of the images through 1 μm and their stack in transmitted light (Figure 2b). The researcher can observe thin film containing blue color wax plates that covered all surface of the leaf, including oil glands seen through the film. Thus, we see that blue pigment lies on the cuticle and cell wall. The surface also fluoresced in blue channel under excitation by laser light of laser-scanning confocal microscope (Figure 2c). In this case, the blue emission was peculiar to both oil glands and the wax film. Azulene artificially connected with cellulose [33] have two maxima of fluorescence 400 and 520 nm. The pigments may be within the film that we see here.

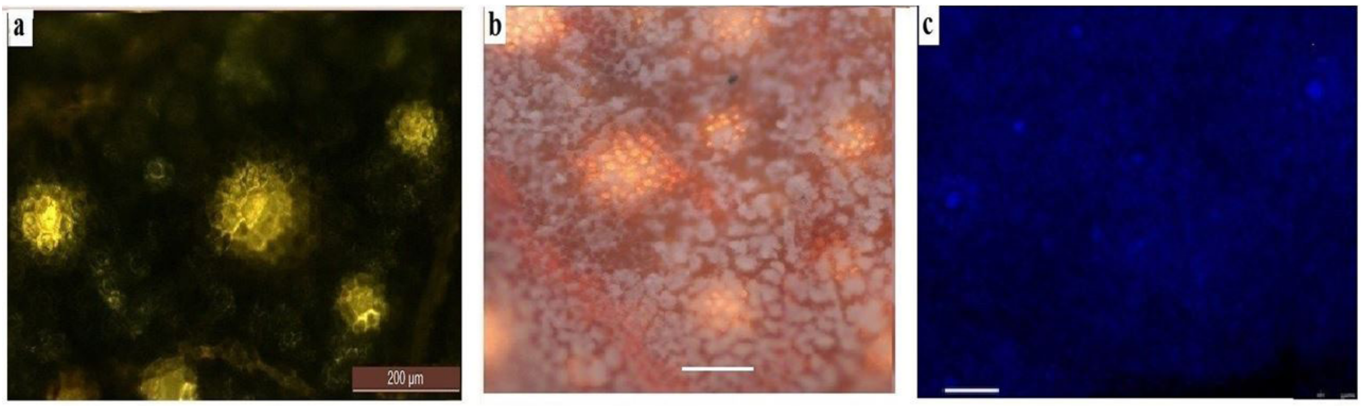


Figure 2: Images of the *Eucalyptus cinerea* leaf surface **(a)** in transmitted light of usual microscope, bar = 200 μm (greenish-yellow oil glands are seen on dark green surface); **(b)** stack of 10 optical slices through 1 μm in transmitted light (RGB-transmitted light of Inviter Evos M5000 microscope), bar = 100 μm (blue wax plates cover all leaf surface, including yellow secretory cells of oil glands); **(c)** autofluorescence excited by laser 458 nm in laser-scanning confocal microscope Leica TCS SP5, image in blue channel, bar = 100 μm (blue fluorescence of both wax plates and oil glands on the surface)

In following experiments, we recorded the absorbance spectra of the leaf surfaces, and one of them is shown on Figure 3a before and after 10 min -extraction of blue pigment with 5 ml 96% ethanol as done in our article [24]. In original spectra (solid line) characteristic maximum 580 nm peculiar to azulenes was seen. This pick disappeared after the pigment extraction with ethanol (broken line). Blue-greenish extract with added sulfur acid up to pH 5 was chromatographied on chromatographic Whatman 1 paper, and the absorbance spectrum most blue band c Rf 0.52 was registered (Figure 3b), although there were more bands (about 4) as described earlier [24]. In the spectrum, we saw several maxima of azulene absorbance at 570-615 nm. Thus, on the leaf surface of Eucalyptus there is the film with azulenes which are possible as antioxidants to defend against ultra-violet radiation and tropospheric ozone which are the origins of reactive oxygen forms damaged cell.

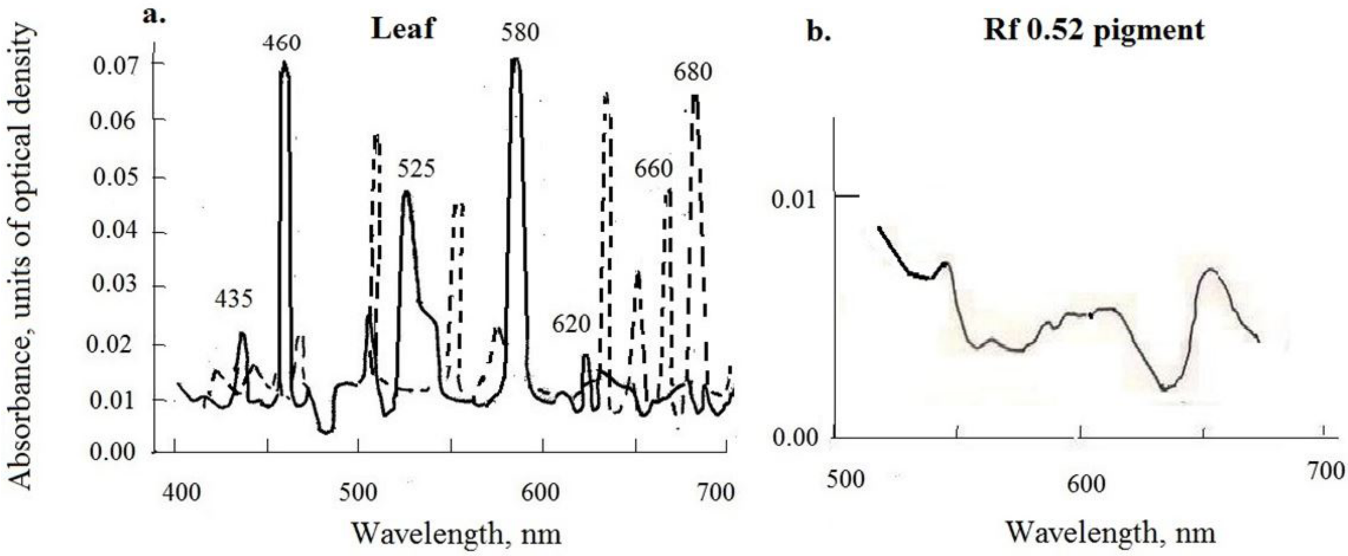


Figure 3: The absorbance spectra of leaf from *E.cinerea* recorded by microspectrophotometer MSF-15; **(a)** Solid or broken lines- before or after the pigment extraction by 96 % ethanol; **(b)** blue band with Rf 0.52 on the papper after the pigment extraction(this extract chromatographed on chromatographic Whatman 1 paper)

Azulenes within cell: Photochemical activity

Earlier it has shown that azulenes accumulated within cell in chloroplasts of clover and pea [20,34]. We could suppose their defensive role like is seen when the compounds lie on the cell surface of cell wall. Second possibility may consist in the influence of azulenes on the photosynthesis through the participation in photochemical activity. The action may be various – from donor/acceptor of electrons to inhibitor and stimulator of electron traffic. Below, a reader may estimate the influence of exogenous azulene on photochemical activity of isolated chloroplasts (Figure 4). Chloroplasts were isolated from leaves of *Kalanchoe pinnata* (Lam.) Pers. (fam. Crassulaceae) which contain small amount of chlorophyll and lack of azulenes unlike chloroplasts of pea and clover where the pigments were observed [20]. The experiments needed to choose the interference filters for actinic light. In classical variants for pea or spinach plastids non-cyclic electron transport was better observed under 650-660 nm, while cyclic transport –under 700-719 nm. However, our practice showed that in our model chloroplasts from *Kalanchoe* photochemical activity with such acceptors as NADP+, potassium ferricyanide and dichlorophenol indopenol was well-seen with filters 555 nm and 670 nm, although data received with 650 nm and 706 nm were similar, but with lesser rates of the reduction of the acceptors. In our work the inhibitors of non-cyclic and cyclic electron transport diuron and antimycin A were used, relatively.

Figure 4 show that azulene stimulated electron transport with all used acceptors more than two times without and with diuron. It is significant because the inhibitor completely blocked non-cyclic transfer of electron. Electron transport enhanced also after inhibition by antimycin A, which decreased cyclic electron traffic. At the excitation by actinic light 670 nm weak non-cyclic transports to NADP+ was observed, there was mainly cyclic transport, but in the conditions azulene stimulated photoreduction of the acceptor.

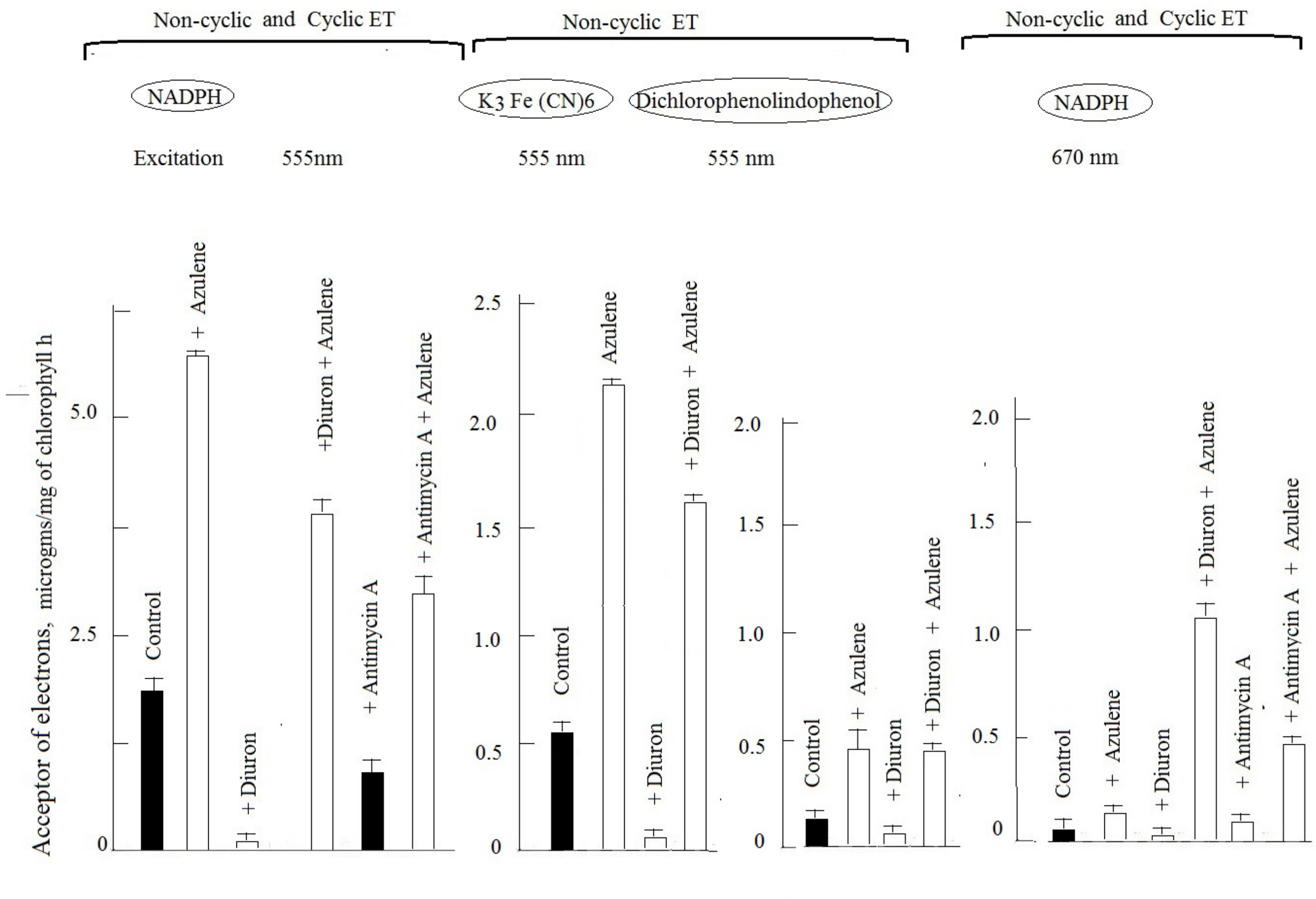


Figure 4: Effects of azulene (final concentration 0.01 mg in 0.5 ml of sample) on the photochemical activity of chloroplasts

Redox characteristics of azulene [33] permit to consider the compound as donor of electrons both in non-cyclic and cyclic transport. According to the works of Plemenkov with co-authors [35], the redox potential of azulene in acetonitrile is + 0.36 V (In cyclic voltammograms of 1, 4-dimethyl-7-ethylazulene taken with a platinum electrode there is a single irreversible oxidation peak Ep = + 0.36 V). The peak current corresponds to the transfer of two electrons per molecule. In the back wave of the voltammogram curves are registered two reduction peaks at the potentials of E p1,red +0.17 and E p2, red = +0.04 V. In our experiments in the electron transport chain during photosynthesis it can be included between cytochrome f (+ 0.36 V) and plastocyanin (+ 0.37 V). Both electron carriers may function in the chains between both photosystems II and I, participating in non-cyclic and cyclic transport. Cytochromes B_6 and B_{559} have no similar possibility due more negative potentials. Some assortment of individual isolated proteins (1 mg/ml of water), which included in the electron transport, was treated by azulene (2 mg/ml of ethanol) on glasses. The blue color of azulene became colorless fast in mixture with cytochrome f (C_{553}) after the missing of electron, weakly it takes place with plastocyanin. Other proteins which have more negative potentials such as ferredoxin and ferredoxin-$NADP^+$-reductase had no effects on azulene blue color. Therefore, the alternative way with azulene is possible. Moreover, it acts as stimulator of NADPH –formation if there is a damage of electron chain or blockade by inhibitors.

Discussion

Analyzing of the data related to the azulenes' occurrence on the cell wall surface we saw bluish plates of wax, which may fluoresce in blue channel excited by laser 405 or 458nm as shown by laser-scanning microscopy. In the absorbance spectrum of the *E.cinerea* leaf recorded by microspectrophotometer/microspectrofluorimeter maximum 580 nm characteristic for most azulenes was observed. This pick disappeared after the ethanol or acetone extraction. In the 15-min extracts azulenes were present [24]. Acute or chronic exposure in ozone had effect mainly on secretory structures – oil gland [24,25], especially well- seen for *Eucalyptus cinerea*. The azulenes in extracts been found in 5-30 min extracts also from the surface of leaves and needles from similar species- *Acacia dealbata, Picea excelsa, Picea pungens, Pinus parviflora, Cedrus atlantica*. In this case, in the short time only small admixture of chlorophyll was extracted, if any. These facts confirm the presence of blue pigments on the surface of cell wall, where they may play the defense role as antioxidants. The pigments became optic filters from ultra-violet irradiation and tropospheric ozone; factors induced formation of reactive oxygen forms such as singlet oxygen, free oxygen radicals, and peroxides. Moreover, they form a barrier from aggressive conquerors (insects, bacteria, viruses). As shown by Japan authors [36] synthesized azulene-related water-soluble compounds can protect cells from UV-induced cytotoxicity. Cytotoxicity of these compounds has been shown against three human normal oral and three human oral cells squamous cell carcinoma cell lines. Synthesized nine water-soluble azulenes demonstrated the anti-UV activity being much higher than that of gallic acid (a component of tannin), epigallocatechin gallate, catalase, curcumin and others. Likely the presence of azulenes on the cellulose cover (exine) in pollen of many species [17] and vegetative microspores of horsetail [19], the pigments play the same role. Azulenes of *Philadelphus grandiflorus, Matricaria chamomilla* and *Tussilago farfara* are accumulated in mature pollen in spring on the certain phase of development [18]. Moreover, self-incompatibility of the pollen in various *Petunia hybrida* clones is connected with the presence of the pigments [18]. In self-incompatible clones, there are only traces of azulenes in the comparison with compatible clones.

The presence of natural azulenes on the leaf surface may have valuable for understanding of tolerance to high UV-irradiation in spring. Although many these questions are not dissolved in experiments, many times we saw blue color of leaves of various species in green-house at high light regimes or herbs of Graminae in spring. When light intensity dropped in artificial or natural conditions, green color prevails, while blue one disappeared. Often in spring red color of anthocyanins in seedlings may be observed. However, in a test for the flavonoids, blue color, different from anthocyanin, was not disappeared under the acid treatment, because it belong to azulenes.

How deeply can azulenes spread into cell wall, it is dependent on size of cuticle and layers of wax, and in its turn - from genetics of the species. In our earlier article [24], we showed longest way of solvent in *Cedrus atlantica* needle- more 1h for extraction of azulenes.The azulenes extracted from ozone-tolerant species were chromatographed on paper and thin-layer silicagel, and several bands of blue pigment were found [24]. Most of them approximately may be related to phenolazulenes [2].

The appearance of chlorophyll in extracts of leaves demonstrates that solvent achieves chloroplasts. What is function of azulenes within a cell, if they are sometimes present in chloroplasts of spring herbs? First of all they play the same protector role, when high sun irradiation acts, like on the leaf cuticle. As antioxidants, pigments store thylakoids from reactive oxygen species. Second role relates to their possible function as donors of electrons in photosynthetic electron transport chain, especially when it damaged. In this case, azulenes may participate in alternative electron transport, by-pass blocked part, when act diuron or antimycin A. Since the azulene-containing fraction was isolated from isolated chloroplasts of pea and clover [19], it is likely that this pigment plays a role in thylakoids. It is possible to be a protective compound, since it has the properties of an antioxidant.

Conclusion

Common protectory role of azulenes as antioxidants in leaves of some studied species is demonstrated and discussed for some suitable models. Azulenes enriched in wax covers in cell wall and wax-less membranes of chloroplast may serve as a defense from reactive oxygen species. This fact was earlier shown for woody species tolerant to UV-irradiation and ozone. Similar azulene-containing plant woody species could be recommended for their introduction along the world. This blue pigment could be donor of electrons in photosynthetic electron transport chain that also give plants with azulenes more chances for their growth and surviving.

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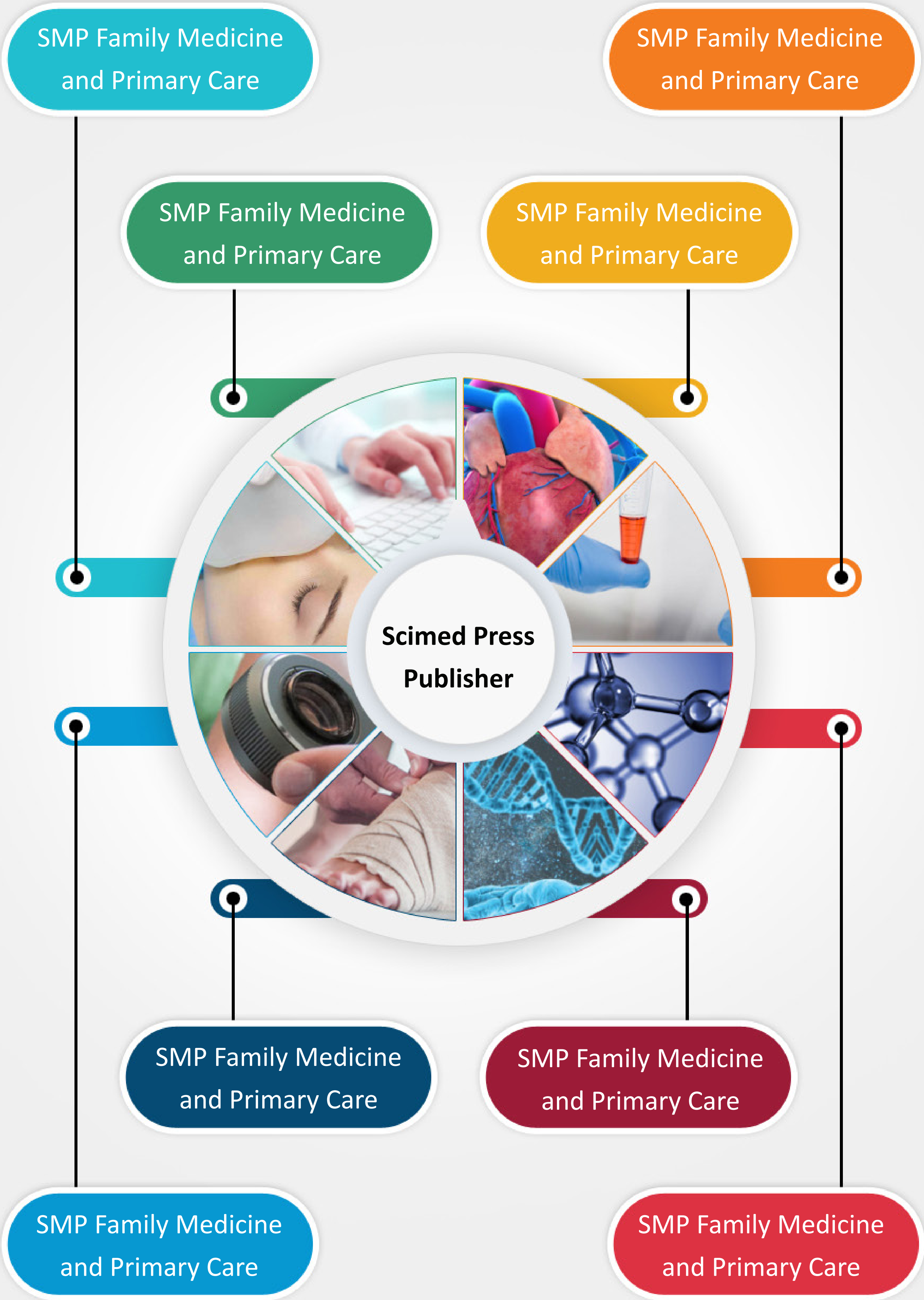
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