



Agricultural Use of Natural Biostimulants – Humic Substances: a review

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Abstract: This work presents the characteristics of humic substances, with particular emphasis on the properties of agricultural importance, i.e., the improvement of soil structure, soil properties and the availability of nutrients. Attention was also paid to the methods of introducing given substances into the soil and the possibility of direct application to the plant surface. The mechanisms responsible for the stimulating and protective effect of humic substances on plant development were explained in detail, including phytohormonal regulation, the mitigation of biotic and abiotic stresses, antioxidant properties and the influence on seed germination and the development of the plant root system. The economic and environmental importance of the application of the substances in question was also emphasized, consisting of maintaining the health of the soil and the quality of plants while reducing the share of chemical fertilizers and reducing the amount of water used. It should be noted that humic substances, like any agricultural product, should be appropriately selected for the type of cultivation, i.e., the optimal dose and method of application should be determined.

Keywords: humic substances, agriculture, biostimulant

1. Characteristics of humic substances

Humic substances belong to the group of naturally occurring organic compounds. They are a mixture of complex and heterogeneous polydisperse materials formed in terrestrial and aquatic environments. They are formed due to the humification process, which involves the biochemical, chemical, and physical transformation of dead organic matter, such as plant, microbial, and animal remains (IHSS 2021). Humification is the second-largest organic process on Earth after photosynthesis, making humic substances a reserve of organic carbon (Weber 2020).



The primary sources of humic substances are:

- the aquatic environment (surface waters, groundwater, sapropel),
- the terrestrial environment (soil, peat, Leonardite, brown coal),
- anthropogenic (compost, sewage sludge, landfill leachate, municipal sewage) (Anielak 2019, Łomińska-Płatek & Anielak 2021, Orliński & Anielak 2021, Pempkowiak et al. 2008).

There are several fractions of humic substances (HSs) based on their solubility in water:

- Fulvic acids (FA) – a fraction soluble in water at all pH values,
- Humic acids (HA) – a fraction soluble in water in an alkaline environment; in an acidic environment at $\text{pH} < 2$, it precipitates,
- Humins / ulmins – a water-insoluble fraction; an intermediate form between humic substances and carbon (Dou et al. 2020).

Organic macromolecules of humic substances consist of hydrophobic nuclei and branched chains with aliphatic functional groups attached. Hydrophobic nuclei have an aromatic structure and are condensed (Anielak 2019). The source of origin and the conditions of formation influence the type of functional groups present in humic substances, which translates into the content and mutual quantitative relations of individual elements. However, it is possible to distinguish certain elements and functional groups characteristic of the substances in question (Pempkowiak et al. 2008). The elemental composition of HSs is dominated by carbon, hydrogen, oxygen, nitrogen, and sulfur. Nitrogen is deposited in the functional groups of amines and amides, while sulfur (the content of which is almost always minimal) is in the sulfonic (SO_3H) and thiol (SH) groups (Anielak 2019, Nardi et al. 2021).

HSs most often contain functional groups in which there is oxygen, mainly carbonyl ($-\text{C}=\text{O}$), carboxyl ($\text{C}(=\text{O})\text{OH}$), and hydroxyl ($-\text{OH}$) groups, including alcohol and phenol. The presence of carboxyl and phenolic groups determines the acidic character and influences the selected properties of humic substances, relevant in terms of agricultural uses or soil remediation (Nardi et al. 2021).

Moreover, micro-spaces were distinguished in the structure of humic substances, acting as molecular sieves. Micro spaces play a vital role in the sorption of low molecular weight compounds, such as organic xenobiotics, herbicides and pesticides. Most organic compounds are substances with complex structures and with various impurities. Humic substances are no exception and, in addition to organic compounds, may contain inorganic substances, such as metals. Therefore, in the process of their extraction, and then preparation for agricultural use, steps should be taken to remove heavy metals and possible pathogens (Anielak 2019).

The article aims to evaluate the properties of humic substances supporting their use as natural biostimulants. In addition, the mechanisms determining the properties of HS were thoroughly discussed to understand their effects on plants and soil better.

2. Humic substances as a biostimulant

Humic substances are a natural component of the soil and a reserve of organic carbon. The presence of HSs in the soil is equivalent to the content of as much as 85-90% of organic carbon, an essential component of organic matter. Decreasing organic matter content in the soil reduces its aeration, which leads to the creation of reducing conditions. The structure of the soil is degraded, which has a negative impact on soil functions and, consequently, the development of plants, their quality and their yield are disturbed. Humic substances influence the regulation of the global carbon cycle and another crucial element, nitrogen (Weber 2020).

Research confirms the positive effect of exogenous humic substances on fertility, influencing the physical, chemical and biological parameters of soils. The soil structure improves - soil aeration is supported by; the formation of aggregates of humic substances and soil particles; the compactness of soil changes; the ability to retain moisture and retain water in the soil is increased; humic substances function as a buffer, maintaining a certain pH level. An essential feature of HSs is their cation exchangeability (CEC). They can sorb or desorb selected cations, thus responsible for their release or removal from the environment. This feature is also crucial in reducing the toxicity of heavy metals present in soils and increasing the bioavailability of nutrients for soil and plants (Rose et al. 2014). HSs also improve the solubility of elements, preventing their leaching, affecting seed germination and the root system development positively (Rouphael & Colla 2018), changing biochemical pathways as primary and secondary metabolisms and affecting endocrine signalling (Canellas & Olivares 2014). It has been shown that as a result of the addition of humic substances to plants, enzymatic defence systems were activated, which alleviated the effects of stress (Pukalchik et al. 2019, Qin & Leskovar 2020).

The action of humic substances on plants occurs in two ways – indirectly, as a modification of soil properties and as a direct impact on physical and metabolic plant processes (Canellas & Olivares 2014). The direct effect is manifested, inter alia, by increasing the activity of growth hormones, producing antioxidants that reduce free radicals and improving chlorophyll synthesis (Rose et al. 2014).

One of the provisions on fertilizers is Regulation (EU) 2019/1009 of the European Parliament and of the Council of 5 June 2019, laying down rules on the making EU fertilizing products available on the market and amending Regulations (EC) No 1069/2009 and (EC) No 1107 / 2009 and repealing Regulation (EC) No 2003/2003. This document lists the functional categories of fertilizing

products (PFC list). The three main categories of agricultural use of humic substances are: biostimulant, fertilizer and organic soil improver. The main function of these amendments is to improve one or more of the following characteristics of the plant or the plant rhizosphere: nutrient use efficiency, tolerance to abiotic stress, quality traits, the availability of confined nutrients in soil or rhizosphere and physical properties, the structure or the biological activity of the soil to which it is added. In addition, the regulation lays down limit values for pollutants (e.g., mercury or nickel) and permissible levels of pathogens, which fertilizer products must not exceed. In Polish law, the requirements for fertilizers are included in the Act of 10 July 2007 on fertilizers and fertilization and in the Regulation of the Minister of Agriculture and Rural Development of 18 June 2008 on the implementation of certain provisions of the Act on fertilizers and fertilization. Legal acts define, among other things, the types of fertilizers, the permissible value of contaminants in fertilizers and the types of pathogens unacceptable in the fertilizer product.

2.1. Improving the structure and physical properties

Biostimulants containing humic acids contribute to the improvement of the structure and physicochemical properties of the soil, especially as a result of increasing the content of organic matter (Malik et al. 2021).

The formation of soil aggregates is promoted by the presence of humic substances that act as a binder that holds the mineral parts of the soil together. Humic substances obtained from various sources are characterized by a different degree of aromatic nucleus polymerization and the type and number of functional groups. These factors affect the nature and strength of bonds with soil mineral components during the aggregation process (Kobierski et al. 2018). The resulting aggregates are characterized by porosity and durability, which results from increased resistance to the washing effect of water (Yang et al. 2021).

Humic substances, as biologically active materials, intensify the release of sugars indirectly through the plant and microbial communities. Carbohydrates probably play a significant role, especially in the initial stages of the formation of soil aggregates (Lykhman et al. 2020). The stable structure of soil aggregates results from a complex of humic substances, carbohydrates, soil mineral particles (e.g., clay) and calcium (Kobierski et al. 2018). The obtained soil structure reduces its erosion and prevents the leaching of carbon and nitrogen (Nardi et al. 2021). In light soils (e.g., sandy), humic substances favour the formation of soil aggregates, thus increasing soil compactness. On the other hand, these substances loosen the structure in heavy soils, improving their aeration and water circulation.

2.2. Retention properties – the improvement of soil water capacity

The use of humic substances in naturally low water capacity soils helps to increase water retention. The higher the HSs content, the greater the water retention. The water-retaining properties of humic substances result from their partially hydrophilic nature (the presence of hydroxyl and carboxyl groups) and porosity (Jaeger et al. 2010).

2.3. Cation exchange capacity

Cation exchange capacity determines the total amount of cations adsorbed by soil colloids at a given pH value, mainly Ca^{2+} , Mg^{2+} , K^+ , Na^+ and H^+ ions. As part of the colloidal soil system, humic substances profoundly influence the cation exchange capacity. Their addition positively affects the cation exchange capacity in the soil, which is one of the fertility indicators (Ateia et al. 2017, Kwiatkowska-Malina 2018). Moreover, HSs increase the specific surface area, thanks to which the cations are not blocked and can be adsorbed in the soil.

The cations are held by electrostatic attraction forces between them and negatively charged clay minerals (e.g., clay) and/or humic particles. Depending on the concentration of cations and the pH, cations may be retained or released into the soil solution (Anielak & Świdorska 2001, Stevenson 1994).

Cation exchange on humic molecules occurs when oxygen functional groups (carboxyl and phenolic) dissociate and render the molecule's surface negative. Cations from the environment are adsorbed as a result of electrostatic interaction (physisorption) and, in turn, attract exchangeable anions in the soil solution. This process is characterized by the zeta potential of the HA molecule: the double electric layer of the surface of dissociated molecules has a negative zeta potential; after adsorption, especially of multi-positive cations, it is positive. Therefore, the relative strength of ionic bonds between cations and anions is the highest for iron, aluminium and calcium ions (multi-positive cations) (Stevenson 1994, Anielak & Grzegorzczuk-Nowacka 2011, Anielak & Świdorska 2001). The ability of humic molecules to bind to cations is fundamental to their ability to chelate micro-nutrients and heavy metals.

2.4. Availability of nutrients

It has been shown that HSs treatments have a beneficial effect on crop efficiency because they contain various oxygen functional groups that make the soil environment relatively acidic (Lehman & Kleber 2015). Macronutrients, such as phosphorus, become more accessible to plants due to the higher solubility of phosphorus-containing particles under acidic conditions (Yang & Antonietti 2020). Attention should be paid to the effectiveness of the application of exogenous HSs in soils depending on the content of organic matter. HSs more enhance the microbial activity of soils with low organic matter content than soils with high

organic matter content. This happens because the stimulating effect is already provided by the native humic substances in organically rich soil (Jung et al. 2021).

The proper functioning of plants depends on access to macro- and micronutrients. The macronutrients necessary for the proper development of plants are carbon, hydrogen, oxygen, nitrogen, phosphorus, sulfur, potassium, calcium and magnesium. However, the group of micronutrients include, among other, iron, manganese, copper, zinc, bor, molybdenum and chlorine (Barker & Pilbeam 2015). The availability of nutrients in the soil and the possibility of their uptake are stimulated by the influence of humic substances on soil processes and plant physiology.

The presence of functional groups in the structure of HSs determines the properties of these substances and allows, among other, the formation of complexes (chelation) and the buffering of soil pH (du Jardin 2015). The formation of complexes is critical due to a deficiency of nutrients, which is a widespread problem in agriculture. Due to the ability to exchange cations, humic substances form soluble complexes with metal cations. This limits the leaching of essential components, making them more accessible to plants. HSs also have an affinity for organic compounds. The small molecular size of nutrients complexed with humic acids also contributes to greater absorption by plants (Nephali et al. 2020).

Phosphorus belongs to the group of macronutrients needed for plant development. However, this element mainly occurs in a form inaccessible to plants due to low solubility. Application to the soil in the form of fertilizer has short-term effects because most phosphorus fertilizer is converted into insoluble minerals in the soil, and only a small percentage is then directly available to plants (Salm et al. 2017). Adding humic substances to the soil positively affects soil nutrition with phosphorus, improving its solubility. HSs contain oxygen functional groups, which make the soil environment relatively acidic. Particles containing phosphorus are characterized by higher solubility precisely under acidic conditions, which makes them more accessible to plants (Jung et al. 2021). Humic substances form complexes with iron and aluminium in the soil solution, and the resulting humus-metal complexes can bind phosphorus. Humic substances can also desorb the phosphorus bound to iron or aluminium oxides by binding to phosphorus sorption sites in the oxides. Improving the availability of a given element also involves disrupting the precipitation of insoluble calcium phosphate (Gerke 2021, Rouphael & Colla 2018). The mechanism which increases the availability of phosphorus also includes the positive effect of humic substances on water capacity and, thus, the ability to dissolve in the soil and the vertical migration of available forms of phosphorus (Guppy et al. 2005).

Iron, as one of the micronutrients, has a significant impact on the growth rate of plants, especially the root part. This element occurs in soil mainly as Fe (III), while plants can take up iron in the form of Fe (II). Humic substances play a crucial role in transforming Fe (III) into Fe (II) in soils. One of the mechanisms that transform the form of iron is its direct reduction through the photocatalytic properties of HSs. Humic substances play the role of redox mediators in the Fe (III) reduction process by microorganisms (Lovley et al. 1996).

The availability of micronutrients can be indirectly improved with humic substances, i.e., by complexing (chelating) nutrients, a buffering soil reaction, increasing water retention and the impact on the rhizosphere, changing the interaction between the soil, plant and microorganisms. The second way to increase the availability of nutrients is the direct action of humic substances, manifested in introducing anatomical and biochemical changes in plants (Jindo et al. 2020b). These substances can directly stimulate plants by modulating the expression of functional genes and proteins (Jung et al. 2021, Lee et al. 2019). Humic acids increase the permeability of the cell membrane, thus contributing to the more efficient transport of mineral compounds to metabolically active sites (Chen et al. 2004). In addition, HSs molecules exhibit hormonal activity, thanks to which they act directly on the root. They are also responsible for activating selected physiological processes in plants (Pukalchik et al. 2019), including seed germination, root genesis and resistance to abiotic stress (Jung et al. 2021, Shah et al. 2018).

The particle size of these substances also influences the way they work in plants. The small particle size fraction can penetrate inside the root cells, acting directly to trigger intracellular signals. In contrast, the large-size fraction binds to external cell receptors to elicit molecular responses in the plant (Nardi et al. 2021, Pizzeghello et al. 2020).

2.5. Biostimulants regulate the hormonal balance of plants

Plant growth and development regulators are organic matter which influences the modification of the physiological functions in the plant. This modification may consist in supporting or inhibiting the processes of its development. For example, during stressful conditions, the hormonal balance of plants is disrupted, and thanks to the use of selected phyto regulators, it is possible to influence the condition of the plant. There are five basic plant hormones. They are auxins, cytokinins, gibberellins, abscisic acid and ethylene. The first three are referred to as growth hormones, affecting cell division or the development of the root system. In contrast, abscisic acid and ethylene are known as stress or ageing hormones (Matysiak & Adamczewski 2009).

Research on the structure of humic substances has shown the presence of such phyto regulators as gibberellins (Muscolo et al. 1999), physiologically active cytokinins (Pizzeghello et al. 2013), abscisic acid (ABA) (García et al. 2016), indole

acetic acid (IAA) and other molecules (e.g. phenylacetic acid, indole-3-butyric acid, carboxylic acids and amino acids) with action similar to IAA (Canellas et al. 2002, Scaglia et al. 2016), aromatic groups with high biological activity, especially phenol-C groups, which account for some IAA-like activity (Savy et al. 2017, Schiavon et al. 2019). Humic substances show auxin-like effects (Rouphael & Colla 2018) and cytokinins (Jindo et al. 2020a, Pizzeghello et al. 2013). One of the primary mechanisms explaining the effect of humic substances on plants is their interaction with auxin, jasmonic acid and abscisic acid, resulting in phytohormonal regulation in the root. These are plant hormones that are responsible, among other things, for reducing biotic and abiotic stresses (e.g., drought and salinity stress). The beneficial effect of humic substances has also been linked to ethylene-dependent signalling pathways, a hormone involved in fruit maturation, seed germination, cell expansion and flower ageing (Jindo et al. 2020a).

Due to auxin-like activity, humic substances increase the activity of H⁺-ATPase in the cell membrane. The activation of ATPase influences physiological reactions, including the rhizosphere's acidification, root growth stimulation, or the activation of transporters for nutrient uptake (Gerke 2021, Jindo et al. 2020b). Therefore, improving the development of the root system contributes directly to the greater efficiency of nutrient uptake.

2.6. Antioxidant properties

Stress factors (e.g., drought, heat, ultraviolet light and the use of herbicides) may induce the excessive production of reactive oxygen species (ROS), which results in the degradation of lipid membrane components as a result of peroxidation and damage to proteins and the DNA of plant cells (Davison et al. 2002). The protective effect of HSs is due to the presence of phenolic fragments that provide antioxidant activity (Kulikova et al. 2018) and increased activity of ROS scavenging enzymes. Furthermore, humic substances can regulate the production of ROS and their accumulation in the roots, thus improving the oxidative metabolism of plant cells (García et al. 2014). In addition, phenolic compounds and carboxylic acids are also responsible for the anti-inflammatory properties of HSs (Table 1).

2.7. Seed germination and the development of the root system

Humic substances contribute to the acceleration of seed germination and the improvement of root growth, especially their lateral proliferation (Canellas et al. 2002, Cha et al. 2017). The phenolic groups in the structure of HSs are responsible for the positive effect of the seed germination process (Bento et al. 2020). Root growth is influenced by the general distribution of certain organic functional groups, mainly phenols and carboxylic acids (Cha et al. 2017).

The mechanisms explaining root stimulation by humic substances include several factors. The first is the presence of hormone-like structures (e.g., auxin) that activate specific hormonal pathways in plants, thereby influencing root development. Another factor is the formation of nitric oxide in the presence of HSs, which results in lateral root development. The third factor is energy metabolism enhancement and increased protein expression (Nunes et al. 2019). Root cell proliferation may contribute to the facilitation of energy acquisition (Jung et al. 2021).

2.8. Secondary metabolism and stress

As a result of the secondary metabolism of plants, a group of compounds is formed that is not directly necessary for their growth and development but are necessary for the plant to survive in a specific environment and under biotic and abiotic stress. HS influences secondary metabolism by changing the gene expression and the content of chemical compounds in plant cells, such as those related to the Krebs cycle, nitrate and phosphorus metabolism, glycolysis and photosynthesis (Jindo et al. 2020b). In addition, studies on the use of humic substances in medicinal plants show that these substances can contribute to an increase in the biosynthesis of secondary metabolites and the activity of bioactive substances from various classes, such as coumarins, anthocyanins and phenolic compounds (monoterpene, terpene, phenylpropanoids and flavonoids) (Jindo et al. 2020b).

Biostimulants based on humic acid can be used to alleviate the effects of salinity and drought (Aydin et al. 2012). Research by Dias et al. (2020) showed the alleviation of salt stress in papaya (*Carica papaja* L.) plants. The soothing effect of humic substances on plants subjected to the stresses includes the reduction of hydrogen peroxide, lipid peroxidation and increasing the proline content. In addition, HS contributes to regulating gene expression, which improves root growth and the physical, chemical and microbiological properties of soil). The ability to regulate osmotic potential by maintaining the state of cell wall tension is also increased (Malik et al. 2021, Rouphael & Colla 2018). The stimulation of thermal defence mechanisms at a molecular level was also confirmed for economically essential plants (e.g., pepper and tomato) exposed to excessive heat (Jung et al. 2021).

Table 1. Influence of humic substances on plants

Effects	Factors	Literature
The antioxidant effect	The ability of phenols and carboxylic acids to scavenge free radicals formed in excessive amounts in conditions of drought and salinity	Aeschbacher et al. 2012, de Melo et al. 2016, Klein et al. 2018
Mitigating the impact of abiotic and biotic stress; (including drought, excessive heat and salinity); phytohormonal regulation	Interaction with drought and salinity hormones (auxin, jasmonic acid and abscisic acid) • Improving plant resistance to salt stress thanks to organic functional groups (phenols and carboxylic acids) • The ability to regulate osmotic potential by maintaining the state of cell wall tension and water absorption under salinity conditions • Stimulating thermal defence mechanisms at a molecular level	Abdellatif et al. 2017, Ali et al. 2020, Cha et al. 2017, de Oliveira et al. 2016, Olivares et al. 2015, Qin & Leskovar 2020, Schiavon et al. 2019
Plant protection	Pest protection • Anti-inflammatory properties and the reduction of plant infections due to the presence of phenolic compounds and carboxyl groups	Olivares et al. 2015, Aeschbacher et al. 2012, Klein et al. 2018
Influence on germination and the development of the plant root system	Phenolic and carboxyl groups accelerate the process of seed germination and root development • Stimulation of cells, acting as messengers that cause physiological effects and a positive effect on the production of chlorophyll A and B and the content of carotenoids • H ⁺ -ATPase activation, increasing the accumulation of auxins in the root and the formation of nitric oxide, resulting in root elongation, and the development of side roots and hairs	Bento et al. 2020, Canellas et al. 2002, Cha et al. 2017, Elmongy et al. 2020, Ghasemi et al. 2015

3. Summary

The global agricultural sector contributes to the global greenhouse effect, including through the use of chemical fertilizers. Agricultural practices based on biostimulants can have a significant impact on environmental protection. They are strongly related to sustainable agriculture and horticulture. Humic substances are natural biostimulants obtained from aquatic and terrestrial environments (e.g., peat and leonardite). Bio-waste and municipal management are prospective sources of these substances. This approach is justified from an economic and environmental point of view.

Studies confirming the positive effect of humic substances on the condition of soil and plant development were cited. Adding these organic substances to the soil contributed to the formation of stable soil aggregates, improved soil aeration, increased soil water capacity (retention capacity) and cation exchange capacity, enhanced soil microbial activity and increased availability of nutrients for plants. Due to the substances in question, the improvement of soil quality and plant development takes place not only indirectly but also directly as a result of phyto-regulation. The hormonal activity of molecules of humic substances enables direct action on the root and the activation of selected physiological processes, including reducing the negative impact of abiotic and biotic stress (e.g., drought, salinity and excessive heat) on plants. Research on the structure of humic substances has shown the presence of phyto-regulators, such as gibberellins, physiologically active cytokinins, abscisic acid, or indole acetic acid. They also exhibit auxin-like effects.

The proper functioning of plants depends on access to macro- and micronutrients. By improving the solubility of elements, humic substances prevent their rinsing. Therefore, plants and their water retention capacity contribute to reducing their consumption in agriculture. Their positive effect on the seed germination process and the development of the root system is also observed. Adding humic substances also reduces the doses of fertilizers used while maintaining the quality of the soil and plants. This helps to reduce environmental pollution and greenhouse gas emissions and reduce the use of chemical fertilizers, thereby lowering production costs and increasing profitability.

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