Chapter 14

MITIGATING ACTIVITY OF HUMIC SUBSTANCES: DIRECT INFLUENCE ON BIOTA

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Abstract

Mitigating activity of HS can be defined as a phenomenon of lowering the adverse effects of contaminants toxicity and of those of abiotic stress factors such as unfavourable temperature, pH, salinity, etc. As a rule, it is related to the detoxifying properties of HS or to their beneficial effects on biota. This review focuses on the latter effects and considers the possible mechanisms of mitigating activity of humic materials in terms of biological activity of HS. The beneficial effects of HS on biota are segregated into the four categories according to the underlying mechanism of their action: an influence on the organism development; an enhancement in nutrient supply; catalysis of the biochemical reactions; and an antioxidant activity. The published data on the mentioned essential biological functions of HS are reviewed.

The given review shows that in spite of the numerous reports published recently to elucidate the mechanisms underlying biological effects of HS, the data reported are quite contradictory. The main reason is the complexity of HS structure. A wide variety of the effects observed can be explained by the polyfunctionality of HS. The general conclusion can be made – all biological effects of HS have been more pronounced under stress conditions independent of what stress factors are involved. Thus, HS can be considered as an environmental modulator mitigating the harmful consequences of stress factors.

1. Introduction

Humic substances (HS) are natural organic compounds comprising from 50 to 90% of the organic matter of peat, lignites, sapropels, as well as of the non-living organic matter of soil and water ecosystems [1-3]. According to the classical definition [4], HS are "a general category of naturally occurring heterogeneous organic substances that can generally be characterized as being yellow to black in color, of high molecular weight and refractory". They are a mixture of complex organic compounds that are usually

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separated into three fractions based on aqueous solubility: humic acids (HA), the fraction of HS that is not soluble in water under acidic conditions ($pH < 2$) but is soluble at higher pH values; fulvic acids (FA), the fraction of HS that is soluble in water under all pH conditions; humins, the fraction of HS that is not soluble in water at any pH value. HS cannot be described by unique, chemically defined molecular structures. They are operationally defined by a model structure constructed on the basis of available compositional, structural, functional, and behavioral data and containing the same basic structural units and the same types of reactive functional groups [5]. So, HS are very often supposed to be just an alkali extract from any organic substrate including sewage sludge, beer factory sludge, raw tea waste, composted grape marc, composted spend mushroom, composted bark, tobacco dust and others. Some authors use term "HS" to define materials which would be more correctly called as “humic-like” substances. This complicates interpretation of the reported data considerably. Despite of that, in this review, we kept the terminology used by the authors of the original papers.

HS have been the subject of numerous scientific studies due both to their mitigating effects on contaminants toxicity to biota [6-12], and to their anti-stress effects under abiotic stress conditions (unfavourable temperature, pH, salinity, et al.) [13-15]. The mitigating effects can be defined as a phenomenon of lowering the adverse effects of both specific and non-specific stress on biota. The detoxifying properties of HS are generally attributed to their capability for binding ecotoxicants. The binding of xenobiotics to HS causes formation of less bioavailable complexes and adducts followed by lowering their toxicity and bioaccumulation. Another phenomenon of importance is acceleration of biotic and abiotic mineralization of organic contaminants in the presence of HS [23-25]. In addition, the detoxifying ability of HS can be underlay by the beneficial effects exhibited onto living organisms. The latter are of particular importance for anti-stressor (or adaptogenic) activity of HS under conditions of the abiotic stress (water stress, unfavorable temperature).

The beneficial effects of HS on living organisms have been numerously reported (see Table 1). They can be provided by either indirect or direct impact of HS. The indirect effects are mostly provided by the HS-driven changes in environmental conditions such as bioavailability of some nutrients (due to increasing solubility), salt balance, physical and physico-chemical soil properties (soil structure, aeration, drainage, water retaining capacity, soil temperature, and others). On the other hand, HS are supposed to influence biota directly. The principal direct effects exhibited by HS onto living organisms include an increase in biomass accumulation, nutrient uptake, biosynthesis, antiviral activity, and others [13, 14, 27-53, 90-96].

In spite of numerous studies on the biological effects of HS, the mechanism of their action remains unclear. The main reason seems to be the stochastic nature of HS. In contrast to common biological macromolecules, which are synthesized by a living organism according to the information encoded in DNA (nuclear acids, proteins, enzymes, antibodies etc.), HS are the products of stochastic synthesis. They are characterized as polydisperse materials having elemental compositions that are non-stoichiometric, and structures, which are irregular and heterogeneous [16]. The above features hamper a use of common biological approaches to study biological activity of HS. This review represents the first attempt to address the possible mechanisms underlying mitigating activity of humic materials in terms of biological activity.

2. Principal components of mitigating activity of humic substances

The ability of HS to reduce toxicity can result from both their influence on metabolic pathways of ecotoxicants and beneficial effects on living organisms (Fig. 1).

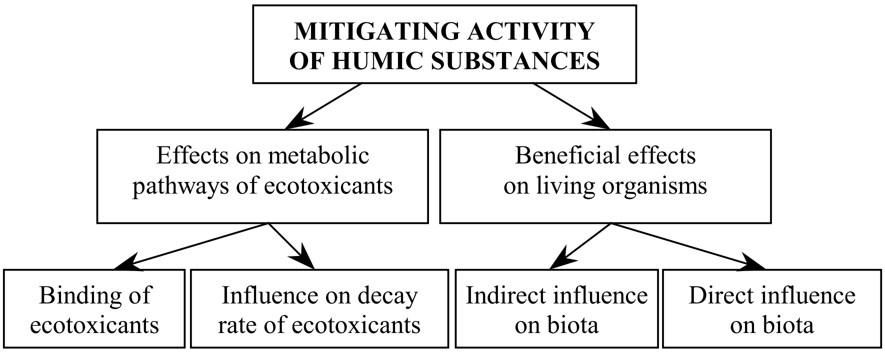

Figure 1. Principal components of mitigating activity of humic substances.

The effects on the metabolic pathway of the ecotoxicants are related to the chemical nature of humics that can be considered as irregular polymers of aromatic polyhydroxyl polycarbonic acids [3]. The peculiar structural feature of HS is a coexistence of both polar and hydrophobic environments in the same molecule. As a result, HS are able to bind both polar and hydrophobic xenobiotic organic compounds, and inorganic ions. The binding to HS causes a change in speciation of xenobiotics followed by a change in their toxicity and bioaccumulation. Based on the binding properties of HS, several remediation technologies were developed [17-22].

Humic materials can also facilitate degradation of some organic contaminants. For example, chemical hydrolysis, followed by degradation by soil microorganisms, could account for most of the atrazine breakdown in soil [23]. Addition of humic material was shown to increase the rate of atrazine hydrolysis. HS can also affect photodegradation, which is important for PAHs, chlorinated aromatic hydrocarbons, chlorinated phenols, and many other pesticides [162]. It was demonstrated that the photolysis rate of 1-aminopyrene could be enhanced by addition of HS [24]. The first order photolysis rate constant of 1-aminopyrene (10 μM) in the phosphate buffer (pH 7.0, 1 mM) containing HA (20-80 ppm) was enhanced by a factor of five compared to that in the absence of HA. Humic materials was hypothesized can transfer energy of oxygen facilitating formation of an excited state oxygen molecule. The single oxygen attacks the organic compound that decays due to indirect photolysis. The similar effect was observed for atrazine [24]. Of importance is, that the photolysis of atrazine was shown to be enhanced by an addition of HA, while the introduced FA caused no effect within 10 days. Recent study has shown that HS played a remarkable role in condensation reactions in the aquatic environment [25]. HS have been found to act as catalysts in Knoevenagel and Claisen-Schmidt reactions accelerating condensation of carbonyl compounds with aliphatic acids. Hence, they may influence the fate of organic pollutants in the environment.

It has been also suggested that these are rather humus-enzyme complexes than HS alone, that are essential for functioning of stable and resilient soil systems. These complexes are considered to be the “crossing-points” between mineral and organic reactions in soil [26]. On the other hand, HS are supposed to be able to influence biota directly. Principal observed direct effects of HS on living organisms are summarized in Table 1.

Table 1. Principal biological effects of HS on living organisms.

Observed effect	References
Plants	
Stimulation of seedling germination and growth	[13, 14, 27-38]
Stimulation of biomass accumulation	[13, 14, 39-46]
Stimulation of nitrogen accumulation	[40-42, 47-53]
Stimulation of mineral elements uptake	[40, 46, 54-61]
Stimulation of biosynthesis of proteins, carbohydrates, chlorophyll et al.	[13, 14, 46, 49, 62]
Stimulation of photosynthesis or respiration	[13, 14, 63-67]
Adaptogenic and detoxifying effects (against excess mineral nutrition, salinity, water stress, unfavourable pH, temperature etc., presence of xenobiotics)	[11, 13-15, 44, 68-73]
Animals	
Stimulation of biomass accumulation	[15, 74]
Immunomodulating activity	[15, 75]
Desmutagenic activity	[68, 76, 77]
Detoxifying ability	[9, 78, 79, 99]
Toxicity	[80, 81]
Microorganisms	
Stimulation of biomass growth and biosynthetic activity	[26, 82-87]
Detoxifying ability	[88, 89]
Viruses	
Antiviral activity	[90-96]

Table 1 shows that, first of all, the direct effects of HS on biota are very versatile; and second, there is no unified mechanism governing biological activity of HS. Of importance is that in contrast to the substances with a well-defined mode of action, HS effects drastically depend on the environmental conditions. David et al. [97] have shown that the most striking examples of HS beneficial effects on plant nutrition were observed under unfavorable growth conditions. Dunstone et al. [98] have found that the largest effects of fulvic acids on stomacal conductivity of wheat were observed under conditions of the sustained drought and hot, dry winds. At the same time, the authors were unable to demonstrate the similar effects of FA on grain yield in droughted plants either in the field or in glasshouse. This elucidates another peculiar feature of HS – instability and poor reproducibility of the biological effects.

Along with the numerous reports on HS detoxifying properties, some data on increasing toxicity of xenobiotics in the presence of HS have been reported as well [80, 81, 100-102]. HS was shown to suppress the immune and inflammatory reactions of cultured human umbilical vein endothelial cells (HUVECs) responsible for endotoxin lipopolysaccharide (LPS) detoxifying. Pretreatment of HUVECs with 100 mg/L HS for two days markedly suppressed the LPS induced expression of adhesion molecules and almost completely inhibited this process at a concentration of 200 mg/L. The authors suggested that HS could be a potential toxin causing blackfoot disease.

3. Beneficial effects of HS on the living organisms

Upon summarizing the discussed above beneficial effects of HS on biota, the following principal ways of HS action could be proposed:

1. HS affect the organism development. Being utilized as a substrate (a source of organic carbon) or nutrient source (N, P, trace elements and vitamins), HS can serve as a moiety of the biosynthesis chains. On the other hand, beneficial effects of HS on the plants are often attributed to hormone-like activity of HS.
2. HS enhance nutrient supply. HS can improve plant growth by increasing uptake of such nutrients as nitrogen, potassium, and some micronutrients.
3. HS catalyze some biochemical reactions. The mechanism of HS interaction with enzymes has not been established yet, - there are no direct evidences that HS can serve as co-oxidants or mediators of enzyme stimulating reactions. The participation of HS in catalysis of biochemical reactions should be thoroughly studied.
4. Antioxidant activity of HS. HS can reduce free radicals resulting from stress such as drought, heat, ultraviolet light and herbicide use. Free radicals are damaging because they are strong oxidizing agents, which damage lipids, proteins and DNA within plants cells.

The comprehensive and recent data on each of the essential biological functions of HS are described below.

3.1. INFLUENCE OF HS ON ORGANISM DEVELOPMENT

3.1.1. HS as a substrate or a nutrient source

The number of microorganisms which are reported to be able of decomposing HS is rather great (Table 2). Still, the information available on the mechanisms of HS transformation and utilization is very scarce.

Of interest is a study on the decomposition of HA isolated from two differently aged sanitary landfills by soil microflora under aerobic conditions [103]. With HA used as a supplementary nutrient source, the level of their utilization was 63.6% for a fresh refuse, and 88.5% - for the refuse disposed for 12 months. When HA was used as a sole source of both carbon and nitrogen, the complete utilization was observed. The decomposition process resulted in alteration of HS properties, namely, in a decrease of molecular weight of humic materials. FTIR spectroscopy has also revealed some changes in HS structure: the complete elimination of C=O band of COOH group, reducing or elimination of C-O stretching in polysaccharides, a strong decrease in absorption of CH_3 and CH_2 groups, and a removal of some aromatic structures [103].

The above results are in good agreement with those reported for HA samples extracted from a mixture of municipal waste and sewage sludge [104]. The yield of microbial biomass substantially increased in the cultures containing HA as a supplementary nutrient source and reached 195% in case of HA from the fresh refuse. At the same time, under the depleted nutrients and low energy source conditions, the same HA inhibited the production of microbial biomass. This effect was attributed to an inhibitory effect of HA decomposition products. The polymerization of low-molecular weight compounds with formation of more stable HS was hypothesized as well.

The numerous attempts to elucidate the mechanism of microbial decomposition of HS were made using a model system of fungi and coal of different ranks [82, 85, 87, 114-117]. These data allowed the authors of this review to propose the scheme of coal HA decomposition by *Basidiomycetes* and *Deuteromycetes* shown in Fig. 2. The effects of these white-rot fungi on the coal-derived HA are determined by the specific features of the fungal ligninolytic enzyme system. The latter consists of lignin peroxidase, manganese peroxidase, other peroxidases, laccase, and supporting enzymes generating H_2O_2. It was shown that depolymerization of lignite HA took place only after its methylation. It seems like lignin peroxidase catalyzed the bond cleavage in the methylated HA and oxidized non-phenolic aromatic groups to aryl cation radicals with further cleavage of C-C and C-O bonds. The predominant products of these enzymatic and enzyme-mediated reactions were methoxylated monoaromatic compounds. Mn peroxidase oxidized Mn(II) to Mn(III). The latter is stabilized due to complexation with organic acids (oxalate, malonate, malate, tartrate or lactate) produced by *Basidiomycetes*. The formed Mn(III) complexes act as high redox potential mediators. Such system – Mn peroxidase and chelated Mn(III) - can be supported by addition of appropriate redox-mediators such as thiols or lipids or unsaturated fatty acids. As a result, the degrading power of this system exceeds that of the enzyme itself. The formation of low molecular weight FA was observed both in the *in vivo* experiments with Mn(II) – amended fungal cultures and in the *in vitro* studies on the coal HA depolymerization using Mn-peroxidase. The correlation between laccase excretion and degradation of coal HA *in vivo* has been also observed for *Trametes versicolor* [110]. Moreover, system laccase and different redox mediators were successfully applied recently to lignin and kraft pulp depolymerization [115].

The ways of HS usage as an organic carbon or nitrogen source by higher organisms are even more sophisticated. Several studies on the uptake of HS by higher plants have been performed. Earlier work relied on the colour changes in the plant organs as an indication of HS uptake [118]. It was found that FA of lower molecular weight can be

Table 2. Some fungi and bacteria degrading humic substances.

Species[1]	Reference
Wood- and straw-degrading basidiomycetes[2]	
Basidiomycete strains RBS 1k, JF 596, i63-2	[105-107]
Clitocybula dusenii	[107, 108]
Fomitopsis pinicola, *Gymnopilus sapineus*, *Hypholoma fasciculare*, *Hypholoma frowardii*, *Kuehneromyces mutabilis*, *Piptoporus betulinus*, *Stropharia rugosoannulata*, *Bjerkandera adusta*	[86]
Lentinula endodes	[109, 110]
Nematoloma frowardii	[87, 108]
Phanerochaete chrysosporium	[82, 111]
Pleurotus ostreatus	[86, 110]
Poyiporus ciliatus, Pycnoporus cinnabarinus	[106]
Trametes versicolor	[109, 110, 112]
Terricolous basidiomycetes native to grassland and/or forest litter	
Agaricus arvensis, *Agaricus bisporus*, *Agaricus porphyrizon*, *Clitocybe odora*, *Coprinus comatus*, *Lepista nebularis*, *Marasmius oreades*	[86]
Collybia dryophila	[113]
Ectomycorrhizal fungi	
Amanita muscaria, *Hebeloma crustuliniforme*, *Lactarius deliciosus*, *Lactarius deterrimus*, *Lactarius torminosus*, *Morchella conica*, *Morchella elata*, *Paxillus involutus*, *Suillus granulatus*, *Tricholoma lascivum*	[86]
Soil-borne microfungi and plant pathogens	
Acremonium murorum, *Botrytis cinerea*, *Chaetomium globosum*, *Cunninghamella elegans* TM 1, Hyphomycetes G28.2&G28.30, *Rhizoctonia solani*, *Scytalidium lignicola*, *Trichoderma* sp.	[86]
Fusarium oxysporum, Trichoderma atroviride	[114]
Consortia of soil microorganisms	
Two consortia of predominantly gram-negative bacteria from black forest soil	[86]
Consortium of microorganisms from sandy brown earth	[103]
Bacteria	
Alcaligenes eutrophus, *Alcaligenes faecalis*, *Bacillus brevis*, *Bacillus cereus*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Xanthomonas campestris*	[86]

[1] Species were named as in original articles.

[2] Classification of fungi into groups was as described in [86].

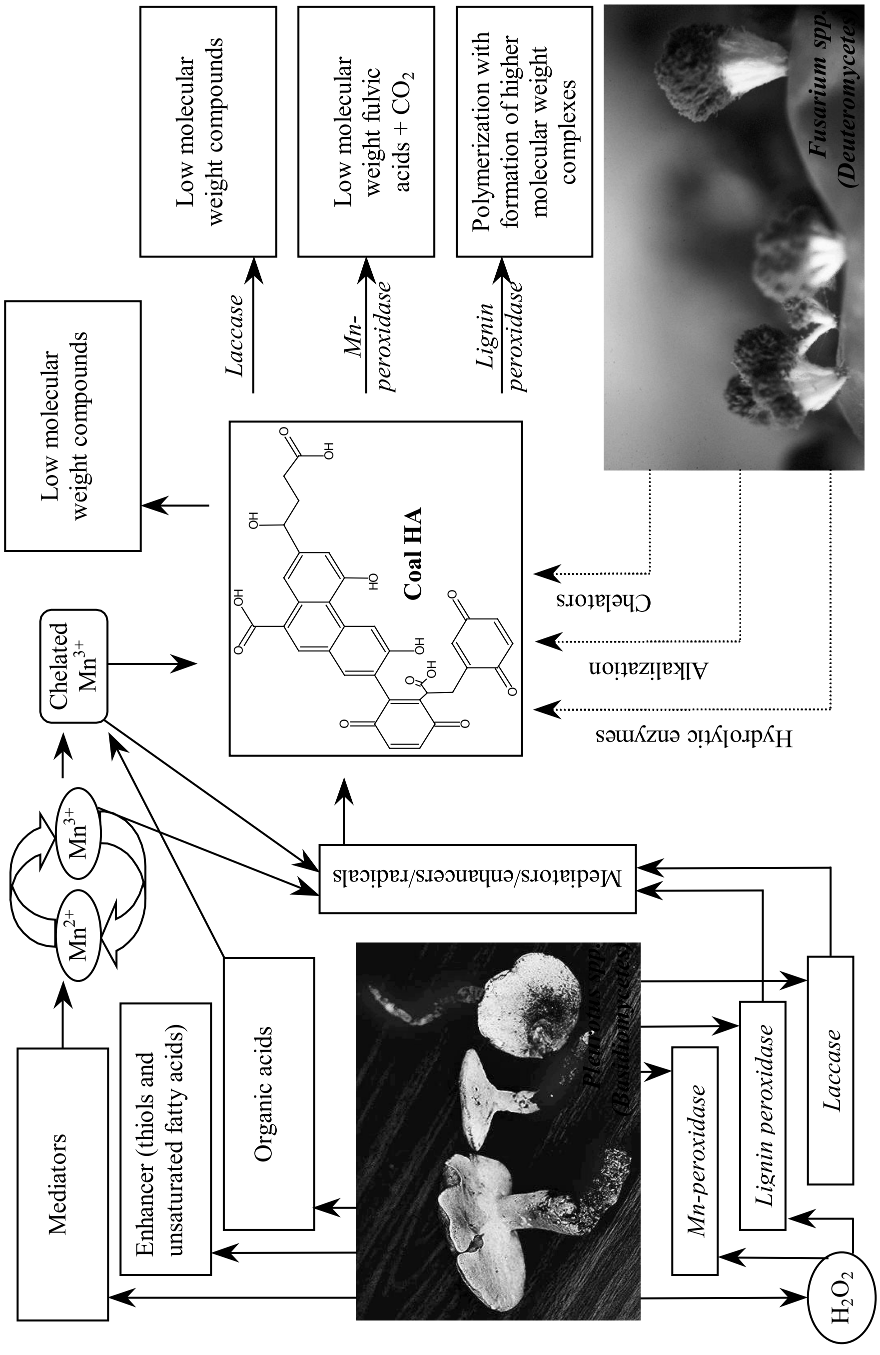

Figure 2. The proposed scheme of coal HA decomposition by *Basidiomycetes* and *Deuteromycetes*.

taken up to a large degree than humic acids. The more recent study used ^{14}C-labeled HS for this purpose [49] and also established that fulvic acids were taken by the plant cells. However, a particular care should be exercised on interpreting the results using the labeled HS. The latter are usually prepared from the substrate incubated with ^{14}C-labeled straw. The extracted HS are not identical to the naturally occurring materials. In addition, low molecular weight compounds from the humic extract can penetrate the plants.

Summarizing the data on HS as a nutrient source for microorganisms, the following conclusions can be made:

- the level of HS utilization by microorganisms under conditions of limited carbon or nitrogen supply is higher than that in a full nutrient solution;
- the efficiency of carbon conversion into microorganism biomass can be enhanced significantly if HS is a sole source of carbon, however, this effect has not been observed under conditions of low nutrient and energy sources;
- the microbial decomposition of HS includes non-enzymatic, enzymatic and enzyme-initiated reactions whose course depend on the specific cell metabolism;
- the long term microbial interaction with HS brings about both decomposition processes and synthesis of newly formed of humic like substances that hampers evaluation of decomposition processes.

3.1.2. Hormone-like activity of HS

Chemical substances identical to or mimicking the action of plant growth hormones have been investigated since the late 1910's. The main classes of those compounds are auxins, gibberellins, and cytokinins. The mode of their action is still not clearly understood. Although it is believed that the growth regulators act directly on the DNA and/or RNA of the cell affecting duplication, transpiration, and translation processes [34]. Interactions among the various groups of growth regulators are complex and complementary to their functions.

The plant hormones can be produced directly by the plant or by the soil microorganisms, especially by those residing in the rhizosphere. Since HS originate from the chemical and biological decomposition of the plant and animal residues and from metabolic activities of microorganisms, they might have a hormonal character. Indeed, in series of papers published since 1914 [27-31], it was shown that HS enhanced plant growth by exhibiting auxin-like activity. In 1917, Bottomley suggested that HS contained growth substances – so called "auximones" [27]. Hillitzer also claimed that HS could act as auxins [29]. Phuong and Tichy reported that humic and, in particular, fulvic acids showed some auxin, gibberellin or cytokinin-like activity [119]. The stimulation effects, however, were much lower compared to those of the real plant hormones. This brought the authors to a conclusion on a lack of phytohormone activity of HS. Cacco and Dell'Agnola detected a hormone-like behavior of HS from podzol using leaf disc senescence test [33]. The activity of HS was also much lower compared to the known plant hormones - indole-3-acetic acid (IAA) or N-6 benzyladenine.

The findings on auxin-like activity of HS have been supported and extended by experiments showing that some humic fractions have a high hormonal activity [33, 35-37]. In particular, it has been shown that low-weight molecular fraction induced morphological changes similar to those caused by IAA [66]. Moreover, this fraction increased IAA-oxidase activity [121]. In further experiments, it has been demonstrated that low molecular weight fraction of HS binds to the IAA cell membrane receptors [122]. However, there is still no direct chemical evidence that hormone-like activity of HS can be provided by the presence of plant growth regulators. Concentration of IAA in HS estimated using immunoassays varied from 0.5 to 3.7% (w/w) [123]. This shows that HS may contain different biologically active compounds – products of biosynthetic activity of microorganisms. For instance, the structure of HS can include polyamines that are plant regulators functioning similarly to the recognized plant hormones. The results of Young and Chen demonstrated that the content of putrescinene, spemidine, and spermine in HS from different sources ranged between 1.54-7.00, 0.39-3.88, 0.48-4.79 nM/g, respectively [38]. The authors concluded that polyamines might explain the hormone-like activity of HS.

Irrespective of principal structural fragments which are responsible for HS hormone-like activity, HS can be considered as a "storage" of hormone-like substances and transferring them to the cell membrane of a plant. Therefore, auxin-like activity of HS cannot be attributed only to their interaction with cell membrane receptors. An influence on the plasma membrane H^+-ATPase and an increase in microelement availability should be considered as well. It has been shown recently that extracts of soil root exudates exhibited a hormonal activity that was lacking in water extracts of soil and in the exudates used for extraction [124]. This confirms an important role of root exudates in decomposition of HS and in forming their hormone-like activity. Hence, the latter is determined by the specific features of soil-plant (root) interaction.

Our experiments with wheat coleoptiles and different fractions of coal-derived HA demonstrated their beneficial effect on coleoptiles growth at all the concentrations tested [120]. Fractionation of HA (CHA-GL02) included separation into hymatomelanic HA (CHM-GL02), a sum of brown and grey HA (CHR-GL02), and fractions of brown (CHB-GL02) and grey (CHG-GL02) HA. Auxin-like activity of different fractions of HA was estimated using bioassay with coleoptiles of wheat (*Triticum aestivum* L.) seedlings. Briefly, wheat seeds were germinated in the dark at 25°C for 72 hours. Then coleoptiles of similar length (about 5 mm) were cut and spited on glass capillaries (3 coleoptiles on a capillary). Upper part of coleoptiles of 4 mm length was preliminary removed to avoid influence of endogenous auxin. Then the length of coleoptiles was measured and capillaries with beaded coleoptiles were placed into the Petri plates added with 5mM K-phosphate buffer at pH 6.0 (blank) or with a solution of HA preparations in phosphate buffer. Then coleoptiles were grown at 25°C for 72 hours and the length of coleoptiles was measured again. The relative increase of coleoptiles length was used as a response. The concentration of HA varied in the range of 5 to 100 mg/L. Results are presented in Fig. 3.

All the HA samples tested showed the beneficial effect on coleoptiles growth at all the concentrations tested. This fact confirms the auxin-like activity of HA. Maximum stimulating activity was observed at the concentration of 10 mg/L and reached 156±9% of the control. The efficicency of different HA fractions was similar except for hymatomelanic and grey HA. The highest effect was observed for the grey HA characterized with the lowest contents of such fragments as carboxylic (COO^-) and aromatic (C_{Ar}) groups and with the highest molecular weight. On the other hand, hymatomelanic HA possessing the highest contents of alkoxy ($C_{Alk}O$) and methoxy (CH_3O) groups and the minimum value of molecular weight, exhibited the lowest beneficial effect on the coleoptiles growth. Statistical analysis of the data set on HA properties and their auxin-like activity have revealed a significant (P =95%) negative relationship between the contents of carboxylic groups in the HA preparations and the value of the beneficial effect (r = –0.96). Taking into consideration a determining role of carboxylic groups in formation of the surface negative charge of HA and the natural negative charge of cell surface, it could be suggested that this is adsorption of HA onto the coleoptiles surface that determines the auxin-like activity of HA.

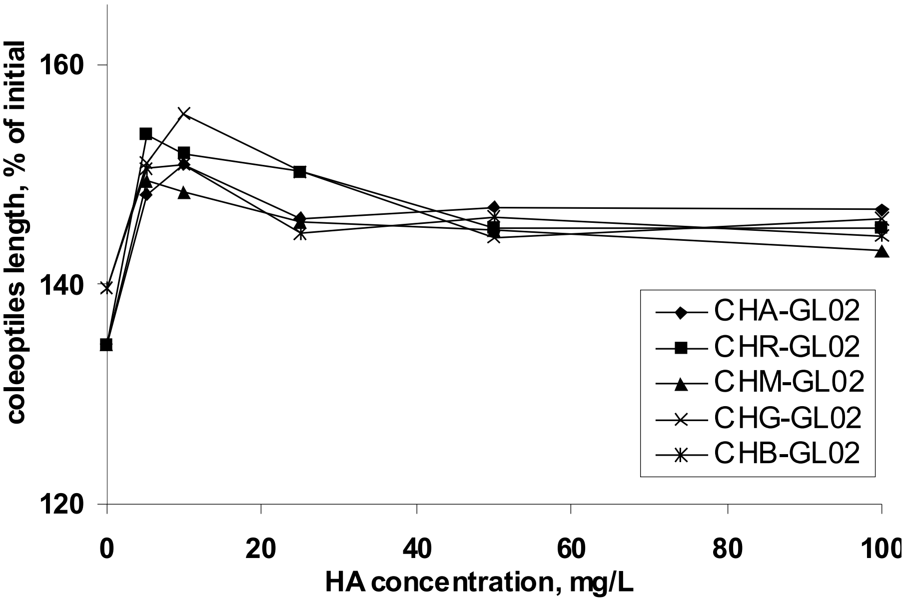

Figure 3. Influence of different fractions of coal HA on the wheat coleoptiles growth.

Summarizing the data on hormone-like activity of HS the following conclusions can be made:

- the level of hormone-like activity of HS is usually lower than that of plant hormones;
- the mechanism of hormone like activity of HS is still poorly understood and needs to be further investigated.

3.2. HS AS NUTRIENT CARRIERS

HS are generally considered to improve plant growth by enhancing nutrient supply [52, 55, 135, 136]. In contrary, some data indicate that there is no significant difference in biomass yield in the presence of HS [58, 70]. This contradiction could be provided by a use of supra-optimum conditions by the latter authors, whereas an impact of HS on the plant nutrition is the most pronounced under the conditions of limited nutrient availability [97]. For example, iron chlorosis, a symptom of iron deficiency in plants, can be corrected by applying HS which play a beneficial role in Fe acquisition by plants [61, 137- 139]. This beneficial effect of HS is attributed to the chelating activity of HS that provides the plant with Fe in easily assimilated form [140]. The presence of HS can also facilitate Fe uptake by plants as a result of HS participation in ion transport [141] and stimulation of membrane associated H^+-translocating enzymes [142, 143].

Humics have a surfactant-like structure containing both hydrophilic domains, such as carboxylic and phenolic groups, and alkylic and aromatic moieties. Because of this amphiphylic character, HS behave as natural surfactants and can adsorb on different natural surfaces including biological membranes [125-132]. Adsorption of HS on biological surfaces has been demonstrated directly, by loss of dissolved carbon from solution [125], and indirectly, by following changes in the electrophoretic mobility of individual cells in the presence or absence of HS [126]. Biological surfaces studied include phytoplankton [125-128], isolated fish gill cells [128], bacteria [129, 130], fungi [131], and plants [132]. Their diversity suggests that the adsorption of HS on biological membranes is a general process. Due to surface-active properties, HS may change the structure and fluidity of the membrane, and cell membrane permeability could be affected [133]. The increase in membrane permeability, in its turn, can result in increasing uptake of nutrients by living organisms [129, 132]. Ermakov et al. found that HA derived form potassium humates adsorbed to the surface of plant cells and increased their extensibility indirectly via reactions in the cell protoplasm [134]. Toughening of the cell walls was found to be stimulated by peroxidase-mediated dimerisation of phenolic groups present in HA and in the cell wall polymers.

Our experiments have also revealed the beneficial effects of HS on the capacity of Fe-deficient tomato and wheat plants to utilize iron under simulated calcareous soil conditions. HS were extracted from peat using standard alkali extraction [144] and cation exchange resin to remove exchangeable Fe. Tomato and wheat seeds were germinated on filter paper moistened with distilled water. After 10 or 5 days for tomato and wheat, respectively, the seedlings were transferred to plastic vessels containing aerated Hoagland nutrient solution either or without Fe supply, and grown for up 40 days. Fe, when added, was supplied at the concentration of 24 μM as $FeSO_4$, $FeSO_4$ in the presence of HS, and Fe-DTPA (an iron complex of diethylenetriaminepentaacetic acid). To simulate calcareous soil conditions, pH of the nutrition solution was adjusted to 8.0 using $CaCO_3$. After 20 days of the plant growing, photosynthesis efficiency in terms of electron transport rate (ETR) and effective quantum yield (Yield) were estimated using pulse amplitude modulation (PAM) fluorometer (PAM-2000, Walz, Germany). After 40 days the plants were harvested and used for length, weight, and chlorophyll content measurements. Data obtained are shown in Table 3.

Both the tomato and wheat plants grown in the nutrient solution without added Fe exhibited visual symptoms of Fe-deficiency (leaf chlorosis). The chlorophyll a/b ratio used as an indicator of light harvesting capacity of the plants under stress [145] was as low as 0.59 and 0.29 for the control tomato and wheat plants, respectively. As a consequence, the biometric parameters of the control plants – length and weight – were substantially suppressed as well.

Table 3. Length, weight, photosynthesis efficiency and chlorophyll content of tomato and wheat plants in the presence of different sources of iron and HS, % of blank .

Treatment	Weight	Length	Photosynthesis		Chlorophyll	
			ETR	Yield	Content	a/b
			Tomato plants			
$FeSO_4$	90±12	97±12	104±11	103±17	142±15	189±14
Fe(III)-DTPA	4649±35	282±18	179±13	149±15	856±29	208±24
HS	110±11	105±10	200±11	154±21	107±11	156±12
$FeSO_4$+HS	4382±56	265±19	189±15	148±17	819±31	221±16
			Wheat plants			
$FeSO_4$	101±5	105±12	97±7	100±8	101±11	95±10
Fe(III)-DTPA	172±12	146±12	218±15	197±12	200±11	205±5
HS	121±15	138±12	344±11	329±13	154±12	130±13
$FeSO_4$+HS	123±15	125±11	212±15	216±14	179±11	198±14

As it can be seen from Table 3, an addition of inorganic Fe ($FeSO_4$) alone did not increase iron-supply of the plants: the detected values of weight, length, photosynthesis efficiency and chlorophyll content were the same as for the control plants. This was to expect due to alkaline conditions (pH 8) of the nutrition media resulting in formation of insoluble Fe(III) hydroxide unavailable for plants uptake. At the same time, simultaneous addition of HS and $FeSO_4$ gave a substantial rise in the length, weight, photosynthesis efficiency and chlorophyll content of the plants. The photosynthetic parameters (ETR and Yield) even reached the values detected for the Fe-DTPA treated plants, whereas the weight, length and chlorophyll content were still lower than in the Fe-DTPA treated plants. The plants grown with addition of the synthetic iron chelate Fe-DTPA did not display any symptoms of chlorosis and were characterized with the highest values of biomass and length.

Of interest is that addition of HS solely led to a partial recovery of the Fe-deficient plants. In particular, the photosynthesis efficiency and chlorophyll content were significantly increased in humus-treated plants. Given that exchangeable Fe was removed during the extraction procedure, it seems like the plants could use constituent (endogenous) Fe of HS. Hence, the effect of enhancing nutrient supply can overlap a function of HS as a nutrient source. Another explanation is a direct influence of HS on the photosynthesis. The corresponding data are reported in the literature [65, 67] that claim the quinonoide structures of HS being able to support electron transport and, therefore, to increase the photosynthesis efficiency. In general, the obtained results have confirmed that treatment of iron-deficient plant with HS can result in increasing uptake of iron and, therefore, in increasing plant biomass. The effect observed seems to be extended for other micronutrients such as Zn and Mn.

Summarizing the data on enhancing nutrient supply in the presence of HS, the following conclusions can be made:

- HS show beneficial effects or no effects on the nutrient supply;
- the most pronounced beneficial effects of HS on the plant nutrition are observed under unfavourable growth conditions;
- the presence of HS under limited nutrition conditions can lead to a partial recovery of the plants.

3.3. HS AS CATALYSTS OF BIOCHEMICAL REACTIONS

The direct participation of HS in biochemical reactions has been observed only in few cases [26, 116]. However, HS can influence biochemical reactions indirectly, nominally, via formation of the HS-enzyme complexes, or as a substrate of enzymatic reaction. The principal processes that can occur in the HS-enzyme system are schematically represented in Fig. 4. Microorganisms and plants are the main sources of soil enzymes [146]. The latter should be segregated into existing in the soluble phase and in the solid phase. It is well known that extracellular soil enzymes can be associated with inorganic and organic colloids and, in particular, with HS. The essential soil enzymes such as urease, protease, phosphatase, hydrolases, laccase, and peroxidase have been detected in soil extracts as complexes with HS [26, 147]. The distribution of enzymes on the surface of organo-mineral particles depends strongly on the particle size as it was shown for invertase and xylanase [148, 149], phosphatase [150] and urease [151]. Moreover, various mineral and organo-mineral surfaces may have an inhibitory influence on the activity of enzymes as it was established for acid phosphatase [150].

The above data indicate that HS can play a role of enzyme carriers facilitating enzyme “immobilization” and improving their long-term stability. Due to the presence of carboxyl, carbonyl, hydroxyl and amide groups in HS structure [1, 2], they can serve as supports for the immobilized enzyme. Invertase was immobilized onto aminopropyl silica activated with soil HS. The immobilization technique yielded an increased amount of and an enhanced activity of the immobilized enzyme [152]. In the context of soil interactions, the formation mechanism of the HS - extracellular enzyme complexes can include an activation of inorganic particles with HS followed by a formation of inorganic particle-HS-enzyme complexes. As a result, upon extracting these complexes from soil, separation of inorganic particles occurs, and only HS-enzyme complexes can be obtained.

Reviewing the data on proteases-HS complexes, it can be noted that their formation depends on both the type of proteases and composition and structure of HS. Proteases able of hydrolyzing N-benzoyl-L-argininamide were shown to be associated with highly condensed humic materials, while N-benzoylcarbonyl-L-phenylalaninyl-L-leucine – hydrolyzing proteases are associated with less-condensed humic materials. Casein-hydrolyzing proteases are generally associated with non-humified organic matter [153]. The study on urease-HS complexes is in line with these data [154]. The effects of two different peat HA fractions, high molecular weight (HMW, 100-300 kDa) and low molecular weight (LMW, 10-20 kDa), on the activity and stability of urease were evaluated. It was shown that HMW fraction of HA significantly inhibited urease activity at pH 6.0, but caused no influence on its activity at pH 7.0 and 8.0. HMW fraction

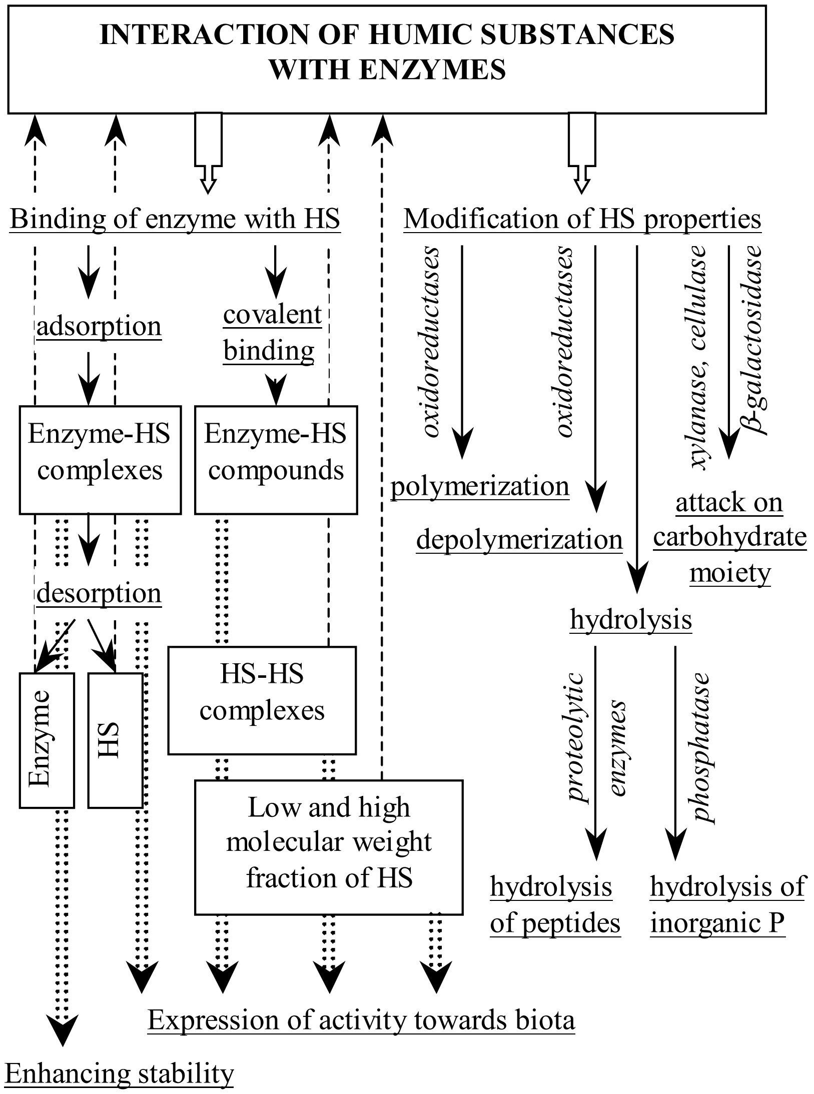

Figure 4. Principal processes of interaction of HS with enzymes.

stabilized urease activity over a period of 11 days of treatments with protease and with Cu^{2+} and Hg^{2+} (two powerful inhibitors of soluble urease activity). On the contrary, the LMW fraction of HA inhibited urease activity at pH 6.0, 7.0 and 8.0, and did not stabilize urease activity in the presence of protease. These results show that the two HA fractions affected differently both the activity and stability of the urease. It has been proposed that the inhibition of the urease by HMW and LMW fractions is mainly due to the presence of two heavy metals which, although immobilized on the HA, are still able to interact with the urease. This conclusion seems to be too simplistic because the different effects of HWM and LMW fractions reported in [52, 122, 137, 154] can be also attributed to complex nature of HS.

The direct interaction of HS with enzymes can be assumed from the wide occurrence of soluble enzymes in soil. The possible modifications of HS by soil enzymes present in the soluble phase are summarized in Fig. 3 which is focused on the direct interaction of oxidoreductase with both natural and synthetic HS. It is shown that depolymerization of coal HA by Mn-peroxidase resulted in formation of fulvic acids and carbon dioxide [87]. The synthetic HA derived from 3-fluorocatechol have also been modified by Mn-peroxidase, but without a significant decrease in the molecular weight [155]. This was attributed to the small molecular weight of synthetic HA. On the contrary, peroxidase and laccase were shown to polymerize HS [116, 156, 157]. It was shown that oxidation of lignite HA catalyzed by horseradish peroxidase led to an increase in molecular weight indicating an enhancement of HA molecular size over time. Polymerization was found to be pH-dependent and occurred to the further extent at pH 7.0 than at pH 4.7. The mechanism of HA – enzyme interaction included the formation of aryl and alkyl ethers. It was also established that HA from a volcanic soil, oxidized coal and leonardite demonstrated the same alterations in the presence of peroxidase and hydrogen peroxide [116]. The direct synthesis of humic-like substances by laccase resulted in formation of high molecular weight compounds similar to soil humic acids according to the data of elemental analysis, size-exclusion chromatography, IR and ^{13}C-NMR spectroscopy [157].

The direct influence of HS fractions (HMW and LMW) on enzymes and on their biosynthesis has been studied mainly using plant materials [50, 52, 141, 142]. Despite the established post transcriptional effect of LMW fraction on protein synthesis in maize seedlings, the mechanism of this effect is not clear [52]. The experiments on the direct effects of HS on enzymes have been conducted using microsomal fraction [50, 141, 142]. It was shown that LMW and HMW fractions stimulated the activity of K^+-stimulated ATP-ase. The confirmation of these results is the stimulation of the H^+-ATPase activity of the isolated plasma membranes with LMW fraction. The induction of laccase biosynthesis by HS (200% compared to the pure fungal culture) during the growth of *Basidiomycetes Coriolus hirsutus* has been shown as well [157]. However, the mechanism of induction has not been studied.

Summarizing the data on HS – enzyme interactions, the following conclusions can be made:

- direct interaction of HS and enzymes is scarcely studied;
- polymerization/depolymerization transformations of HS as a result of intereactions with oxidoreductases are established.

3.4. ANTIOXIDANT ACTIVITY OF HS

Protection of cells, tissues and biological macromolecules against reactive oxygen species including free radicals is of great importance for living organisms. Oxidative stress arises in a biological system after an increased exposure to oxidants as a result of a decrease in the total antioxidant activity of the biological system. The main role in protection of the biological systems play specific compounds called antioxidants. The general description of these groups can be given in terms of their antioxidant activity – ability to interrupt radical-chain oxidation processes or to decrease the amount of free radicals (particularly, reactive oxygen species).

The study of antioxidant activity of HS is of a great interest. However, the measurement of the antioxidant activity of HS causes a lot of methodological difficulties. The first is HS interaction with system components producing free radicals, the second is the complexity of HS structure. The attempt to measure antioxidant activity of HS using voltammetry is described in [158]. The authors used method of electrochemical generation of oxygen superoxide radical and measured antioxidant activity of some standard antioxidants (vitamin C, glucose, resorcinol, and catechol) as well as of the plant extracts and of HS (water extracts from peat). The main problem in interpretation of their data is that no characteristics of HS were given, and correlation with standard units of antioxidant activity was not provided. However, among the samples of plant origin and of the standard antioxidants, HS demonstrated the highest value of antioxidant activity comparable to that of phthalocyanine Co, phthalocyanine Ni and phthalocyanine Cr. The data obtained allowed to propose the mechanism of interaction based on the dismutation of oxygen superoxide radical to dioxygen:

$$O_2 + e \rightarrow O_2^- + R \xrightarrow{k} O_2 + R^-$$

Therefore, HS can play protective function in ecosystems interrupting radical reaction and preventing damages of cell membranes and biological macromolecules.

Main interest to HS antioxidant activity is provoked by the role of HS in the environments exposed to the factors causing the formation of free radicals ($OH\cdot$, $O_2H\cdot$, $ROO_2\cdot$), nominally, drought, heat, herbicide use, and ultraviolet radiation. Relationship between the structure of HS and their reactivity toward molecular ozone (O_3) and hydroxyl radicals ($OH\cdot$) has been established [159-161]. The positive correlations of the scavenging activity of HS versus their concentration and the amount of C=C bonds in their structure were observed in radical reactions with $O_3\cdot$ and $OH\cdot$. Ozone consumption and $OH\cdot$ scavenging rate constants were found to be positively correlated with the content of aromatic carbon, and inversely correlated with that of aliphatic carbon. The data obtained [159] allow to conclude that ozone reacts preferentially with aromatic constituents of HS and, specifically, with the electron enriched aromatics. In compliance with these findings, the statistically significant relationship was found between oxidation rate parameters and C/H ratio, - indicator of the unsaturation degree of the structure. So, the chemical nature of HS exerts a strong control over their reactions with ozone.

The study of MnO catalyzed ozonation of atrazine in the presence of HS revealed that HS at the concentration of 2-6 mg/L (DOC) might scavenge hydroxyl radicals produced during ozonation decreasing the oxidation efficiency of atrazine. However, when HS were present at low concentrations (1 mg/L DOC) both the manganese species and HS initiated and promoted the formation of hydroxyl radicals that enhanced the destruction of atrazine.

The following conclusions can be drawn:

- the radical scavenging effects are displayed at high concentrations of HS,
- the antioxidant activity of HS is strongly dependent on their structural features such as enrichment with unsaturated moieties.

4. Conclusions

Reviewing the data on mitigating activity of HS, the following principal ways of HS action were proposed:

- influence on the organism development;
- enhancing nutrient supply;
- interactions of HS with enzymes and catalysis of biochemical reactions;
- antioxidant activity.

The complexity of HS structure provides a wide variety of the observed biological effects. This is true for HS interactions with xenobiotics, nutrients, enzymes, membranes, etc. Therefore, the study of biological effects of HS neglecting either possible interaction of HS with components of biological system or their structural complexity leads to conflicting data and misinterpretation. In order to overcome the problem, one should standardize the experimental design and use characterized preparations of HS.

Another specific feature of biological activity exerted by HS is that the most pronounced effects on living organisms and on the components of the ecosystems can be observed under stress conditions induced by both specific and non-specific stress factors. Hence, HS can be considered as environmental modulators mitigating the adverse effects of stress factors.

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