

Autofluorescence of secretory cells in allelopathic *Eucalyptus* species studied by luminescence and confocal microscopy

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ABSTRACT

Autofluorescence of leaf glandular structures of important allelopathic and medicinal trees manna gum (*Eucalyptus viminalis* Labill.) and silver dollar (*Eucalyptus cinerea* F. Muell. ex. Benth) were studied using luminescence and confocal microscopy. The emission depended on the wavelength of excitation and differed in the species. Glands of both test species fluoresced weakly in ultraviolet or violet light. Excitation of blue light (450-490 nm) induced green emission from yellow glandular cells of *E. cinerea*, which appears to be due to some flavonoids present in *Eucalyptus* oil. Use of laser-scanning confocal microscopy made it possible to see the structure of glands and their connection with oil ducts in both test species. In the fluorescence spectra recorded for glands of both species, we found one characteristic maxima in range of 535-540 nm, peculiar to some flavonoids. Hence, both these fluorescent methods were recommended for analysis of intact *Eucalyptus* secretory cell in allelopathic and pharmaceutical studies.

Key words: Autofluorescence, *Eucalyptus cinerea*, *Eucalyptus viminalis*, flavonoids, laser-scanning confocal microscopy, luminescence microscopy, phenols, secretory cells.

INTRODUCTION

The eucalyptus plants are endemic to south-eastern Australia, but now they are grown widely in many countries, because these are very useful to reclaim the marshes and prevention of malaria. Among the allelopathically active and medicinal woody species, *Eucalyptus* genus (family: Myrtaceae) have oil secretory structures (1,19). Their oil is phytotoxic (4), however, the leaves of various *Eucalyptus* species are allelopathic and do not decompose easily in soil (4,8,10,11,22).

Hence, there is a need to study the secretory structures of *Eucalyptus viminalis* Labill. and *Eucalyptus cinerea* F. Muell. ex. Benth trees, as these are cultivated widely in all subtropics, and their oil glands have been investigated only in few studies (2,3). Mainly, the publications focus on the phenolic compounds present in the oil glands. The sub-dermal secretory cavities (glands) embedded in the leaves of *Eucalyptus* (Myrtaceae) were once thought to be the exclusive repositories of monoterpene and sesquiterpene oils. Recently, leaf secretory cells of *Eucalyptus cinerea* were also objects of ozone treatment (14,15,17). However, the fluorescent characteristics of the secretory cells of *Eucalyptus* genus have not been studied yet. The compounds/allelochemicals present in the secretions of many plants may fluoresce and possess characteristic maxima in the fluorescent spectra to identify the compounds in mixtures (12,14), including model systems (13,14). Various colours of the

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emission and its intensity permits us to identify the secretory cells among non-secretory ones. This type of fluorescence of intact cells without histochemical staining is called autofluorescence or self-fluorescence. This investigation aimed to use the luminescence microscopy, including its confocal modification, to study leaf oil glandular structures of *Eucalyptus cinerea* and *Eucalyptus viminalis*.

MATERIAL AND METHODS

The fresh and dried leaves of *Eucalyptus viminalis* Labill. and *Eucalyptus cinerea* F. Muell. ex. Benth. were collected from the Sochi National Park (Dendrarium and cultures plantations) Russia on September 9-10, 2019-2020.

Autofluorescence : Autofluorescence of living cells was observed and photographed using actinic light of luminescence microscope *Leica* DM 6000 B (Germany-Austria-United States) and its modified laser-scanning confocal microscope *Leica* TCS SP-5 described previously (18). All experiments were done on the glass slides at room temperature 20-22° and showed images of natural emission colours under luminescence microscope and in pseudocolours under laser-scanning confocal microscope. The fluorescence spectra were also recorded with the laser-scanning confocal microscope *Leica* TCS SP-5. The sources of exciting light in the luminescence microscope were a mercury lamp for visual observation and in confocal microscope - laser Argon/2 (wavelengths 458, 477, 488, 514 nm), HeNe1 (543 nm wavelength) and HeNe2 (633 nm wavelength) were used. Some experiments were done on glass with or without glycerine on confocal microscope (objectives x 10, 20 and 40).

Fluorescence spectra : The fluorescence spectra were recorded using the above-mentioned laser-scanning confocal microscope *Leica* TCS SP-5 (Germany) and 3-channels: blue, green and red were used. Individual cells or parts of the secretory structures were first identified as a region of interest (shortly ROI). The intensity per cell was determined by integrating the emission from the ROI. Optical probes on the slices were marked by rings, rectangles and free form figures.

RESULTS AND DISCUSSION

We studied the autofluorescence of oil-containing glands of two eucalyptus species. Purified oils do not have ability for light emission (14). However in nature, oil secretory cells often contained various compounds (16) that fluoresce because they contain fluorophores.

1. Luminescence microscopy. We studied the secretory cells of our objects to observe and took photographs of their surface (Figs. 1 and 2). As shown in transmitted light, glandular structures were more abundant in *Eucalyptus cinerea*, than in *Eucalyptus viminalis*. In first case, we observed that green-yellow coloured glands have contacts with ducts, but these were not clearly seen on the surface of *E. viminalis*. These glands were present at different depths of leaf. These glands sizes also differed and those of *E. cinerea* were two folds larger. Some structures of *E. cinerea* had red colour as phenolic compounds, perhaps anthocyanins (Fig. 1). The red interior was seen after the rupture of the gland. Another picture showed

the autofluorescence during the investigation. Excitation by ultraviolet 340-380 nm or violet 355-425 nm light showed the absence of fluorescence in green-yellow coloured structures and in the middle of red-coloured ones (only the bundle cells of gland emitted as blue or blue-green) in *E. cinerea*. This picture also showed that glands with red secretion are open and seen under ultraviolet light 340-380 nm and violet light 355-425 nm. Lack of fluorescence in the middle of red-coloured cells may be due to the presence of tannins with weak emission (12,23). The excitation by light at 515-560 nm showed weak red fluorescence. The excitation by light at 515-560 nm showed weak red fluorescence. The excitation by ultraviolet (340-380 nm) or violet (355-425 nm) light made glands of *E. viminalis* looked as dark spots surrounded with bright blue or blue-green fluorescing non-secretory cells (Fig. 2). Although at last, actinic light weak green colour of the gland was also seen.

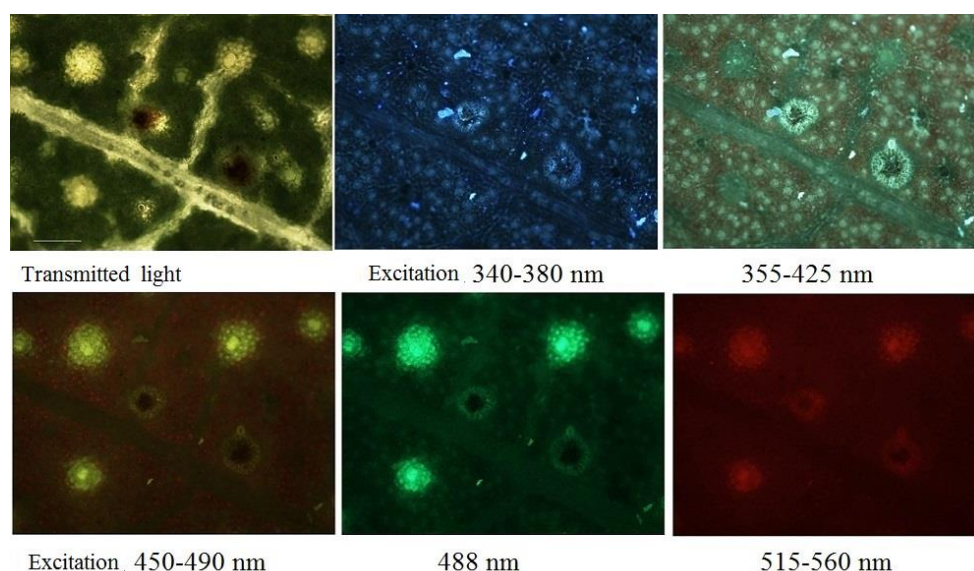


Figure 1. The images of the surface *Eucalyptus cinerea* under luminescence microscope Leica DM 6000 B at various excitation of the fluorescence. Bar = 100 μ m.

More different pictures were seen in yellow-orange or red actinic light. In *E. cinerea* leaf, light 450-490 nm or only 488 nm induced yellow or yellow-green-fluorescence (Fig. 1), while red-colored glands (in transmitted light) had weak emission. Unlike *E. cinerea*, glandular cells of *E. viminalis* looked dark as reported earlier (Fig. 2). Only surrounding cells emitted in yellow or green. At excited light of 515-560 nm, glands of both *Eucalyptus* samples did not fluorescence, and surrounding cells were red-fluoresced due to chlorophyll emission. Thus, we can suppose that various emissions from glandular structures were related to different compositions of secretions in the species.

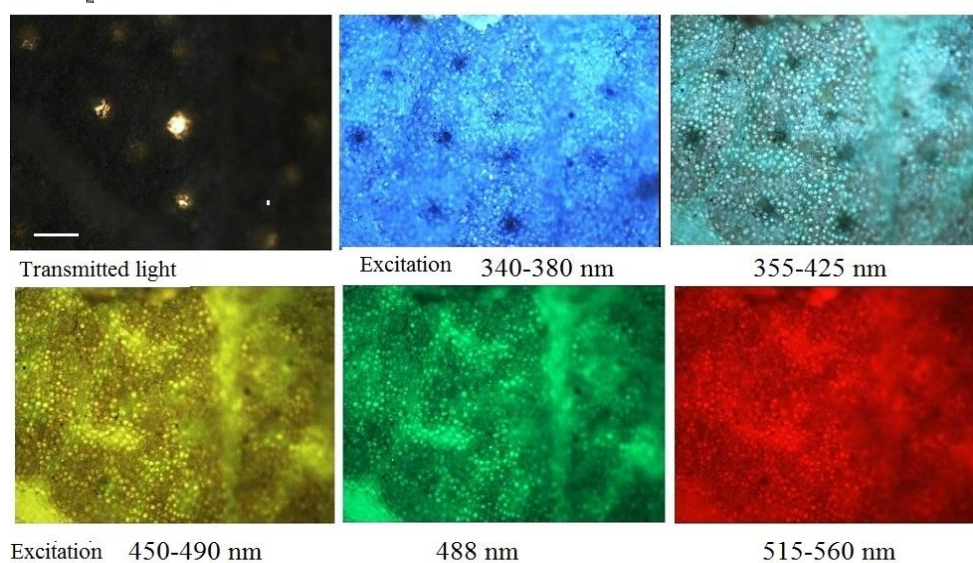


Figure 2. The images of the surface of *Eucalyptus viminalis* under luminescence microscope Leica DM 6000 B at various excitation. Bar = 250 μ m.

In review (2), many phenols were present in various secretory structures of *Eucalyptus*. Fluorescence of the compounds varies from blue, blue-green, yellow (for main flavonoids) or even red for some anthocyanins (12). The *eucalyptus* spp, especially *E. cinerea* are rich in terpenes and terpene-containing oils (21). The *Eucalyptus* essential oils are antimicrobial against bacteria (*Streptococcus pyogenes*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*) and yeasts and were found in the dried autumn and winter leaves. We have little information about the fluorophores in *Eucalyptus*. Goodger *et.al.* (3) reported that the sub-dermal secretory cavities (glands) embedded in the leaves of 11-*Eucalyptus* species (excluding the *E. cinerea* and *E. viminalis*) are the exclusive repositories of monoterpene- and sesquiterpene-containing oils, but non-volatile compounds were present in foliar glands. Their secretory structures contain numerous flavanones. In particular, glands of 4-species in subgenus *Eucalyptus* contains the biologically active flavanone pinocembrin (3). This flavanone was detected in glands of 11 sub-species of *Eucalyptus* using Electro-Spray Ionization Liquid Chromatography and has great potential in medicinal use. Another important finding was that glands of some members of *Eucalyptus* subgenus also contained flavanone *O*-glucosides and flavanone- β -triketone conjugates. In addition, glands also contained free β -triketones, β -triketone heterodimers and chromone *C*-glucosides. However, there is no fluorescence for some flavanones such as pinocembrin (9). Perhaps, visible green fluorescence in our case with *E. cinerea* belongs to other flavonoids. Unlike this species, leaves of *Eucalyptus viminalis* contains the hydrolysable tannins, galloyl esters and ellagitannins (20). The excitation by violet light at 420 nm showed that tannins might have fluorescence in blue-green region 480-500 nm (12). Thus, the emission of glands depended on the wavelength of excitation and differed in the species. Seen under

luminescence microscope, glands of *E. cinerea* coloured in yellow or yellow-red did not fluoresce under ultraviolet or violet light, except cells of bundle around the secretory structures. Excitation of light 450-490 nm induced green emission of yellow glandular cells, which appears related to some flavonoids present in the oil, while glands coloured in red or have red secretion did not emit. Unlike *E. cinerea*, glands of *E. viminalis* did not fluoresce at all in our conditions, perhaps, due to the presence of non-fluorescent phenols. However, the surface cells around glands fluoresces here at all excitation parameters from blue to red light.

2. Laser-scanning confocal microscopy. We used the laser-scanning confocal microscope to find the glands fluorescence and registered the spectra from individual secretory structures. This technique permitted us to see: (i). all images in 3-light channels (blue, green and red); (ii) images from optical single slices and (iii) their sum or summa, assembling the optical slices images in visible fluorescing structure (in one channel or in all 3-channels).

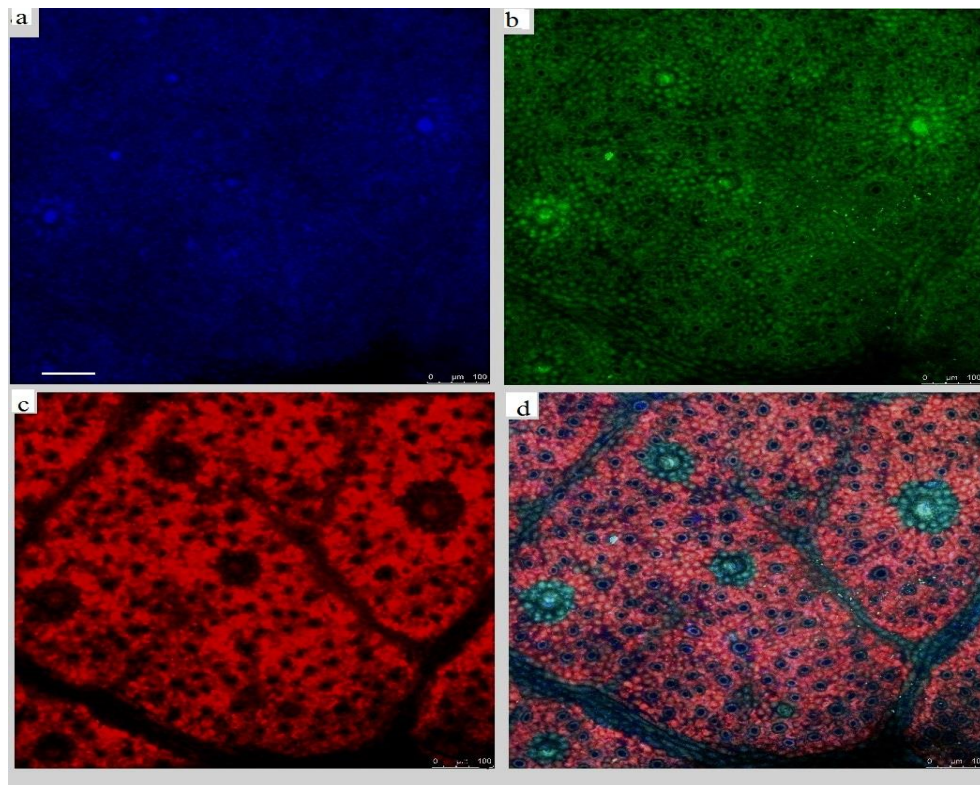


Figure 3. The images of the surface of *Eucalyptus cinerea* under confocal microscope *Leica* TSP-5 at laser excitation 458 nm in three channels –blue, green, red (a-c) and summa (d). Bar = 100 µm.

The laser-scanning confocal microscopy showed us the structure of glands and their connection with oil ducts in both test *Eucalyptus* species. Seeing the images in three

channels with laser-excitation 458 nm, we found the main emission from glands of *E. cinerea* as green (Fig. 3 b). This technique also showed small blue fluorescence in blue channel (Fig. 3a) and also the connection between oil glands and oil ducts (Fig. 3 c). Summary of images in 3-channels are seen in Fig. 3d. Comparison of images of single gland optical slices and assembled images of slices named as stack in green and red channels (Fig. 3 b and c), showed bright green fluorescence in first channel and no emission in second channel.

Red chlorophyll fluorescence was observed outside the glandular structures (Fig. 3 c). Within the glands, only oil was present, and chlorophyll was absent. Thus, green emission was stronger in the middle of the secretory structure, while the bundle cells around the gland had less emission. Thus a single gland looks as the vessel with oil secretion.

Confocal microscopy also permits us to record the fluorescent spectra from certain parts of images. In the fluorescence spectra of glands and ducts, we recorded characteristic maximum at 535-540 nm (Fig. 4). One can see ROI (research of interest) parts in images and spectra: Numbers 1 and 2-for glands and number 3-for duct. Glands and ducts have the same maxima 535-540 nm (weak contribution of chlorophyll maxima at 680 nm was also seen). This confirmed similar secretion content in both secretory structures. Moreover, ROI in the middle of gland (image 1) and some cells of bundle (image 2) are almost identical. Around secretory cells and ducts, fluorescence belongs mainly to chlorophyll – big maxima 675-680 nm in the emission spectra (Fig. 4, number 4).

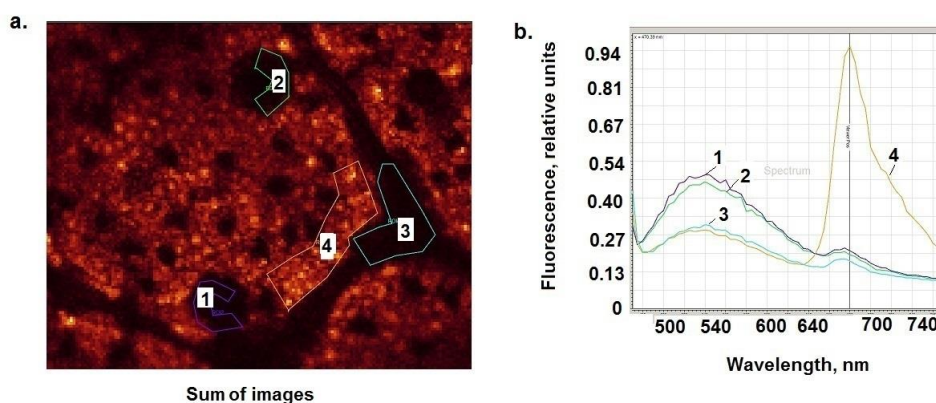


Figure 4. The ROI of images (a) and spectra (b) of the surface of *Eucalyptus cinerea* under confocal microscope *Leica* TSP-5 at laser excitation 458 nm. Bar = 100 μ m. 1: Middle of gland and 2: Bundle cells of glands, 3: Duct, 4: Surface out glands

Usually, maxima 535-540 nm is peculiar to some flavonoids and alkaloids (12). But we do not know those compounds, which emits in mixture with oil of *Eucalyptus cinerea*. Presence of alkaloids in these test species is not known. The occurrence of monoterpenes like non-fluorescent 1,8-cineole in eucalyptus oil is known (21). Although some other monoterpenes and sesquiterpenes, present in oils, fluoresce in the range of 415-460 nm (12). As possible fluorophores, flavonoids may fluoresce in region of 450-480 nm and blue-green part at 490-520 nm (12,23). Unidentified flavonoids shown earlier by histochemical staining with special dyes, are present in the glands of *E. cinerea* (2). Among the fluorescing

flavonoids and coumarins, emission maxima in the 530-550 nm region was peculiar to rutin and esculetin (12), but their presence in eucalyptus genus is not established yet. However, a new problem arises: how phenol or alkaloid compounds interact with oil(s). Due to the interaction, the fluorescence becomes absent or changed as compared to emission of single individual substances. Some model systems alkaloid-oil or phenol-oil were studied earlier only in few experiments (12,14). Analysis of fluorescence of similar mixtures needs to be studied in future.

Confocal microscopy gives the information about the concentration of fluorescing compounds in single gland, using the optical slices. We have found the data of two channels - green and red (Fig. 5) and combined the Images and spectra. The gland fluoresced only in green channel, when was excited by light at 458 nm, and the emission was absent in red channel. The received spectra with maxima 540 nm as seen in Fig. 4 were identical for any slice up to the end of gland. This showed the homogeneous mixture of oil and fluorescing phenolic component in the secretion in glandular structure.

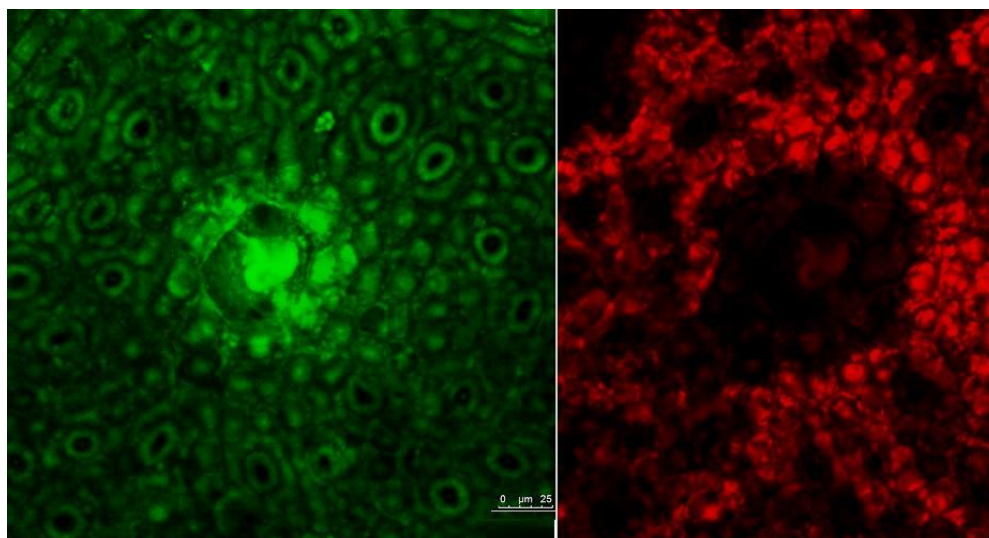


Figure 5. The images of gland optical slices through 2 μm from leaf of the *Eucalyptus cinerea* in green and red channels. Under confocal microscope, slices were collected in the total image – stack. Bar = 25 μm .

In comparison to usual luminescence microscopy, the confocal microscopy gave more information in our experiments on *Eucalyptus viminalis* (Figs. 6 and 7). In three channels, there was weak emission. However, fluorescence was dark blue, mainly in the centre of every gland (Fig. 6a). The emission was significant in green (Fig. 6b). The absence of the secretory structure emission in red channel (Fig. 6c) showed us the clear location of ducts in contact with the glands. These connections were visible in red channel as dark structures. In green channel, we also showed the connections between glands and ducts in green color (Fig. 3b). It was also well-seen in summary images in three channels (Fig. 6d).

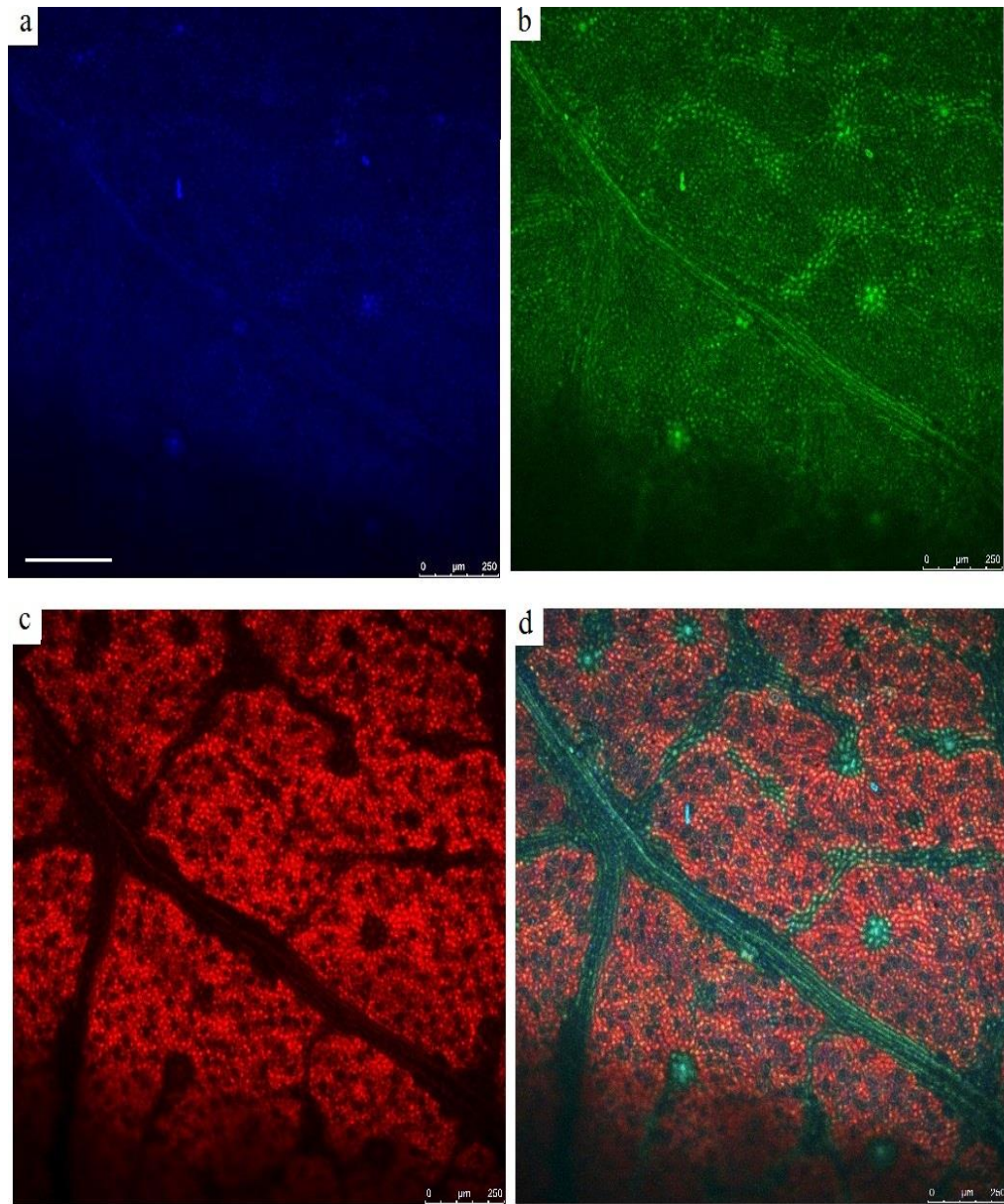


Figure 6. The images of the surface of *Eucalyptus viminalis* under confocal microscope *Leica TSP-5* at laser excitation 458 nm in three channels (a-c) and their summa (d). Bar = 250 µm.

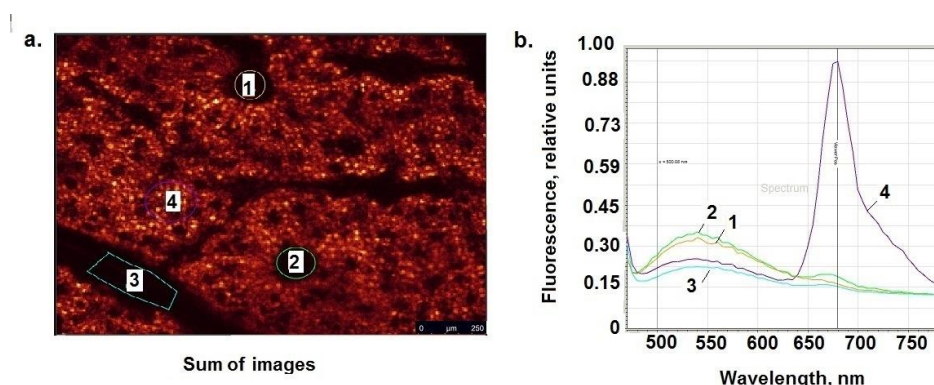


Figure 7. The ROI of images (a) and the spectra (b) of the surface of *Eucalyptus viminalis* under confocal microscope *Leica TSP-5* at laser excitation 458 nm. Bar = 250 μm. 1 and 2 : Glands, 3: Duct, 4 : Surface out glands

Stack of optical slices (All assembled images from each slice) seen in 3-channels provides the possibility to receive the fluorescence spectra from certain parts of leaf surface ROI. Fig. 7 showed that the glands (images 1 and 2) and ducts (image 3) have one and same maxima 540 nm as seen in *E. cinerea*. The chlorophyll was minimal here. On the contrary, surrounded red fluoresced cells have maximal peak at 680 nm, peculiar to chlorophyll. In *E. viminalis*, the compounds which fluorophores emit at 500-560 nm, appeared to be terpenoid phenolaldehydes isolated from leaves (7). However, there is little information about the phenolics contents of *E. viminalis* and *E. cinerea*. Flavonoids were found in many *Eucalyptus* species (5) which may fluoresce. Besides, other compounds were polyphenols that had weak emission in blue, not in green.

Thus, oil glands of both *Eucalyptus* species: *E. viminalis* and *E. cinerea* have similar structure and contents as seen in spectral characteristics. We conclude that differences between these *Eucalyptus* species may be in fluorescing phenols containing oil or part of terpenoid molecules of oil (terpenophenols) in secretory structures. However, this will be studied in future investigations.

CONCLUSIONS

Fluorescent methods not used to study the leaf *Eucalyptus* secretory cells were found of interest for allelopathy, pharmacy and structural botany. Autofluorescence of intact leaf cells from *E. cinerea* and *E. viminalis* can be used in the laboratory to find compounds and their contents. The fluorescent methods allowed us to study the structure and content of secretory systems of two *Eucalyptus* species: *E. viminalis* and *E. cinerea*, rich in oils, based on their autofluorescence characteristics. Variations in the excitation light wavelength on leaf glands and ducts showed the presence of compounds (possibly phenols) fluorescing, mainly, in the green range 535-540 nm (confirmed by both usual luminescence and confocal microscopy) and only little emission in blue (confocal microscopy). Chlorophyll was seen in non-secretory cells that surrounded the secretory structures.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest : The Authors declare no conflict of Interest.

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