SPECTROSCOPY OF CHROMOPHORIC ORGANIC SUBSTANCES RELEASED BY SOIL MICROSCOPIC FUNGI INTO AQUEOUS MEDIUM

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ABSTRACT

Spectral properties of chromophoric organic matter released by microscopic fungi into aqueous medium were measured and compared with that for natural humic substances (HS) and commercial humate preparation in water. Chromophoric organic matter released by various fungi strains showed absorption and fluorescence spectra similar in main spectral features for samples with different pigmentation of mycelium: decreasing absorbance values towards longer wavelengths with a shoulder at 280-290 nm and fluorescence emission spectra with two overlapping bands (UV fluorescence of phenolics and proteins within 300-350 nm and blue fluorescence of fungal polymers). After two weeks of cultures growing in aqueous humate solution its fluorescence characteristics became more similar to that of HS in natural water. Wavelength of emission maximum and quantum yield of humic-type fluorescence band were found to be excitation wavelength dependent because of increased heterogeneity of HS in culture medium compared to initial commercial humate solution. Transformations in HS composition caused by microscopic fungi can be monitored and characterized using spectral measurements.

INTRODUCTION

Microscopic fungi are among the most diverse organisms in the world (i), representing very important functional and structural component of biological ecosystems (ii). Fungal communities play a significant role in human well-being and ecological processes. Nowadays, these microorganisms are widely used in biomedical research and biotechnology. In addition to direct benefit (sources of antibiotics) or adverse effects (agents of disease), microscopic fungi can impact many environmental processes, particularly those associated with the decomposition of organic matter. Fungal natural habitats include soil, water, and various organisms.They are present in almost all regions and climates, even under extreme conditions. However their role in geochemical cycling usually remains underestimated.

The processes of soil organic matter transformation and regularities of utilization of plant residues by fungi were described in (iii,iv,v,vi). Fungi affect the soil properties in different ways: via transformation of organic matter, via soil structure status, acidity and temperature, as well as via the regulation of soil microbiota functioning. Fungi play an important role in plant litter decomposition through nutrient recycling and humus formation in soil because they attack the lignocellulose matrix in litter that other organisms are unable to assimilate. Biodiversity and potential functions of microscopic fungi in aquatic ecosystems were shown in (vii,viii). While it is under debate whether microscopic fungi are capable to produce humic substances directly (ix,x), undoubtedly they play essential role in lignin degradation and humic substances turnover (xi,xii).

Humic substances (HS) are natural organic compounds comprising 50 to 90% of the organic matter of peat, coal, and sapropel (i.e., sludge accumulated at the bottom of lakes), as well as of the nonliving organic matter of soil and water ecosystems (xiii). Being the products of stochastic synthesis, HS are characterized as polydispersed substances having non-stoichiometric elemental composition with irregular and heterogeneous structures. Thus, it is not possible to assign an exact structure to HS. Instead, they are operationally defined using available compositional, structural, functional, and behavioral data (xiv).

It was established that some microscopic fungi are capable to produce dark brown polymers in the presence of dead plant biomass, and thereby might contribute to the pool of humic substances in a salt marsh estuaries (xv,xvi).The fungal polymers resemble humic substances obtained from dead plant biomass or from salt marsh sediments in their elemental composition, elemental ratios, and spectral characteristics (UV, visible, FTIR) (xvii). Comparative studies of fungal polymers - melanin and humin-like substances (HLS) produced by individual and mixed cultures of basidiomycetes - showed their similarity in elemental composition and physicochemical properties to natural HS (xviii,xix). Melanins are polymers of phenolic and/or indolic nature. The position of HLS among other natural polymers formed under similar conditions, such as melanins and HS, is still not determined (18). According to the IR data and 13C-NMR spectroscopy fungal melanin and HLS were close to soil humic acids, and fungal HLS displayed higher similarity to natural humic acids compared to fungal melanins. Studies of the dynamics of laccase production suggested that the HLS were produced via extracellular degradation of lignin macromolecules with involvement of laccase, an extracellular oxidase of basidiomycetes (19).

Interaction of microorgamisms with non-living organic matter in aqueous and terrestrial bioceonoses is an intriguing problem for our environment (xx,xxi). However the effect of HS on microscopic fungi has not been studied much. The influence of commercial HS potassium humate on some physiological characteristics of microscopic fungi has been studied in (xxii) using cultures with differently pigmented mycelium. It was shown that growth rate for the colony grown on medium containing 0.1% or 0.02% of potassium humate in relation to that for the control colony grown on medium without humate decreased for black and deep-brown colonies and predominantly increased for light-colored colonies. Growth rate of non-pigmented strain *Fusarium moniliforme* did not show noticeable changes in growth rate.

Fluorescence spectroscopy is the effective method to study dissolved organic matter naturally occurring in water (xxiii,xxiv,xxv,xxvi) and commercial HS (xxvii,xxviii). First results on investigation of HS transformations caused by microscopic fungi were reported in (xxix). The objective of the recent work was detailed study of fluorescence properties (change of emission maximum, fluorescence quantum yield) of chromophoric organic substances released by soil fungi into water without and in presence of commercial humic substances.

METHODS

We have analyzed the following soil fungal strains with different pigmentation of mycelium: *Fusarium moniliforme* (non-colored)*, Alternaria alternata* (dark pigmented)*, Phoma glomerata* (dark-brown pigmented)*, Cladosporium cladosporioides* (black pigmented)*, Geomyces pannorum* (brown-pigmented) and *Mycelia sterilia* (orange-brown pigmented)*.* The fungi species were cultivated in liquid Czapek medium with addition or without addition of potassium humate Powhumus (produced from brown coal leonardite) in concentration of 0.2 g/l. All cultures grew up for 2-6 weeks in 200-ml glass flasks in darkness at temperature of 25 degrees. Thereafter filtered culture fluid was used for further spectral investigations.

Absorption spectra were measured for filtered samples without dilution using UV-Vis spectrophotometer Unico 2804. Fluorescence emission spectra were registered for excitation wavelengths 270, 310 or 355 nm using luminescence spectrometer Solar CM 2203. The choice of excitation wavelength was based on previous reports on studying spectral components of chromophoric dissolved organic matter (25,xxx). For fluorescence measurements fungal exudates were diluted in 10 times. All spectra were registered in standard quartz cells with 1-cm optical path.

The fluorescence quantum yield (QY) for each excitation wavelength was calculated using a reference sample with known quantum yield. As a reference sample the solution of quinine sulphate was used, because of similarity of its fluorescence band to HS spectral band, both in shape and in location of the maximum. The quantum yield of quinine sulphate dissolved in aqueous solution of sulphuric acid in water with concentration 0.05 mole/l is 0.546 (xxxi).

RESULTS AND DISCUSSION

Absorption spectra

Typical absorption spectra of filtered fungal exudates are featureless, with a monotonic decline with wavelength increasing from 200 to 700 nm (Figure 1). In some wavelength ranges certain spectral features have been observed. We attribute them to absorption of light by phenolics or quinones (a shoulder located around 280-290 nm) and by carotenoids or melanin pigments (the band spreading from 400 to 500 nm typical for brown-pigmented cultures).

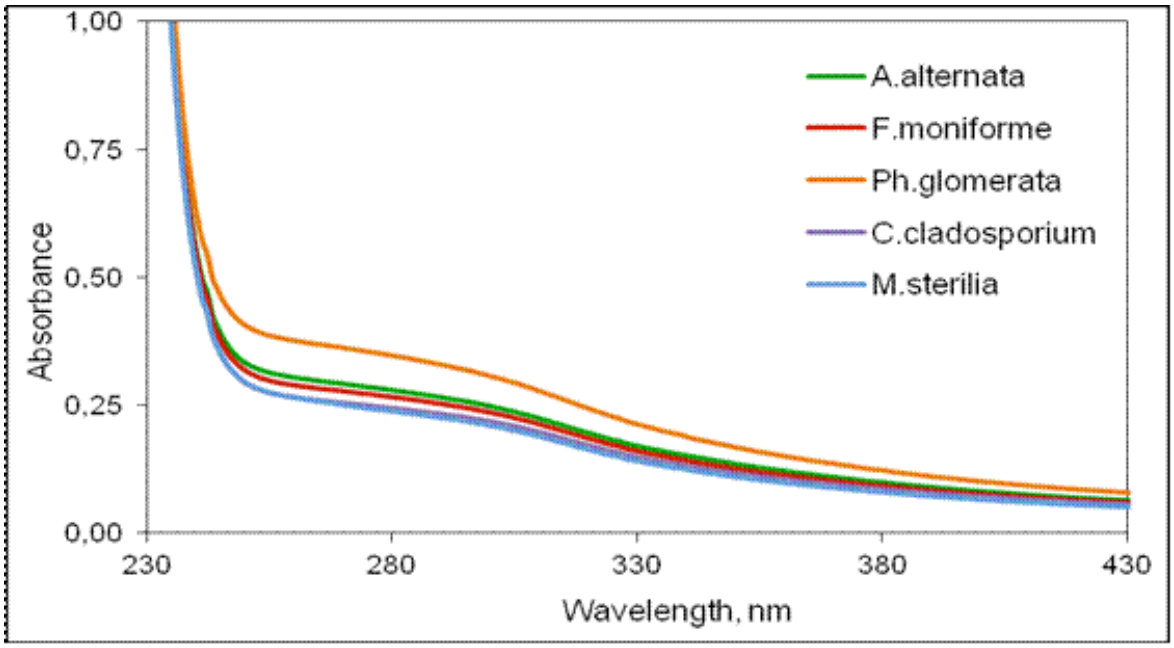

Figure 1: Absorption spectra of fungal exudates.

Fluorescence spectra

Fluorescence of fungal exudates with addition or without addition of potassium humate was observed in the 280-600 nm spectral range (Figures 2-3). Typical fluorescence spectra of fungal exudates excited at 270 nm consist of two overlapping bands: the UV peak with maximum around 300-350 nm attributed to fluorescence of phenolics or protein complexes and the blue emission band called as humic-type fluorescence. As it was preciously shown in experiments with natural water samples and aqueous soil extractions (25-28), typical fluorescence of natural HS has emission maximum position in the range 420-460 nm, while fluorescence emission band of commercial humates is shifted to longer wavelength region (500-520 nm) compared to natural HS.

Emission maximum position of natural HS samples depends on excitation wavelength λ_{ex}. The wavelength of maimum emission excited at λ_{ex} = 310 nm shifts towards shorter wavelength compared to that excitated at 270 nm and 355 nm. This phenomenon was observed for all types of natural HS (26,30) and is known as “blue shift” of DOM fluorescence (xxxii,xxxiii). In contrast, the position of emission maximum for commercial HS does not depend on excitation wavelength (27-28).

After fungi growing in the humate-containing medium their fluorescence spectra consist of two broad overlapping bands: UV fluorescence of phenolics or proteins and the wide band around 425-470 nm conditional on fungal metabolite products (Figure 3). With λ_{ex} = 310 nm the position of the second band is shifted towards shorter wavelengths compared to fluorescence of the same sample excited at 270 nm or 355 nm. This resembles the spectral features of dissolved organic matter occurring in natural water (26,30). This spectral behavior can not be explained by simple addition of fluorescence bands for humate and fungal metabolites, since blue fluorescence of fungal polymers is not enough strong to give such an effect.

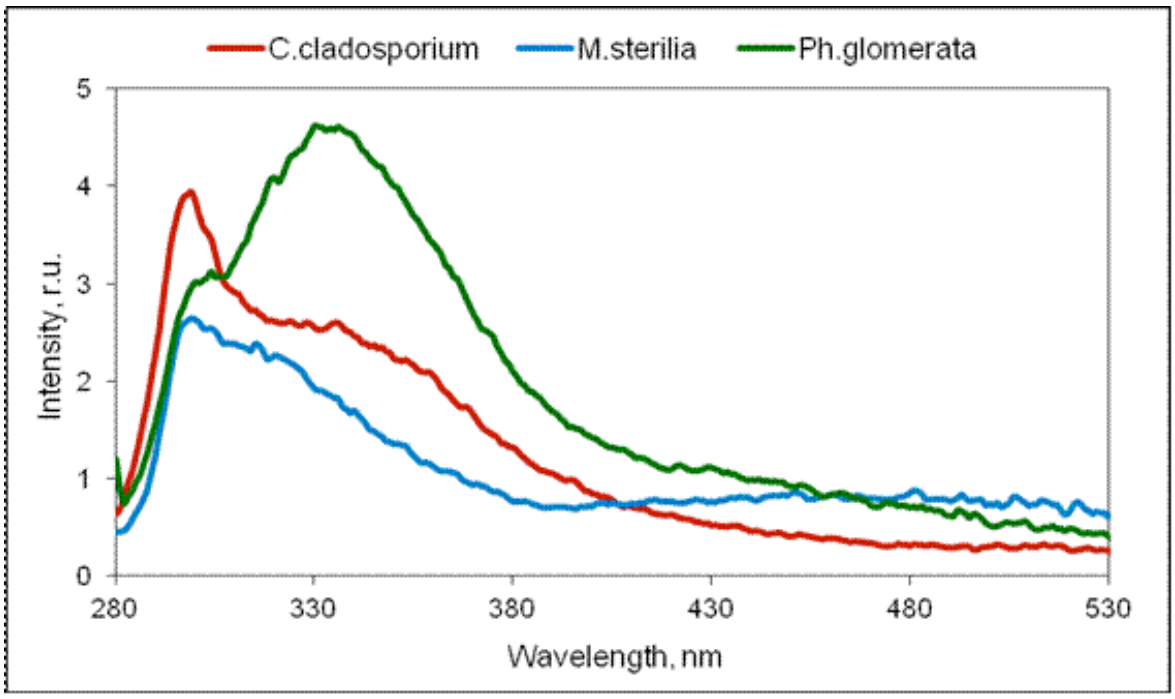

Figure 2: Fluorescence spectra of fungal exudates excited at 270 nm.

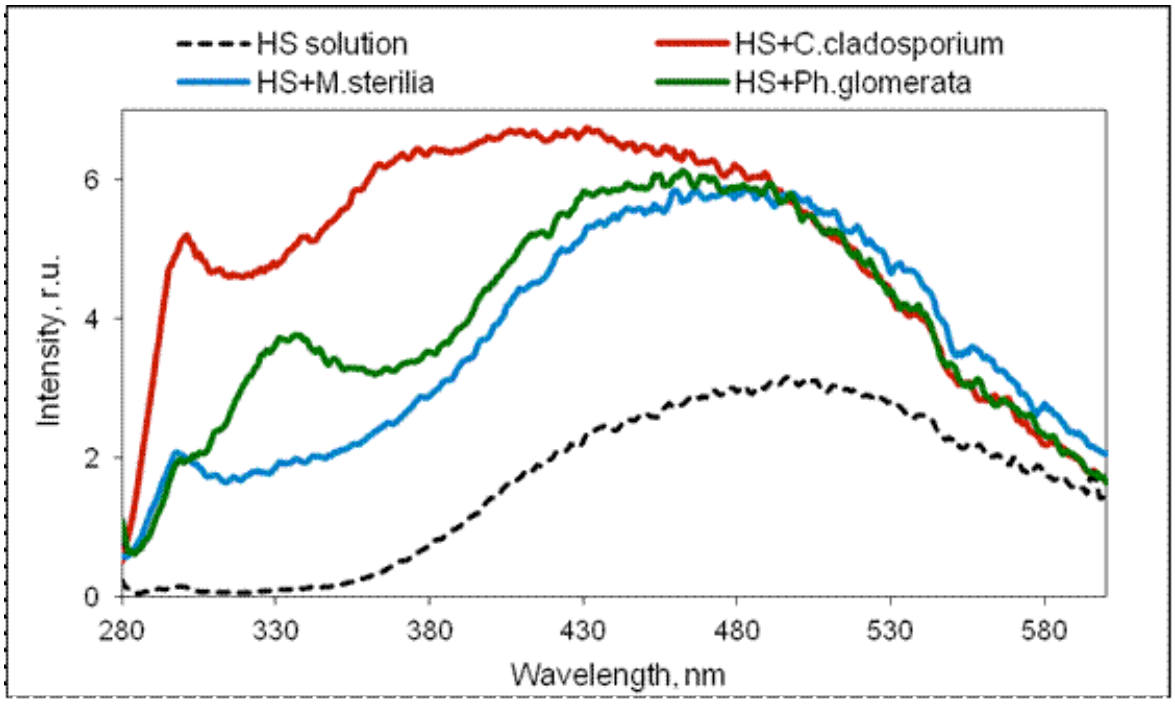

Figure 3: Fluorescence spectra of fungal exudates for cultures grown with addition of humate excited at 270 nm.

Fluorescence quantum yield (QY)

The fluorescence QY value varies within 2-4% for natural water samples, 0.1-0.3% for aqueous soil extractions, and 0.4-1.2 for commercial HS samples dissolved in water (27-28). Fluorescence QY of most of natural HS increases along with excitation wavelength rising from 270 to 355 nm (28).

Our experiments revealed that fluorescence properties of HS solution were essentially changed after growing of fungi culture in it (see Table 1 and Figure 3). Fluorescence QY of HS modified by fungi increased, and emission maximum was essentially shifted towards shorter wavelengths compared to that for original solution of commercial humate.

Moreover, the spectral behaviour of humic-type fluorescence along with rising excitation wavelength has been changed after growing of fungi cultures. For original HS solution fluorescence QY was slightly decreasing with λ_{ex} rising, but after fungi growing during at least two weeks the culture medium containing commercial HS demonstrated fluorescence QY increasing along with λ_{ex} rising. Emission maximum wavelengths for initial HS solution were similar for λ_{ex} = 270, 310 or 355 nm, while after 14 days of cultures growing in it emission maximum became dependent on excitation wavelength.

Table 1: Fluorescence quantum yield (QY) and emission wavelength λ_{em} for commercial HS solution and fungal exudates for cultures grown with and without addition of HS.

QY and λ_{em} (for given λ_{ex})	*HS solution*	*C.cladosporium CF*		*Ph.glomerata CF*		*M.sterilia CF*	
		without HS	*with HS*	*without HS*	*with HS*	*without HS*	*with HS*
QY (λ_{ex}=270nm)	0,6%	2,4%	3,3%	1,3%	1,9%	1,5%	1,4%
QY (λ_{ex}=310nm)	0,5%	1,7%	5,2%	0,5%	1,9%	0,9%	2,1%
QY (λ_{ex}=355nm)	0,4%	5,0%	7,2%	1,0%	2,1%	2,3%	3,4%
λ_{em} (λ_{ex}=270nm)	498	UV	414	UV	423	UV	459
λ_{em} (λ_{ex}=310nm)	501		423		435		446
λ_{em} (λ_{ex}=355nm)	500		451		465		473

These findings we explain by transformation of refractory HS by fungi cultures during their growing from bigger to smaller macromolecules. Microscopic fungi utilize macromolecular compounds with longwave fluorescence emission and produce smaller ones with emission shifted towards shorter wavelengths. Dependence of fluorescence QY and λ_{em} on λ_{ex} reflects heterogeneity of composition of humic-type substances in the culture medium. Thus, spectral characteristics of potassium humate solution became similar to such characteristic of natural HS.

For confirmation of the received data we have made experiments to compare fluorescence of dissolved organic matter in natural waters and commercial humic acids. Test samples were taken from Moscow and Setun rivers. Further river samples were filtered through cellulose acetate filter UAM-50 (Vladipor, Russia) with 5 nm pore size to get low-molecular weight fraction. As a result of investigations we submit the list of spectral characteristics (see Table 2) of HS in initial water sample and low molecular weight fraction. Humic acid Aldrich was dissolved in water to receive the samples of commercial HS.

Once again we have observed that emission maximum of commercial HS solutions is shifted to longer wavelength region compared to that of natural HS in water. So-called “blue shift” was observed for natural water with change in excitation wavelength from 270 to 310 nm, and it did not occur for humic acid solutions. We can resume that that for low molecular weight fraction of riverine HS the fluorescence QY value is higher than the same value for the initial natural HS, which proves the hypothesis that microscopic fungi break macromolecular HS compounds producing smaller ones with higher fluorescence QY.

Table 2: Fluorescence quantum yield (QY) and emission wavelength λ_{em} for natural HS samples (dissolved organic matter in natural water and its low molecular weight fraction) and commercial humic acids.

QY and λ_{em} (for given λ_{ex})	*Initial natural HS*			*Low molecular weight fraction of natural HS*			*Commercial humic acids*	
QY (λex=270nm)	1,8%	2,7%	2,6%	2,2%	4,0%	3,9%	1,3%	1,3%
QY (λex=310nm)	2,5%	3,2%	3,4%	3,0%	4,8%	3,9%	1,2%	1,2%
QY (λex=355nm)	3,6%	3,9%	5,0%	5,0%	7,2%	5,7%	1,0%	1,1%
λem (λex=270nm)	446	446	438	450	436	439	478	477
λem (λex=310nm)	428	421	420	424	420	423	475	475
λem (λex=355nm)	448	445	445	451	447	445	476	477

CONCLUSIONS

Soil fungal strains with different pigmentation of mycelium (from non-colored to dark-pigmented) were grown for few weeks in aqueous medium with and without addition of potassium humate commercially produced from brown coal. Chromophoric organic matter released by various fungi strains into aqueous medium showed absorption and fluorescence spectra similar in main spectral features for various fungi strains: decreasing absorbance values towards longer wavelengths with a shoulder at 280-290 nm and fluorescence emission spectra with two overlapping bands (UV fluorescence of phenolics and proteins and blue fluorescence of fungal polymers). After two weeks of microscopic fungi growing in humate solution (concentration 0.2 g/l) its fluorescence characteristics became more similar to that of dissolved organic matter in natural water. Wavelength of emission maximum and quantum yield of humic-type fluorescence band were found to be excitation wavelength dependent. Our experiments revealed microbial degradation of coal-originated commercial humate to HS of smaller molecular size and increased heterogeneity. We resume that transformations of humic substances by fungal cultures can be monitored and characterized using spectral measurements.

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