

THE POTENTIAL USE OF HUMIC ACIDS

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ABSTRACT

The potential use of humic acids has received a great deal of attention during the last decade. Many research workers have suggested the advantage one can expect in the application of humic acids to provide a better growth of plants. Although their characteristics are subjected to considerable variation according to the sources from which they were extracted, the common effect of their application shows very convincing phenomena. However, such information are still limited in Indonesia resulting a somewhat ignorance among the scientists in the country about the remarkable potential of humic acids application. The objective of this discussion is, therefore, to elaborate basic information regarding the extraction techniques, characteristics variation, potential use as organic-N sources in the form of amino acids for carrier component of microbial inoculants and tissue culture media of plants, ion uptake enhancer, promoter of proteins and nucleic acids synthesis, and enzyme activator.

Keywords : Humic acids, organic N, amino acids, microbial inoculant, tissue culture

INTRODUCTION

Basically, organic matter can be grouped into two major classes, i.e. non-humified and humified materials. The former consists of carbohydrates, amino acids, protein, lipids, lignin, nucleic acids, pigments, hormones, and a variety of organic acids. The latter are products that have been formed during the decomposition process from the nonhumified constituents, and includes a group of complex compounds, such as fulvic acids (FA) and humic acids (HA) and their hydroxybenzoic acid derivatives (Tan, 1986).

Humic acids appear to be complex macromolecules of aromatic units with linked amino acids, peptides, amino sugars, aliphatic acids and other organic constituents (Stevenson, 1982). The aromatic rings are the di- and triphenol type, contain both free OH groups and quinone double linkages, and are bridged by O, S, and N atoms, as well as by NH₂, CH₂, and other groups (Stevenson, 1982). Barak & Chen (1992) stated that HA's are commonly assumed to be polyelectrolytes, i.e. long-chain ionizable macromolecules. They showed that humic macromolecules have equivalent radii ranging from 1.1 nm at 10% ionization to 0.24 nm at 90% ionization.

Compared to other humic materials, HA has received greater attentions among the scientists. Documentations have been established worldwide varying from characterization of HA isolated from different materials (Deiana, *et al.*, 1990, Lobartini *et al.*, 1991 & 1992, Tan *et al.*, 1992, and Goenadi, 1997), oxide adsorption (Shulthess & Huang, 1991, Fontess *et al.*, 1992), soft-weed herbicide (Goenadi, 1995) to its potential as organic N sources used in the callus development (Goenadi & Sudharma, 1995). The possibility of diverse application of soil HA is encouraged by the findings that the substances are not only consisting of major nutrients such as C, H, O, N, and S, but also containing great variety of amino acids in which the types and numbers are dependent upon soil and/or crop types (Orlov, 1985). However, such information is so little available in Indonesia resulting a very limited attempt in exploring the potential use of soil HA.

This manuscript is, therefore, aimed to present basic information regarding to extraction technique, characteristic variation, and the possibility of using HA's as organic N sources, ion uptake carrier, promoter of proteins and nucleic acids syntheses, enzyme activator, carrier construction of microbial inoculant and formulation of tissue culture media.

EXTRACTION AND FRACTIONATION OF SOIL HUMIC SUBSTANCES

Technique for humic substance extraction and fractionation has been developed by Schnitzer (1982) (Fig. 1) based on the principles where humic materials are soluble in dilute base, and thus dilute NaOH is a logical extractant. The action of $\text{Na}_4\text{P}_2\text{O}_7$ is thought to depend on the ability of the P_2O_7 ion to interact with polyvalent cations bound to soil organic matter to form either insoluble precipitates or soluble metal complexes and on the formation of a soluble salt of the organic matter by interacting with cation (Na^+) of the extractant.

The use of alkaline extractants has been criticized by Bremner (1950) since it may modified the nature of the HA. However, the evidences showed by Schnitzer & Skinner (1968) indicated that there seemed no damages or modification in the chemical structure and characteristic of soil organic matter after extraction with dilute alkali under an atmosphere of N_2 at room temperature. Extraction with 0.1 N NaOH yielded higher organic matter than that obtained by higher NaOH concentrations, but that 0.5 N NaOH extracted organic matter with low ash content. When $\text{Na}_4\text{P}_2\text{O}_7$ is used as extractant or in the extraction mixture, it is practically impossible to remove the phosphate from the extracted organic matter, and this causes problems with its subsequent characterization (Schnitzer, 1982).

Tan *et al.* (1991) questioned the need of using N_2 gas in the extraction procedures. They believed that the N_2 gas can be replaced by air to meet condition of extraction. It is obvious that if the use of N_2 can be deleted, not only will the speed of extraction be increased, but the procedure can also be simplified considerably. Using topsoils of

subtropical Ultisols and Alfisols, they found no evidences indicating differences in amounts of HA's attributed to NaOH extraction under air and N_2 gas as well as in contents of C, H, O, N, and S (Table 1). On the other hand, the total acidity showed higher values for HA's extracted under a N_2 atmosphere than under air. The latter was accompanied by correspondingly larger COOH and phenolic-OH concentrations. They finally concluded that the lower carboxyl and phenolic-OH group contents in HA's as a result of extraction under air do not support the idea of increased of humic matter by NaOH. Supporting evidences were obtained from cross polarization magic angle spinning (CPMAS) ^{13}C Nucleic Magnetic Resonance (NMR) and infrared spectra of both extraction methods (Fig. 2).

The standard procedure of organic matter fractionation is employing the solubility phenomena of soil extracts in aqueous solutions at various pH levels and in ethanol. The three principal humic fractions are HA, FA, and humin (see Fig. 1). Humic acids are by definition substances soluble in dilute alkali but are precipitated by acidification of the alkaline extract. The humic fraction that remains in solution, when the alkaline extract is acidified, is fulvic acids. In another word, FA's are soluble in both dilute alkali and dilute acid. On the other hand, humin is the humic fraction that cannot be extracted from the soil or sediment by dilute base and acid. Although not chemically discrete, these fractions differ nonetheless in chemical and physical characteristics. Working with fractionated humic extracts has been considered more *advantageous rather than with crude humic extracts*, particularly if extensive chemical investigations of these materials are the subject of the study (Schnitzer, 1982).

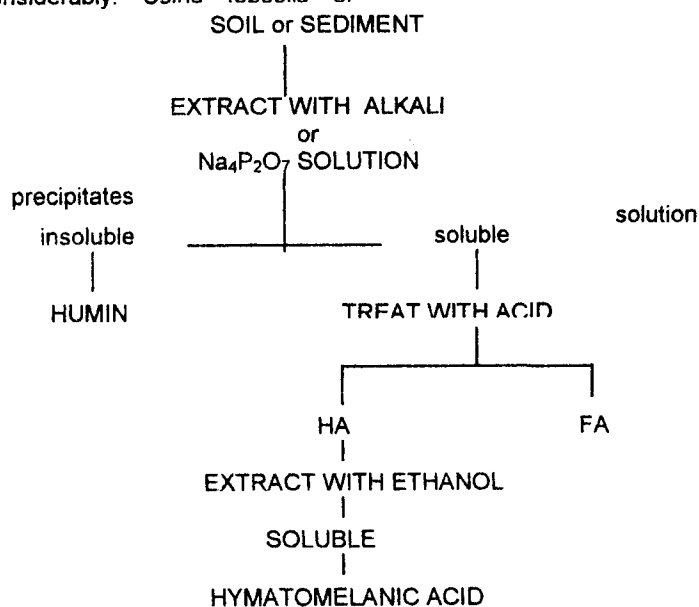


Figure 1 Extraction and fractionation of humic substances (Schnitzer, 1982).

Table 1. Humic acids contents and their elemental compositions as affected by extraction in air and N₂ gas atmosphere (Tan *et al.*, 1991).

Origin	HA	C	H	O	N	S	Total Acidity	COOH	Phenolic-OH
	(mg/g)	(..... g/100 g) (..... me/g) (..... me/g)							
Cecil-Air	5.7	52	5.2	39	4.1	0.4	4.5	3.9	0.7
Cecil-N ₂	7.1	51	5.5	39	3.9	0.5	5.3	4.3	1.0
Onaway-Air	1.8	51	5.1	38	4.5	0.4	4.3	4.1	2.5
Onaway-N ₂	1.3	51	5.1	39	4.9	0.5	4.9	6.3	2.2

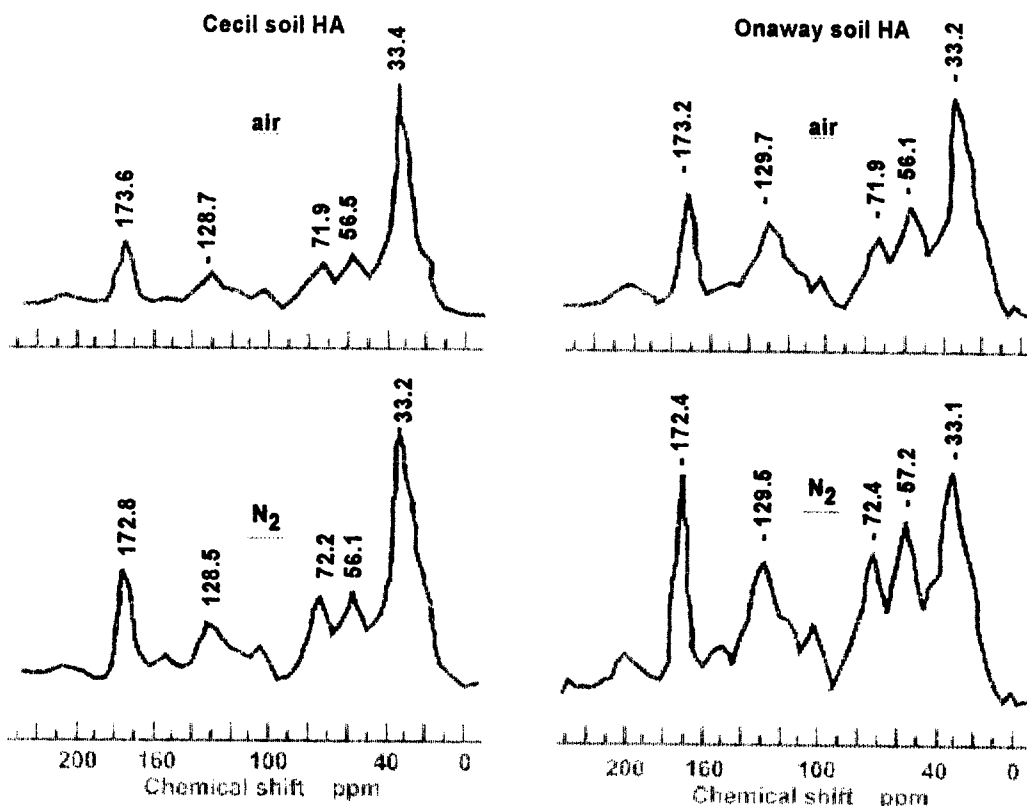


Figure 2. CPMAS ¹³C NMR spectra of HA from Cecil (Ultisols) and Onaway (Alfisols) extracted under air and N₂ gas atmosphere (Tan *et al.*, 1991).

Humic acids can be purified by replacing the extraction and fractionation procedures on freeze-dried materials. The main objective of such purification is to minimize the ash content. An additional objective is to remove low molecular weight organic molecules that are not structural HA constituents. Other purification procedures involve dialysis to reduce salt contamination and ultrafiltration to separate fine clay particles. The

removal of low molecular weight constituents, i.e. aliphatic, aromatic, and phenolic acids, from high molecular weight HA's has been proposed by molecular exclusion gel chromatography using Enzacryl gel. Besides, purification was suggested by Lobartini *et al.* (1991) by passing the HA solution through a H-saturated cation exchange (Dowex 50-X8) column.

TECHNIQUES IN CHARACTERIZATION OF HUMIC MATERIALS

Chemical Methods

Schnitzer (1982) outlined procedures for chemical analysis of HA's. This type of analysis provide information on the distribution of the major constituent elements. Carbon and O are the major elements of HA amounting to 50-60% and 30-35%, respectively. With lower percentage, HA contains 4-6% H, 2-6% N, and 0-2% S. Functional group analyses can shed light on the occurrence of major reactive or functional groups in HA's. From both types of analyses, the distribution of the major elements in each functional group can be determined. The major functional groups in HA's are rich in O and are often referred to as O-containing functional groups (Schnitzer & Khan, 1972). These functional groups consist of CO₂H, phenolic and alcoholic OH, and C=O. Ionizable hydrogens in aromatic and aliphatic CO₂H groups and in phenolic OH groups are considered to be responsible for the total acidity or exchange capacity of HA's.

Ultra Violet Spectrometry

In acid, neutral, and alkaline aqueous solutions, absorption of HA yields in general uncharacteristic spectra in the UV and visible regions of the electromagnetic spectrum (Schnitzer & Khan, 1978). However, the ratio of absorbances at 465 and 665 nm (E_4/E_6) is often used for characterization of HA. Values below 5 are indicative of HA's, whereas higher ratios are characteristics of FA's. Chen *et al.* (1977) showed that the magnitude of the E_4/E_6 ratio is inversely related to the particle size or molecular weight of the HA's. A low ratio indicates a large particle size, whereas a high ratio is indicative of the reverse. Also, the E_4/E_6 ratio is independent of the HA concentrations, at least in the range of 100 to 500 ug/ml.

Infrared Spectroscopy

Infrared spectra of HA's are characteristically showing bands at the following frequencies: 3400 cm⁻¹ (H bonded to OH), 2900 cm⁻¹ (aliphatic C-H stretch), 1725 cm⁻¹ (C=O of CO₂H, C=O stretch of ketonic carbonyl), 1630 cm⁻¹ (aromatic C=C (?) H-bonded C=O of Carbonyl, COO⁻), 1450 cm⁻¹ (aliphatic C-H), 1400 cm⁻¹ (COO⁻¹, aliphatic C-H), 1200 cm⁻¹ (C-O stretch OH deformation of CO₂H), and 1050 cm⁻¹ (Si-O of silicate impurities) (Schnitzer, 1982). The spectra obtained from IR analyses provide the evidences of the presence of O-containing functional groups, such as CO₂H, OH, and C=O in the HA's. This technique has been suggested to be useful in gross characterization of HA's of diverse origin, for the evaluation of the effects of different chemical extractants, chemical modifications, and formation of derivatives. It can

also be used to ascertain and characterize the formation of metal-humate and clay-humate complexes, and to indicate possible interactions of organic compounds (e.g. pesticides, herbicides) with humic acids.

C Nuclear Magnetic Resonance (NMR) Spectroscopy

This technique was developed in the 1980's and complete discussion of its application on HA's in the solution state was presented by Wilson (1990). Fig. 3 shows a typical spectrum of HA's in the solution state ¹³C NMR (a), and the pattern of the sample after hydrolysis with hot, 6 M HCl (b) (Schnitzer, 1990). The spectrum provides an inventory of the different components of the humic acids. The presence of unsubstituted aliphatic C (i.e. C in straight-chain, branched and cyclic alkanes, alkanolic acids, and other aliphatic components) is indicated by signals in the 0-50 ppm of the spectrum. Carbon in proteinaceous materials (amino acids, peptides, and proteins) shows resonances between 40 and 60 ppm, and C in carbohydrates gives signals between 61 to 105 ppm. Signals between 106 and 165 ppm are due to aromatic C, while those near 155 ppm arise from phenolic C. The strong signal between 170 and 180 ppm comes from C in carboxyl groups, with possibly some overlapping from phenolic, amide, and ester carbons. It is obvious that the ¹³C NMR spectrum provide a detail information regarding variety of components and structure presence in HA's whose determinations by other methods would either be laborious and time-consuming or not possible at all. This technique becomes standard method in characterization of HA's (Deiana *et al.*, 1990; Hernandez *et al.*, 1990; Malcolm, 1990; and Tan *et al.*, 1992).

CHARACTERISTIC VARIATION DUE TO ORIGIN DIFFERENCES

The contents of HA as well as its characteristics may vary considerably from soil to soil and/or the organic sources from which it was extracted. Tan (1986) showed that large amounts of HA's are present in Alfisols and Mollisols in contrast to those lower values found in Ultisols and Vertisols. This corresponds to the higher organic matter content in the former compared to the latter. Malcolm (1990) concluded that HA of soils are different from those of streams in terms of molecular size and weight, N content, ¹⁴C age, and CPMAS ¹³C-NMR spectra. The latter have a molecular size of 0.9 to 1.2 um and a molecular weight range between 1500 and 3000 daltons. In contrast, the soil HA's have a higher molecular weight ranging from 1500 to in excess of 50000 daltons. Soil HA's have a strong tendency to aggregate, therefore, they exhibit a broad range of molecular sizes. The N contents of soil HA's are usually near 4%, which is twice of that of stream HA's.

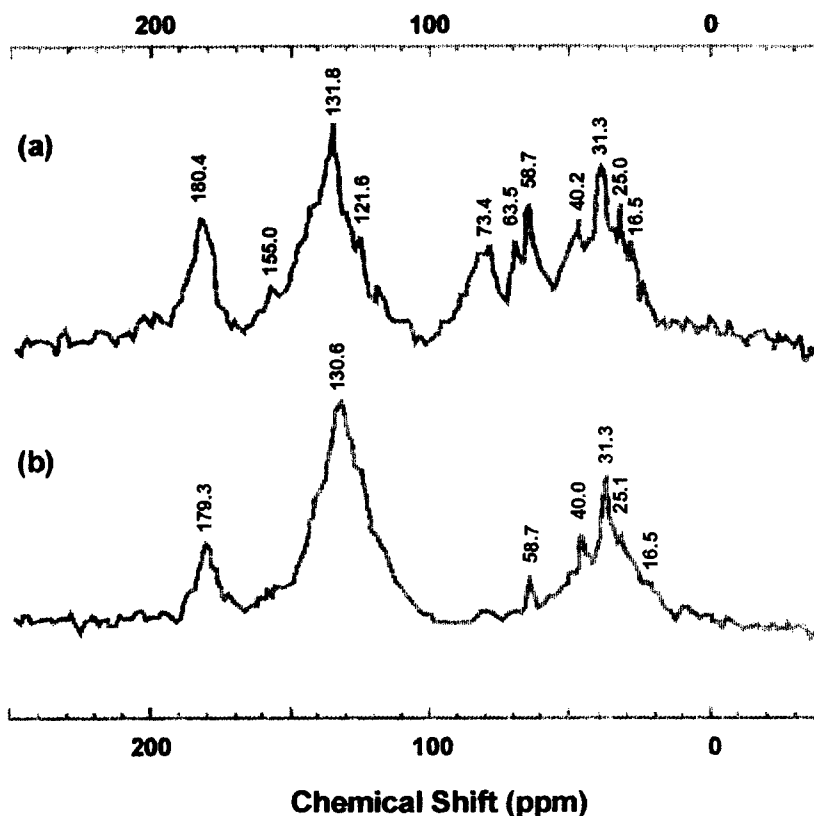


Figure 3. Solution-state ^{13}C NMR spectra of: (a) humic acid extracted from a Mollisol Ah horizon, (b) same humic acid after hydrolysis with hot, 6 M HCl.

The above is supported by the findings reported by Lobartini *et al.* (1991). Studying the humic matter from swamps, streams, and soils in the southern United States, these workers are of the opinion that HA's of respective origin are different considerably. The humic matter isolated from swamp and river water comprised 30-40% HA (NaOH), whereas that from soils was 80%. They found that the HA from swamp was more aromatic than that from river water.

Characterization of humic acids already present in three organic wastes, i.e. sewage sludge, manure, and worm compost, was conducted by Deiana *et al.* (1990). They found that humic acids extracted from sewage sludges contained the highest percentage of aliphatic carbon, associated with polysaccharides and proteinaceous structures, and has characteristics resembles to those of aquatic humic acids. On the other hand, humic acids from manure and worm compost are similar to those originating from soils. These results were based on FT-infra red and ^{13}C - and ^1H -NMR's analyses.

Humic acids contain considerable amounts of amino acids. The amount variation is attributed to differences in soil type and/or crops. Analyzing a

sod-podzolic soil (most probably Ultisols) with different land use types, Orlov (1985) reported that most of the amino acids found in the HA's was dominated by aspartic acid-serine-glycine. Coelho *et al.* (1985) presented amino acids data analyzed from four Brazilian soils (Table 2). The data indicate that aspartic acid, glycine, and glutamic acid are the major components of amino acids found in the soil investigated.

POTENTIAL USE OF SOIL HUMIC ACIDS

General Agricultural Uses

Most studies in the potential use of HA's has been focused on the effect of HA application on the plant growth. Complete literatures have been available documenting research in this subject. Chen & Aviad (1990) made a thorough discussion in the use of HA's to stimulate plant growth. Favorable effects of humic acid on plant growth under both laboratory and field conditions in term of length and fresh and/or dry weights of shoots and roots, number of lateral roots, root initiation, seedling growth after germination, microbial population and activity, nutrient uptake, and flowering were recorded. The mode action of HA on plant growth

Table 2. Distribution[†] of amino acids in humic acids from Brazilian soils and melanins from fungi grown on glucose media with sodium nitrate as the N source (Coelho *et al.*, 1985).

Amino acid	Red yellow podzol (forest)	Red yellow podzol (sorghum)	Dusky red latosol (legumes)	Red yellow latosol (tropical savanna)	Dark red latosol (savanna)	<i>Hendersonula toruloidea</i> melanin	<i>Aspergillus glaucus</i> melanin
Acidic							
Aspartic acid	12.3	10.6	14.6	12.6	14.4	10.8	10.3
Glutamic acid	9.6	8.3	8.5	9.5	9.0	7.9	10.3
Basic							
Arginine	1.8	2.2	1.7	1.7	1.7	2.2	2.4
Histidine	4.2		2.6	1.5	2.2	2.6	1.6
Lysine	3.8	3.7	3.7	3.7	4.2	2.6	3.1
Neutral							
Alanine	8.1	8.6	10.6	10.3	9.8	9.8	8.5
Glycine	13.1	12.2	14.0	11.9	11.0	10.8	11.0
Isoleucine	2.8	3.3	3.1	3.3	3.1	3.6	3.6
Leucine	3.7	5.6	5.4	5.5	5.3	6.3	5.1
Phenylalanine	3.3	3.0	1.9	2.7	2.5	2.2	2.7
Proline	3.7	4.5	4.5	3.9	2.5	6.6	6.9
Threonine	3.9	4.5	5.8	5.3	4.0	7.2	6.7
Tyrosine	2.5	3.9	2.5	3.0	3.1	1.1	2.5
Serine	3.4	4.3	4.9	5.0	4.3	7.5	7.3
Valine	4.6	5.7	4.9	4.9	4.7	5.7	5.3
Sulfur-containing							
Cysteic acid	0	0.6	0	0.6	0.6	0.2	0.2
Half cystine	0.6	0	0	0	0	0.7	0.1
Methionine	0.4	0.8	0.6	0.9	1.0	0.4	0.2

[†] Relative molar distribution = $\frac{\text{-amino-N each amino acid} \times 100}{\text{total amino and imino acid-N}}$

can be specifically distinguished from that of conventional application of organic matter into the soils. Specific effects of HA on plant growth include (a) solubilization of micronutrients (e.g. Fe, Zn, Mn) and some macronutrients (e.g. K, Ca, P), (b) reduction of active levels of toxic elements, and (c) enhancement of microbial populations. There is a question whether the plants are able to absorb HA's directly or indirectly. Using ¹⁴C-labeled HA, Vaughan & McDonald (1976) proved that direct uptake was evidenced by subcellular components of beet roots. The greatest amount of radioactivity was associated with cell walls and smaller levels with mitochondria and ribosomes. It was suggested that the only low molecular weight fractions of the aromatic core are biologically active (Vaughan & Ord, 1981). This study also revealed that low molecular weight of HA fractions are taken up both actively and passively, whereas HA of molecular weight > 50000 daltons is taken up only passively. Although, in the later study, Vaughan *et al.* (1985) concluded that mostly low molecular weight fractions of HA were taken up actively by plants, the chemical nature of these fractions remains to be clarified.

Role of Humic Acid Application

The potential-uses of HA will be related mostly as organic N-containing substances, nutrient carrier

into cell membranes, its effect on m-RNA production and enzyme synthesis and development, and plant hormone-like activity.

Source of organic-N. Stevenson & He (1990) stated that the N of humic substances exists in several forms, some being labile and readily utilized by microorganisms (e.g. H. bonded peptides and proteins) and some being recalcitrant, or nearly so. Significant amounts of the N associated with humic acids cannot be accounted for it known compounds. This N may occur in the following types of linkages as : (a) a free amino (-NH₂) group, (b) an open chain (-NH-, =N-) group, (c) part of a heterocyclic ring, such as an -NH- of indole and pyrole or the -N= or the -N= of pyridine, (d) a bridge constituent linking quinone rings together, and (e) an amino acid attached to aromatic rings in such a manner that the amino acid is not released by acid hydrolysis. These diverse N forms lead to the assumption that HA may be used as nutrient source for microbial inoculants. At present, a study has been being conducted by the author to determine possibility of using zeolite/bentonite-humic substance mixtures as a carrier for *Azospirillum* sp. and P solubilizing bacteria. The preliminary data shows that zeolite-humic substance mixture give a better quality as microbial carrier rather than bentonite-humic substance mixtures. The reason for this could be

attributed to a higher adsorption of microbial suspension by bentonite clay particles. In contrast, zeolite has pore spaces which are apparently convenient for the microbes to remain viable.

Effects on membranes. The facts that the presence of HA's stimulate ion uptake led to the conclusion that these materials affect membrane permeability (Vaughan & McDonald, 1976). The mode of action of HA on membranes is not clear, but it is probably related to the surface activity of humic substances (Chen & Schnitzer, 1978), resulting from the presence of both hydrophilic and hydrophobic sites. Based on this, Chen & Aviad (1990) concluded that the HA may interact with the phospholipid structures of cell membranes and react as carrier of nutrients through them.

Proteins and nucleic acid synthesis. Interactions of humic substance with the synthesis of nucleic acid have been reported. Changes in ribonucleic acid (RNA) synthesis were observed for excised pea roots by Vaughan & Malcolm (1979). These authors concluded that HA influences the production of *m*-RNA which is essential for many biochemical processes in the cell. Low concentrations of 10 mg/L HA enhanced the phosphorylase synthesis in the root of wheat. Humic substances also influence the development of catalase, *o*-diphenoloxidase, and cytochrome in tomatoes (Stanchev *et al.*, 1975), and invertase and peroxidase in beets (Vaughan *et al.*, 1974). However, very limited data, if any, have been reported in relation to the use of HA for perennial crops.

Enzyme activity. The notion that humic substances may act as growth hormones led many scientists to investigate their influence on enzyme activity, particularly effects on indole-3-acetic acid (IAA) metabolism. It is evidenced that humic substances inhibit IAA-oxidase, thereby hindering IAA destruction. Unfractionated HA was more effective than other fractions in suppressing IAA destruction (Mato *et al.*, 1972). Effects of humic substances on the activity of other enzymes, such as phosphatase, invertase, choline esterase, and peroxidase, have been reported as a positive correlation with humic concentrations (Chen & Aviad, 1990). However, the mechanisms by which humic substances affect enzyme activities are not completely understood. Butler & Ladd (1969) suggested that HA may inhibit pronase activity by either competing with the substrate for the catalytically active sites on the enzymes. Competition in chelation of Fe by humic material was also suggested as inhibitory to peroxidase activity (Vaughan & Malcolm, 1979). Apparently, there is a number of different mechanisms that are related to the reactivity of functional groups on the humic materials and which vary depending on the specific enzyme.

Growth promotor in tissue-cultured plants. A study conducted by Irianto (1991) confirmed the assumption in which HA can act as growth hormone. The addition of 160 ppm HA on excised seed embryos of *Pinus Elliottii* Engelm. grown in a B&L medium increased the uptake of P, K, Ca, and S by the callus bodies resulting in the increased weight of the bodies. In the other experiment, he found that application of 160 ppm HA increased plant size and its fresh weight of embryo-cultured pine. Elemental analysis by EDAX indicated that P, K, and S concentrations in roots were increased over the control. It is obvious that moderate amounts of HA stimulated better performance of pine seedlings developed in tissue cultures. The effect of crude HA concentrations extracted from Air Sugihan Histosols of South Sumatra on the initiation and growth of shoots and calli of selected industrial/medicinal crops showed that the HA's replace the function of conventional benzyl adenine in the solid and liquid media and even resulting in a more vigorous shoots and remarkable shortening of shoot initiation period (Goenadi & Sudharma, 1994).

CONCLUSIONS

1. Humic acids as a major fraction of humic substances can be extracted from soils, sediments, and other humified materials.
2. Both conventional and sophisticated methods are presently available to perform detail studies on humic acids.
3. Potential uses of humic acids include their application as : (a) organic N-containing substances, (b) nutrient carrier into cell membranes, (c) promoter of proteins and nucleic acid syntheses, and (d) growth hormones.
4. As a source of organic N, HA's may be used as a nutritional component for the formulation of microbial carriers, whereas their positive effects on ion uptake, proteins and nucleic acid syntheses, and hormone-like activity will open a new application perspective in plant tissue cultures.

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