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Coloured allelochemicals in modelling of cell-cell allelopathic interactions

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ABSTRACT

Modelling of cell-cell interactions using microscopy is based on the observations of direct interactions of acceptor cells as biosensors (unicellular plant generative and vegetative microspores) with pigmented and fluorescing components of secretions released by the donor cells of allelopathic plant species. Pigmented secretions enriched in red anthocyanins (glucosides of pelargonidin from *Hippeastrum hybridum* petals), blue azulenes (synthetic azulene and azulene extracted from leaves of *Artemisia absinthium)* and yellow alkaloids (rutacridone from *Ruta graveolens* roots) serve as tool to study the mechanisms of cell-cell interactions. The compounds may stimulate or inhibit the germination of pollens, vegetative microspores and seeds. Test allelochemicals penetrate into the acceptor cells and get bound with DNA-containing organelles (nuclei and chloroplasts) that change the colour or fluorescence of cellular structures. Coloured allelochemicals were used as histochemical dyes to determine the cellular mechanisms during the allelopathic interactions.

Key words: Acceptor cells, allelochemicals, *Artemisia absinthium,* biosensors, cell-donors, DNA, *Equisetum arvense,* fluorescence, germination, *Hippeastrum hybridum, Matricaria reticuta*, model systems, *Philadelphus grandiflorus,* pollen, *Ruta graveolens*, seeds, *Trifolium pratense, Vallota speciosa*, vegetative microspores

INTRODUCTION

Currently the modelling of allelopathic relations occurs via ecological approach with the help of mathematical apparatus (2) or the effects of various phytotoxins are used to understand the main biochemical and physiological processes in plants (6,7). There are also special publications about the toxic and non-toxic interactions within phytoplankton or between the phytoplankton and zooplankton (22,24). There are also approached to analyse the plant unicellular microspores interactions based on the cellular spectral characteristics (13,15,16).

The cell-cell interactions in allelopathy are studied using various microscopic methods e.g. pollen-pollen interactions are analysed by microspectrofluorimetry or laser-scanning confocal microscopy (12,14,16,20,21). This technique is suitable to determine the allelopathic mechanisms between the diferent plant species, when one cell (a donor cell) releases a molecule that is received by another cell (an acceptor cell). Cell-cell

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interactions at the cellular level can also be used to test the behavior of acceptor cell undergoing the actions of extracts from allelopathic plants or individual components of secretions from donor cells. Using microscopic methods, we can directly observe: (a) the transport of compounds analyzed into an acceptor cell selected as a model (biosensor), (b) changes in the autofluorescence or colour of both interacting cells and (c) the subcellular location where the allelochemical acted within the acceptor cell (model) and interaction with certain compartments.

For such investigations, suitable sensitive model-biosensors (acceptor cells) and pigmented (fluorescent) allelochemicals from donor cells are received. As models, plant unicellular microspores - both generative (pollens of seed-bearing plants) and vegetative (spore-bearing species) are recommended (13,15-17). In pollen allelopathy, the allelopathic effects on a cellular level are successfully studied (9,20,21).

Highly fluorescent pigmented secondary products released by the allelopathic plants are suitable biomarkers for such microscopic observations (12-14). Candidates for similar studies may be red pigments (anthocyanins), blue pigments (azulenes) and yellow alkaloids. Anthocyanins are common in petals of flowers, leaves and roots (16,18) and in pollen grains of many species (25). Azulenes are present in leaves and petals of flowers (18) and pollens (16,19). The yellow alkaloid rutacridone and its glucoside are concentrated mainly in roots and have bright emission with maxima at 580-590 (11). The presence of above coloured allelochemicals in plant secretions made them potentially useful to study the allelopathic interactions at a cellular level.

Modelling of cell-cell interactions under microscope may consist of adding the excretions (mainly water or ethanolic extracts enriched in color allelochemical) or individual allelochemical to the acceptor cell (model) served as a biosensor and an observation of behavior of cell receiving the chemosignal. This research aims to show the usefulness of coloured allelochemicals [flavonoids anthocyanins (pelargonidins), sesquiterpene lactones (azulenes) and alkaloids (rutacridone)] to study the mechanisms of cell-cell interactions in unicellular biosensors using the microscopic observations.

MATERIALS AND METHODS

1. Plant material: The donor cells and acceptor cells (Table 1) were collected (i). from different ecosystems (Grasslands, meadow, parks and groves) during June-August 2008-2010 near Oka river, Russia (without asterisk in Table 1) or (ii). from green-house plants (with asterisk in Table 1) and (iii). from the isolated cellular organelles.

Cells: Biosensors were pollens of various species and vegetative microspores of horsetail. The methods for their germination on slides (subject glasses) put on the Petri dishes were described earlier (13,15). Results were expressed as mean ± SEM (n = 400-500 microspores per one variant; P =0.95). Each value is the mean of 4-5 independent measurements as per Student multiple analysis test.

Organelles: Isolated nuclei and chloroplasts were also used as models of intracellular

Table 1. List of acceptor cells and donor cells used for the modeling

Donor Cells			**Acceptor Cells (Biosensors)**	
Allelochemicals	Plant part	Plant Spp.	Plant cell	Plant Spp.
Azulenes, mainly chamazulene	Leaves	*Artemisia absinthium* L., Common wormwort (Asteraceae)	Pollen	*Hippeastrum hybridum* L. (Herb.)*, KInight's star (Amaryllidaceae)
Alkaloid rutacridone	Roots	*Ruta graveolens* L., Rue *(*Rutaceae)	Pollen	*Vallota speciosa* (L. f) T. Durand et Schinz, Scarborough lily* (Amaryllidaceae)
Anthocyanins pelargonidin and its glucosides	Petals	*Hippeastrum hybridum* L. (Herb.)*, Knight,s star (Amaryllidaceae)	Pollen	*Plantago major* L., Plantain (Plantaginaceae)
			Vegetative microspores	*Equisetum arvense* L., common horsetail (Equisetaceae)

* Species grown in green-house

organelles. Nuclei cannot be isolated from the microspores due to their hard envelope; hence, they were isolated from the soft non-pigmented tissues of petals of mock-orange *Philadelphus grandiflorus* Willd. or matricary (wild chamomile) *Matricaria reticuta* L. as described below. Petal sample (20-40 g) was homogenized for 1 min at 56,000 rpm in 0.05 M potassium phosphate buffer, pH 7.25 containing 0.2 M sucrose. The homogenate (50 mL) was filtered through three nylon meshes and centrifuged at 800 *g* for 2 min. The pellet with large tissue particles and anther fragments was discarded. The supernatant was centrifuged on sucrose density gradient (0.2, 1 and 1.5 M) in the same buffer at 10,000 *g* for 5 min. The pellet and the bottom 1.5 M fraction were discarded and the upper ones were analyzed. The purest fraction of nuclei with intact membranes was at the 1-0.2 M interface. All fractions were stained with fluorescent dyes Hoechst 33342 ("Serva") emitting in the blue (460 nm) and acridine orange ("Serva") emitting in the green (520-530 nm).

Chloroplasts were isolated from *Trifolium pratense* L., red clover (Fabaceae), with mannitol in the medium as per the method of Robinson *et al.* (10).

2. Microscopy of cells and organelles

Luminescence microscopy: Fluorescence of intact cells under luminescence microscope was excited by ultra-violet (Filters UVS-6 with maximal transmission at 370 nm and SZS -7 with maximal transmission 380 nm from "LOMO" corporation, Sankt-Peterburg) or 405-436 nm (Filters VS1-6 with maximal transmission at 400 nm and SZS -7 from "LOMO" corporation, Sankt-Peterburg) photographed under luminescence microscopes "*Carl Zeiss CD-28* with the same filters and *Carl Zeiss* A-10 Axio Imager (Axio Imager with a complete set of zero-pixel shift Semrock GFP, YFP, CFP, and Cy3 filters) as well as under luminescence microscope *Leica* DM 6000 B that allows visualization of optical slices. Filters of AX-10 Imager included filter wheel 1 = 428301-9901-000 and filter wheel 2 = 428301-9901-000.

Microspectrofluorimetry: Emission of intact cells under luminescence microscope combined with the registration system of microspectrofluorimeter was measured as

described earlier (15,16) in form of fluorescence spectra by microspectrofluorimeter MSF 1 or shown as histograms registering the emission in dual beams at 530 nm (green or blue/green emission) and at 640nm (red emission) by dual-beam microspectrofluorimeter MSF-2 [made by the Institute of Biological Techniques, Pushchino]. The emission intensity (I) in two different spectral regions (in blue-green with maxima 530 nm or blue with maxima 460 nm and in red with maxima 640 nm) of the whole microspore cells was recorded on a dual-wavelength microspectrofluorimeter MSF-2 at room temperature 20-22^{O}C. Results were expressed as mean ± SEM. The relative standard deviation (RSD) was 5-6% (n = 400-500 microspores per one variant; P =0.95). Each value is the mean of 3 or 4 independent measurements followed by SD.

Confocal microscopy: Structural images of fluorescent cells were obtained by laser-scanning confocal microscopy on the glass slides at room temperature 20-22^{O}C as published earlier (16,17,20,21). The fluorescence image of cells was also seen by the water immersion with laser -scanning confocal microscopes (LSCM) LSM510 NLO "Carl Zeiss *Karl Zeiss*" and *Leica* TCS SP-5. The excitation by three types of lasers: Argon/2 (l458, 488, 514 nm), HeNe1 (543nm) and HeNe2 (l 633 nm) were used for the emission in the Zeiss microscope and for Leica TCS SP-5 microscope laser wavelength was 405 нм. Excitation by lasers at wavelengths of 458, 488, 543 and 633 nm, resulted in emission fluorescence at 505-630 nm, 650-750 nm and 650-750 nm, respectively. Three photomultipliers can catch the fluorescence, separately or simultaneously by use the pseudocolor effects. The image analysis was done with the computer softwares LSM 510 and Lucida Analyse 5. Pseudocolors were according to the excitation wavelength blue for 488; green for 543 and red for 633. Layered images of the optical slices along the objects were in their pseudocolors.

3. Reagents: The reagents were water and ethanolic extracts (as models of natural secretions from donor cells) from the allelopathic plants and individual components of secretions (see formulae below). The sample from plant material of species was extracted at room temperature with solvents (1:10 w/v) water or ethanol or chloroform (for *A. absinthium* leaves) for 20-30 min, and the extracts were centrifuged at 1,000 *g* for further analysis. The absorbance spectra of extracts were recorded at Specord M-40 (Karl Zeiss) and the fluorescence spectra - with Perkin Elmer spectrofluorimeter 350 MPF-44B (UK) in 2, 4 and 10 mm cuvettes. Water extracts of anthocyanins were primarily purified on silicagel thin-layer plates and used for the experiments. To compare the effects with the water fraction, the ethanol extract of the pigments enriched mainly in glucosides of pelargonidin were isolated from petals of knight star's *H. hybridum* as per Byamukama *et al.* (5) were pelargonidin 3-*O*-(6"-*O*-*α*-rhamnopyranosyl-*β*-glucopyranoside) and cyanidin 3-*O*-(6"-O-α-rhamnopyranosyl-*β*-glucopyranoside) were identified. There were no differences in the spectral characteristics between water extracts of anthocyanins and the fractions of individual glucosylated pelargonidins, therefore, silicagel-purified water fraction of anthocyanins was used to stain the cell. Azulene was purchased from "Merck/Fluka " (Darmstadt, *FRG* /Buchs, *Switzerland*), whereas, azulene fractions enriched in chamazulene were isolated from leaves of common wormwort *A. absinthium* L. as per the procedure described for pollen (19). Rutacridone glucoside was isolated as per previously published method (3) from *R. graveolens* roots. Formulae of coloured

allelochemicals used and their spectral characteristics are shown on Figs 1 and 2. Kits of nucleosides as well as ribose and deoxyribose for modelling with nucleic acid components were given from "Serva".

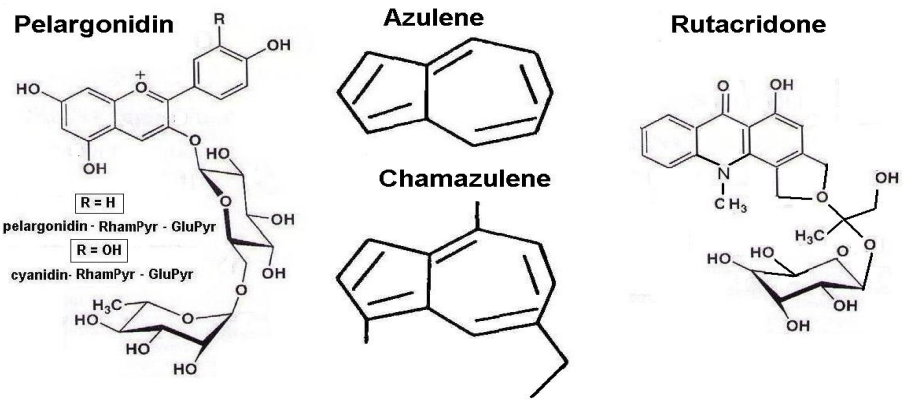

Figure 1. Formulae of allelochemicals used

4. Statistical analysis: Total 100 plant microspores or isolated organelles were examined on each slide under usual or luminescence microscope. Counting was done in four or five replications (number of slides per treatment). The special statistic Student-t program was also used. Results were expressed as mean ± SEM. The relative standard deviation (RSD) was 5-6% (n = 400-500 microspores or isolated organelles per one variant; P =0.95).

RESULTS AND DISCUSSION

Treatments with coloured allelochemicals induced various changes in the acceptor cells (Fig.3). Either colour or the fluorescence or both colour and fluorescence were changed. We described the results for every allelochemicals used.

Modelling with pelargonidin: When the pollen of *H. hybridum* was used as acceptor cells treated with anthocyanin pelargonidin, the nucleus of viable cells (cells able to germinate and form a pollen tube) stained blue (Fig. 3, Image 1) that peculiar to basic form of the allelochemical cyanidin (see Fig. 2 where the maximum colour in red at pH 6.0-6.5 with 520-530 nm maxima and in blue at pH > 7.0 with 590-600 nm maxima in the absorbance spectra). Unlike viable pollen grains, non-viable ones did not change colour under transmitting light of microscope (Fig. 3, Image 2). The green fluorescence of same samples under luminescence (Fig. 3, Images 3-5) and confocal (Fig. 3, Image 6) microscopes was observed. The optical slice of viable pollen (Fig. 3, Image 4) showed the location of emission only on the surface that correlates with the lack of fluorescence of basic form of pelargonidin (cyanidin). A red emission (peculiar to acidic form of anthocyanin) could be seen in the centre of non-viable pollen (Fig. 3, Image 5), whereas, the surface fluoresced in green.

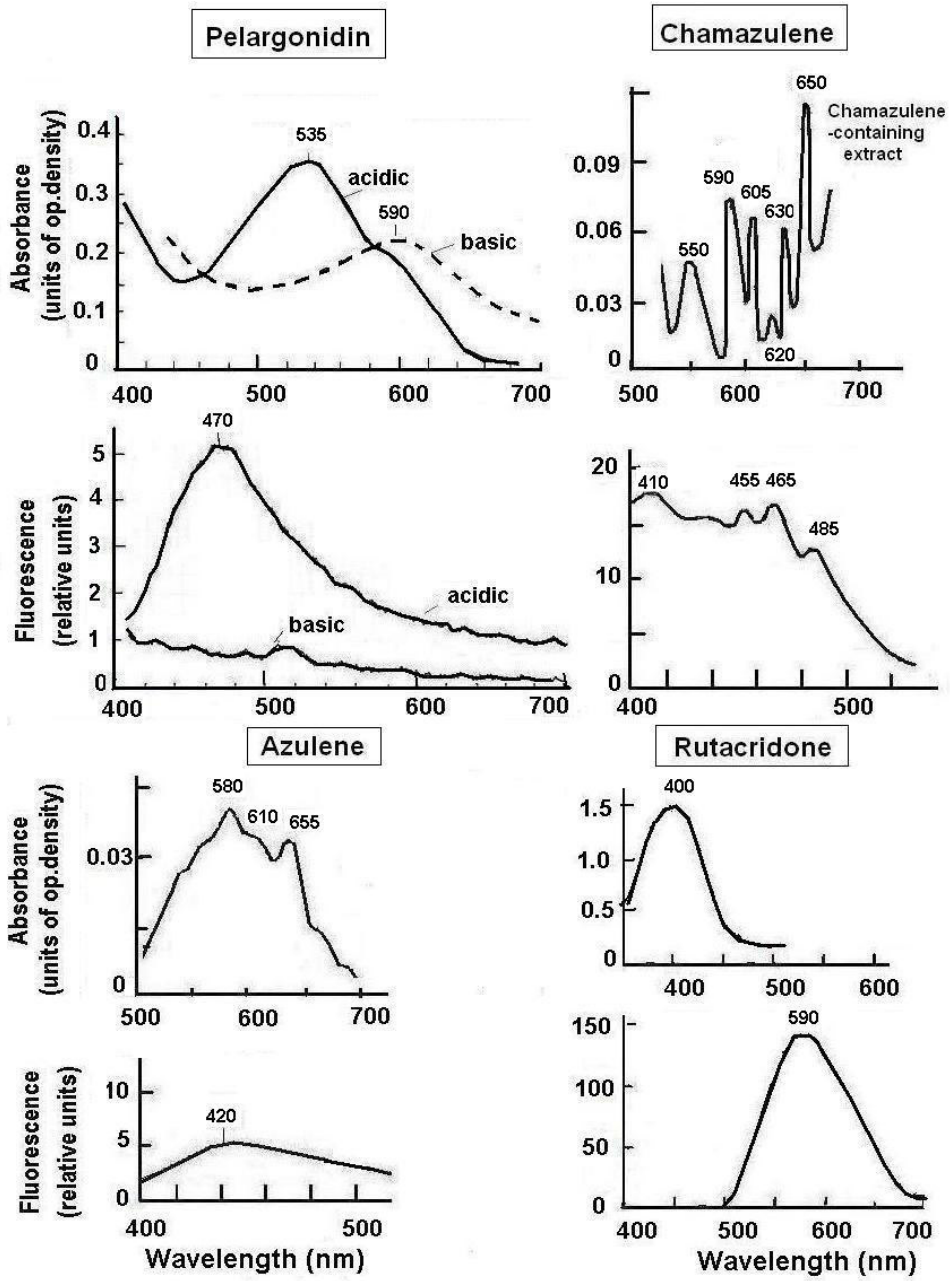

Figure 2. Absorbance and fluorescence spectra of coloured allelochemicals (10^{-5} M) used. Acidic form anthocyanin - pelargonidin - R = H, Basic form cyanidin - R = OH.

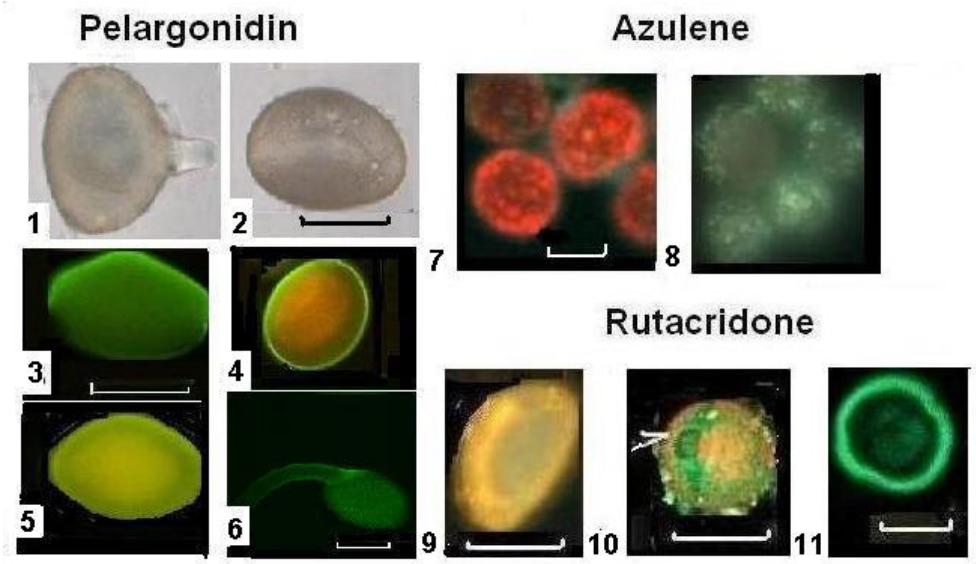

Figure 3. Images of acceptor cells after staining with coloured allelochemicals (10^{-5} M). Images 1-6: After treatment with pelargonidin (Control samples without the treatment have no green fluorescence). Images 1 and 2 of *H. hybridum* pollen are seen under usual microscope in transmittent light, Images 3-5: Under luminescence microscope (5 – optical slice), 6- pollen and pollen tube – under *Leica* laser-scanning confocal microscope after the treatment with anthocyanin pelargonidin (Bars = 50 µm). Images 7 and 8 : Fluorescence images of vegetative microspores from *Equisetum arvense* untreated and treated with azulene under luminescence microscope (Bars = 20 µm); Images 9, 10 and 11: Fluorescing images of the pollen from *H. hybridum* under luminescence microscope (Bar = 50 µm) and vegetative microspores (nuclei are shown with arrows) from *Equisetum arvense* under *Carl Zeiss* laser-scanning confocal microscope (Bar = 20 µm) and isolated nucleus from *P. grandiflorus* petals under *Carl Zeiss* laser-scanning confocal microscope (Bar = 3 µm) treated by rutacridone, respectively. Untreated microspores and isolated nuclei had no visible green fluorescence.

The fluorescence spectrum (excitation by laser 405 nm) of pollen surface stained with anthocyanin had maximum 450 nm and a shoulder at 520 nm (Fig.4). Common view of the object fluoresced in green (Fig. 3. Image 6). Thus, pelargonidin was able to penetrate into cell and colour the nucleus. The staining was compared with the biological effects of anthocyanin (Table 2). The germination of all microspores used as acceptor cells from *Plantago major*, *H. hybridum* and *Equisetum arvense* were stimulated by pelargonidin glucosides, which correlated with the results of cell staining for the nucleus. The stimulation was very high both in *P. major* pollen tube growth (435 % of control) and in vegetative microspores of *E. arvense* (342 % of control); while the rate of pollen germination for *P. major* and *H. hybridum* was about 18 %.

Allelopathic stimulation of microspores germination in our experiments suggests a possible role of allelochemicals in plant-plant interactions (4). Moreover, a decrease in red fluorescence was also observed in vegetative microspores of *Equisetum arvense*, which had chloroplasts. Isolated chloroplasts from red clover *T. pratense* were treated with anthocyanin to determine whether there was any interaction between this molecule and

Table 2. Effects of pelargonidin (10^{-5} M) on the germination of plant microspores during 2.5 h

Treatment	Germination Index of *Plantago major* pollen	Pollen tubes length (μm)	Germination Index of *Hippeastrum hybridum* pollen	Germination Index of *Equisetum arvense* vegetative microspores
Control	0.67 ± 0.02 a	20 ± 2 a	0.54 ± 0.02 a	0.33 ± 0.03 a
Pelargonidin	0.79± 0.03 b	107 ± 10 b	0.64± 0.03 b	1.46± 0.09 b
Stimulation (%) over control	17.9	435	18.5	342

(Germination Index = Numbers of germinated / Numbers of analysed cells); *Each value is the mean of 400 cells analysed per treatment (4 slides per treatment as independent measurements) followed by SD. Numbers in columns followed by the same letters are not different at P 0.95 as per Student multiple range analysis test.

these organelles. A decrease in the organelles' emission at 640-680 nm was also observed (Table 3). The target of the anthocyanin action is proposed to be DNA (8,23). Thus, the possible mechanism of allelopathic anthocyanin interaction with acceptor cells involves the penetration into the cell and binding to the cell wall and DNA-containing structures.

Table 3. Fluorescence intensity (at 640-680 nm) of isolated chloroplasts from red clover *T. pratense* treated with 10^{-5} M pelargonidin (excitation 420-430 nm).*

Treatment	Red emission (relative units)
Control	0.93 ±0.06 a
+ pelargonidin	0.23 ±0.02 b

*Each value is the mean of 400 cells analysed per treatment (4 slides per treatment as independent measurements) followed by SD. Numbers in columns followed by the same letters are not different at P 0.95 according to Student multiple range analysis test.

Modelling with azulenes: Azulene is known to modulate the germination of plant microspores (13,16), in particular bind to cell wall and nucleus of pollen grains (16,19). The extracts with chloroform from chamazulene-containing species *A. absinthium* and *Achillea millefolium* that after drying mixed with water inhibit the germination of pollen from *H. hybridum* and *V. speciosa* (17). Pure azulene and a chamazulene-containing extract were used in these experiments. Staining vegetative microspores of horsetail *Equisetum arvense* (chloroplast-containing models) with azulene induced blue (excitation 360-380 nm) or blue-green (excitation 420-430 nm) fluorescence of nucleus and chloroplasts (i.e. DNA- containing structures) instead of red fluorescence seen in the control [Fig. 3, (Images 7 and 8)]. The blue fluorescence intensity was also seen in the fluorescence spectra of pollen nuclei of *H. hybridum* and *V. speciosa* (Fig. 4). Besides, isolated chloroplasts treated with azulene increased the blue fluorescence intensity and a decrease in red emission of organelles (Table 4) which is consistent with the previous observation that allelochemicals may bind to plastids. The possible target of azulene is DNA-containing structures because the compound interacts with nuclei (Fig. 4). The fluorescence of cell nucleus in the presence of azulene was shown earlier (13,14). Nuclei isolated from white petals of *P. grandiflorus* and *M. recutita* stained with azulene also emitted in blue. The extract from *A. absinthium* enriched in chamazulene also induced nuclei blue fluorescence (Fig. 4).

Table 4. Fluorescence intensity of isolated chloroplasts from red clover *T. pratense* treated with 10^{-5} M azulene (excitation 360-380 nm). *

Treatment	Blue emission at 430- 460 nm (relative units)	Red emission at 640-680 nm (relative units)
Control	0.01 ±0.001 a	0.21 ±0.010 a
+ azulene	0.02 ±0.002 b	0.07 ±0.014 b

*Each value is the mean of 400 cells analysed per treatment (4 slides per treatment as independent measurements) followed by SD. Numbers in columns followed by the same letters are not different at P 0.95 according to Student multiple range analysis test.

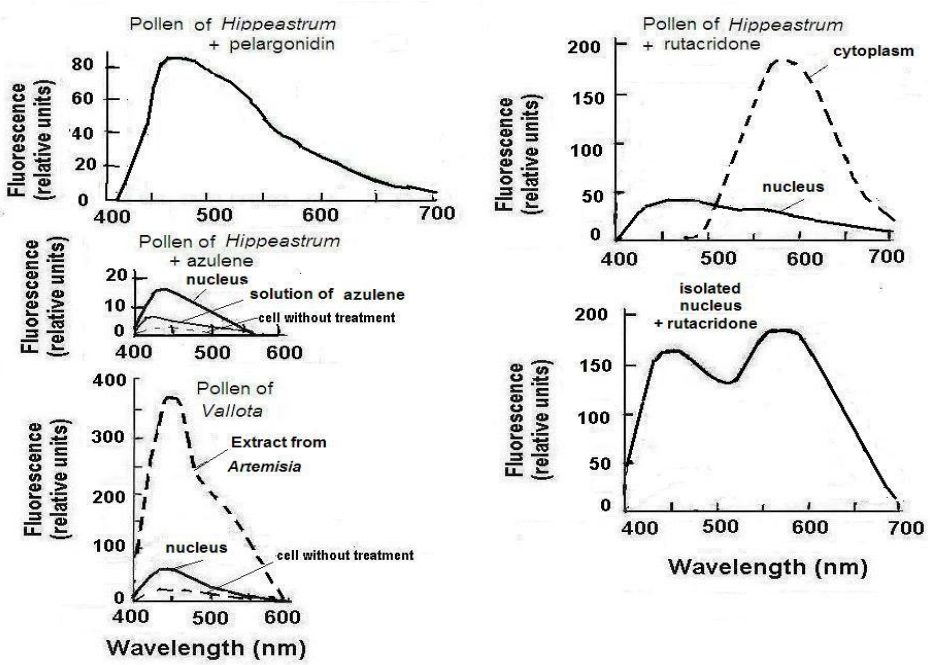

Figure 4. Fluorescence spectra of acceptor cells and isolated petal nuclei from *P. grandiflorus* stained with allelochemicals (10^{-5} M).

Modelling with rutacridone. Yellow alkaloid rutacridone is the active allelochemical of rue roots that may participate in root-root interspecies interactions (1) and inhibit the development of seeds. Treatment with rutacridone led to binding with nucleus of *H. hybridum* (11). The analysis of rutacridone interaction with acceptor cells (Figs. 3 and 4) showed the shift of orange fluorescence with maximum 580-586 nm to green emission in the region of nucleus in pollen of *H. hybridum* or the nuclei and chloroplasts of vegetative microspores of *Equisetum arvense*. Similar results when this alkaloid was used to stain isolated nuclei from white petals of *P. grandiflorus* and *M. recutita*. The nuclei fluoresced green (Fig. 4) and had two fluorescence spectrum maxima at 460-480 nm and 585-590 nm, whereas only one maximum 585-590 nm was observed in cytoplasm of cells or in the rutacridone solution. The staining of isolated chloroplasts with rutacridone decreased their

red fluorescence and enhanced the green emission (Table 5) which support the hypothesis that such allelochemicals can bind to chloroplasts, possibly at a DNA binding site (11).

Table 5. Fluorescence intensity of isolated chloroplasts from red clover *T. pratense* treated with 10^{-5} M rutacridone (excitation 420-430 nm)

Treatment	Green emission at 520 nm (relative units)	Red emission at 640-680 nm (relative units)
Control	0.01 ±0.001 a	0.41 ±0.020 a
+ rutacridone	0.02 ±0.002 b	0.16 ±0.010 b

*Each value is the mean of 500 cells analysed per treatment (5 slides per treatment as independent measurements) followed by SD. Numbers in columns followed by the same letters are not different at P 0.95 according to Student multiple range analysis test.

Modelling of allelochemical interactions with components of nucleic acids: The interaction of allelochemicals with the components of nucleic acids - nucleosides, i.e. pyrimidine and purine bases bound to ribose or deoxyribose: thymidine, cytosine, adenosine and guanosine as well as ribose (deoxyribose) itself - was studied using the films of compounds on the subject glass (Fig. 5). Modelling with the films (their solid systems fluoresce in region 380-410 nm) was due to several circumstances, viz., (a) in nature nucleic acids are in solid form and (b) in the solutions of the nucleosides and sugars their fluorescence was very small if any.

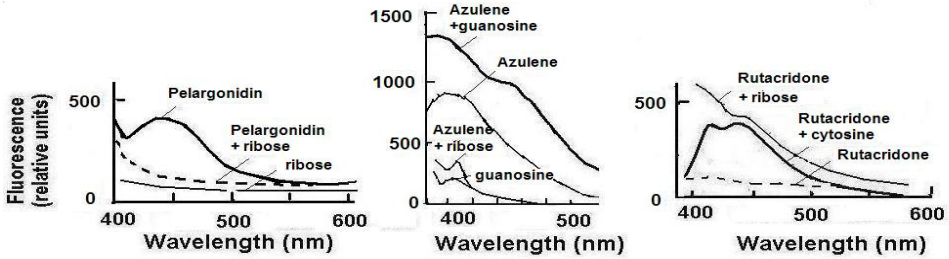

Figure 5. Fluorescence spectra of films from (2 mg) every nucleic acid components treated with 10^{-5} M allelochemicals. The emission excitation was 360 nm.

If the films of the above-mentioned components of nucleic acids were stained with pelargonidin (Fig. 5) at short time-exposure (< 1 h) only in treatment with ribose (deoxyribose) the anthocyanin blue fluorescence was quenched. This correlates with data of whole cell staining (Fig. 3), when the nucleus transformed fluorescent pelargonidin to non-fluorescent cyanidin. The binding site of cyanidin is proposed to be the phosphorus between two deoxyribose residues of the DNA near the contact of ribose (deoxyribose) with nucleotides (8). Anthocyanin-DNA copigmentation complex becomes the protection against oxidative damage (23). Unlike results with pelargonidin, among the films staining by azulene only guanosine increased the fluorescence at 420-430 nm (Fig. 5), while other nucleosides did not interact. DNA–azulene complex emits in blue-green with 440 and 520 nm maxima (13). Guanosine may be considered as a potential target for the allelochemical. Another picture was after the staining by rutacridone (Fig. 5). In this treatment, blue-green

emission was observed mainly for cytosine (maxima 415 and 445 nm in the fluorescence spectra) and in small degree for ribose (smooth peak at 445 nm was seen), while solution of rutacridone had no maxima in the spectral region. Green (or blue-green) fluorescence of nuclei (Fig. 3) and pure DNA (11) was also observed in the presence of rutacridone. Cytosine appears to be one of targets for rutacridone on the nucleic acid. Tested allelochemicals may be considered as histochemical dyes and also used for indication of DNA- containing cellular structures.

CONCLUSIONS

Modelling of cell-cell interactions using coloured allelochemicals and unicellular models (biosensors) can be useful for the analysis of allelopathic mechanisms at cellular level. Colour and fluorescence of acceptor cells served as indicators of cellular reactions after adding the allelochemicals from donor cells. The compounds bound to the surface or/and penetrate and act on cellular organelles. As shown with luminescence microscopic techniques, the anthocyanin pelargonidin and sesquiterpene lactone azulene reacted both with cellular surface (cell wall) and after the penetration into the acceptor cell - with nucleus and chloroplasts (DNA- containing organelles). On the other hand, the alkaloid rutacridone reacted mainly with nucleus and chloroplasts. DNA was the potential target for these allelochemicals, in particular as sugar ribose (deoxyribose) for pelargonidin, guanosine for azulene and cytosine for rutacridone. Therefore, these coloured allelochemicals could be developed as useful histochemical dyes to study the cellular mechanisms involved in allelopathic interactions. orgasm

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