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Comparison of some chemical and physico-chemical properties of natural and model sodium humates and of the biological activity of both substances in tomato water cultures

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Abstract

Natural humate from compost and the model sunstance obtained from p-benzoquinone were dissolved in an acetone-water mixture and subjected to chromatography on a column of aluminium oxide. Similar fractions were obtained which were chemically and spectrophotometrically investigated for the content of functional groups. The particle size of these substances was determined by filtration on Sephadex molecular sieves. Experiments were performed with tomato in water cultures on stagnant nutrient solution. The biological activity of the corresponding fractions of natural and model humates was found to be analogous. The results are discussed and confronted with functional groups content and particle size of the respective substances.

It has been reported in our earlier papers (Gumiński and Gumińska, 1953; Gumiński et al., 1965) that the favourable effect of natural humate manifested in the case of iron deficiency may be reached by treatment with model humate obtained e.g. from p-benzoquinone. Natural humate was fractionated by the method described by Unger (1965). It appeared that there is a distinct correlation between the ability of iron binding and solvatation of the obtained fractions and their biological activity in water cultures with stagnant nutrient solution where plant suffered of iron deficiency (Gumiński and Sulej, 1967).

The question, therefore, arose whether model humate prepared from p-benzoquinone can be fractionated similarly, as the natural substance, and if so, wheter the corresponding fractions will exhibit a similar bio-

logical activity in cultures deficient in iron. Therefore comparative studies were undertaken with special attention to the occurence of characteristic functional groups and to the colloidal particle size in the particular fractions.

MATERIAL AND METHODS

The material for experiments consisted of tomato seedings germinated in sand and watered with tap water. After development of the first leaf the plant were transferred to water cultures.

The following nutrient solution was used: $Ca(NO_3)_2 \cdot 4H_2O$ — 0.71, KNO_3 — 0.57, $MgSO_4 \cdot 7H_2O$ — 0.28, $(NH_4)_2HPO_4$ — 0.14, $Fe_2(SO_4)_3 \cdot nH_2O$ — 0.12 g/dm³ distilled water with microelements added: H_3BO_3 — 1.54, $MnSO_4 \cdot 4H_2O$ — 1.09, $ZnSO_4 \cdot 7H_2O$ — 0.57, $CuSO_4 \cdot 5H_2O$ — 0.12, H_2MoO_4 — 0,09 mg/dm³ solution (macroelements after H a m p e, 1938 and microelements after D e l w i c h et al., 1961).

Glass jars (1 dm³), protected from light with paint, were filled with the solution described above. In each jar 4 seedlings were placed and after 3 days the weakest one was removed. The particular experimental combinations consisted of 5 jars containing 3 plants each. Two combinations served as control without humate: one with medium daily stirred and aerated by pouring out the liquid and other with stagnant nutrient solution. Solution with humate or its fractions was never stirred (stagnant). The plants were left for about 3 weeks in a greenhouse under summer daylight. Then the dry mass of plants was determined.

Natural humate was obtained from leaf compost according to the method described in the above quoted papers of the present authors (with use of HCl and NaOH). The product consisted of a mixture of sodium salts of so-called humic and hymatomelanic acids.

Model humate was prepared as follows: 5 g of p-benzoquinone was dissolved in 500 cm³ of 0.1 per cent NaOH and boiled for a short time. The mixture was left overnight and then treated with diluted HCl which caused precipitation of the colloidal floks. After several hours the mixture was centrifuged, the brown solution was filtered off and the sediment was washed on a filter with distilled water.

The substance thus obtained both natural and model was stored in dry state as a dark brown powder. Before use it was dissolved in highly diluted sodium hydroxide so as to obtain a colloidal solution with pH about 7.5.

For humate fractionation a mixture of acetone and water in a 2 : 1 ratio and a chromatographic column with aluminium oxide were used. The procedure described by G u m i ń s k i and S u l e j (1967) was followed.

The influence of the particular fractions on the maintaining of iron in the nutrient solution was tested as in the above named paper. This consisted in estimating the iron content in the suface layer of the solution filling the cylindrical vessel. The determination was repeated after 1, 5, 10, and 24 h.

For determining the functional groups the ion exchange method and spectrophotometry in infrared were applied. The content of carboxyl groups was determined by method of ion exchange with calcium acetate after Fuchs, and the content of hydroxyl groups derived from phenols by the method of ion exchange with barium hydroxide according to Syskow and Kucharenko in the modofication by Ihnatowicz (1952). For examination of the infrared spectra a Zeiss UR 10 spectrophotometre was used and the technique of pressing the samples with potassium bromide.

Ash content in the particular fractions was also determined. If it was high, centrifugation of the fractions eluted from the chromatographic column (with diluted sodium hydroxide) was repeated and dialysis was performed.

The size of humate particle was determined using a column filled with molecular sieves Sephadex G 100 and additionally G 50 (fine). As standards served: (1) dextrane blue, molecular weight 100 000, (2) I. G. globulin — 147 000, (3) phosphoglyceraldehyde dehydrogenase — 117 000, (4) albumin from bovine blood fraction V — 65 000, (5) pepsin — 35 000, (6) cytochrome c — 17 000-13 000, (7) insulin — 5 000.

After determining the "zero" capacity with the use of dextrane blue, the proteins were placed on the column and their maximal concentration was determined by the tannin method so as to obtain a standard curve. With Sephadex 50 only cytochrome c and insulin were used as standards.

A column 50 cm high was used. It was eluted with NaCl 0.9 per cent solution brought to pH 7.8 by means of NaOH. The addition of NaOH was necessary for washing out the humates which require an alkaline medium. Separation was run on a fraction collector with an automatic drop counter. Maximal colour intensity of the humates was considered as indicating their highest concentration. The colour was estimated on a "Specol" photocolorimetre at 480 nm wave length.

For biological experiments 50 mg of humate per 1 dm^3 of nutrient solution was used.

RESULTS

In contrast to natural humate, the model substance dissolved completely in the acetone-water mixture. Thus no model fraction number I (insoluble) was obtained according to classification adopted previously by

fractionation of natural humate (Gumiński and Sulej, 1967). Column chromatography, however, gave similar results with both substances, one brown fractions passing trough the column was obtained in each case and three fractions with gradually paler colouring, absorbing on aluminium oxide (fractions III, IV and V).

Abiological experiments, concerning the influence of the particular fractions on the rate of iron precipitation in the nutrient solution, revealed similar effects produced by the corresponding fractions of both substances. Estimation of iron in the surface layer of the solution was done at definite time intervals. It shoved that iron was efficiently maintained in the solution by unfractionated humates and fractions II of both substances whereas both the natural and model fractions adsorbing on aluminium oxide did not prevent coagulation and precipitation of iron compounds to the bottom of the vessel. Moreover, fraction I, present in natural humate only, also maintained iron in the solution

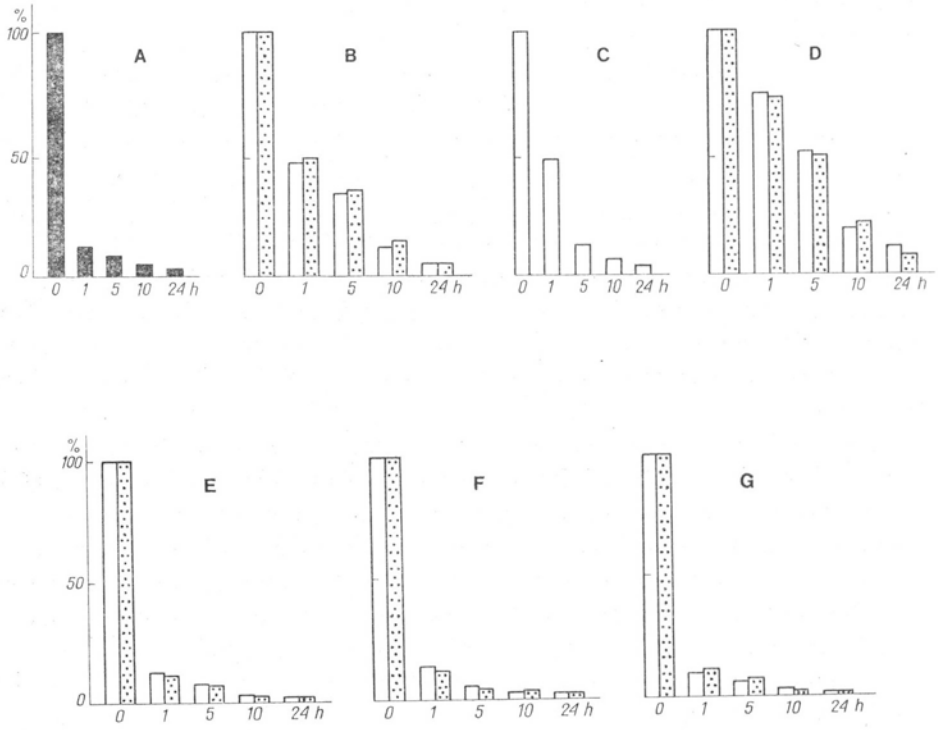

Diagram 1. Influence of humate fractions on maintenance of iron in the surface layer of the nutrient solution in a cylindrical vessel. Time — hours, iron content — per cent. In 1st experiment nondialysed substances:

A — without humate (control), B — unfractionated substances, C — fraction *I*, D — fraction II, E — fractions III, F — fractions IV, G — fractions V.

Black bars refer to control without humate, clear ones to natural humate, dotted ones to model humate

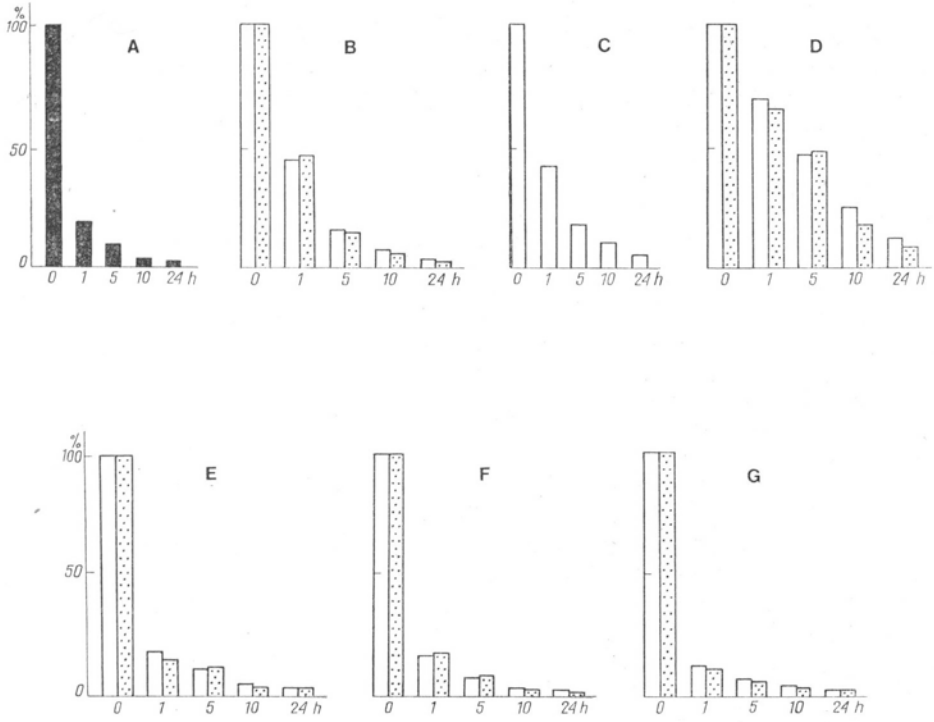

Diagram 2. Notations the same as in diagram 1, but refer to substances used in the second experiment (dialysed)

(diagrams 1 and 2). The fractions were different in ash content which was highest in the fractions adsorbing on aluminium oxide. Repeated centrifugation and dialysis greatly reduced the ash content.

In connection with the different ash content two biological experiments were carried out: with non dialysed and dialysed substances. Furthermore in the second vegetation experiment the iron dose was diminished to one half in order to make its deficit in the stagnant nutrient solution more acute.

The results of these experiments demonstrated a similar biological effectiveness of the corresponding fractions of both humates like in the test for maintenance of iron in the nutrient solution. It namely, appeared that only those humic substances which were capable of maintaining iron in solution effectively prevented chlorosis and made normal plant growth possible in the cultures with stagnant medium. On the contrary, the fractions ineffective against iron precipitation did not give favourable biological effects. These were the fractions absorbing on aluminium oxide. In the first experiment in which non dialysed substances were

used, the latter even inhibited plant growth as compared with the control plants (in stagnant nutrient solution) quite distinctly. In the second experiment with dialysed substances there was no distinct inhibition, however, also in this case the influence of this fractions was unfavourable to the plants.

Table 1

Water cultures of tomatoes — plants grown 20 days in June
Humates non dialysed

Treatment	Dry weight in mg (means of 5 replicates)
A. Nutrient solutions stagnant	
Without humate (control cultures 1).	215±6.4
Natural humate — whole	852±6.5
— fraction I	795±7.4
— fraction II	743±7,8
— fraction III	88±3.4
— fraction IV	100±3.7
— fraction V	94±4.2
Model humate — whole	872±5.2
— fraction II	705±3.8
— fraction III	70±3.1
— fraction IV	74±3.3
— fraction V	59±2.7
B. Nutrient solutions aerated	
Without humate (control cultures 2).	861±5.5

Table 2

Water cultures of tomatoes — plants grown 15 days in July
Humates dialysed

Treatment	Dry weight in mg (means of 5 replicates)
A. Nutrient solutions stagnant	
Without humate (control cultures 1).	150±8.0
Natural humate — whole	525±11.2
— fraction I	542±12.3
— fraction II	560±7.7
— fraction III	142±3.2
— fraction IV	110±2.4
— fraction V	115±4.3
Model humate — whole	365±12.0
— fraction II	338±8.2
— fraction III	110±6.1
— fraction IV	118±7.5
— fraction V	106±8.3
B. Nutrient solutions aerated	
Without humate (control cultures 2).	505±12.2

The determinations of dry weight of the plants are listed in tables 1 and 2. If we compare these tables it is seen that in the first experiment the model humate equalled in effectiveness the natural one, whereas in the second experiment is was less effective. In both cases, however, the similarity of action of the corresponding fractions of both substances was significant.

For analysis of the content of phenolic hydroxyl groups and carboxyl groups unfractionated humates were used as well as fractions I and II of natural and fractions II, III of model humate. It was not possible to obtain sufficient amounts of other fractions for determination of the above mentioned functional groups. Two batches of the substances used in the two biological experiments were used for the determinations. From among the fractions absorbing on aluminium oxide only model fraction III was used from the first batch (less contaminated with ash substances) after subiecting it to dialysis. A high content of ash components made quantitative determination of carboxyl groups impossible and after dialysis the quantity of recovered material was not sufficient for analysis (with exception of fraction III from the first batch of model substance).

The results shown in table 3 indicate that unfractionated humates, independently of their origin, are characterized by a similar quantitative ratio of the -OH/-COOH groups. Fraction I, occurring only in natural humate contained somewhat less — OH and -COOH groups but their ratio was similar as in the unfractionaed humates. Fractions II passing through the column exhibited a high ratio of -OH/-COOH groups. Fraction III adsorbed on the column contained a relatively large amount of carboxylic groups and the -OH/-COOH ratio was low.

Table 3

Content of phenolic-hydroxyl and carboxyl groups in humates in re-count to dry ash free matter and the ash content in humates (percent)

	Series 1				Series 2			
	-OH groups	-CO-OH groups	-OH/CO-OH ratio	ash content	-OH groups	-CO-OH groups	-OH/CO-OH ratio	ash content
A. Natural humate								
whole	9.45	14.29	0.66	6.79	9.64	10.50	0.92	7.04
fraction I	7.59	11.31	0.67	15.80	7.76	9.81	0.79	17.04
fraction II	6.90	1.88	3.67	11.90	5.78	1.08	5.25	21.01
B. Model humate								
whole	7.94	13.99	0.57	1.29	7.50	11.34	0.66	0.83
fraction II	10.16	2.64	3.85	8.38	9.85	5.70	1.73	25.86
fraction III	7.76	22.34	0.35	5.04*	—	—	—	41.70

* after dialysis

Spectrophotometric analysis in the infrared showed the following absorption maxima expressed in wave numbers:

3400 cm^{-1} — phenolic hydroxyl groups — in all substances,

2960 cm^{-1} — nonaromatic (aliphatic and alicyclic and hydroaromatic) carbon and hydrogen bonds — only in natural substances (absent in the model ones),

1730—1710 cm^{-1} — carbonyl groups of acidic character — in natural and model unfractionated substances, and in fraction I present only in natural humate,

1600 cm^{-1} — aromatic carbon bonds and quinoid structures in all substances,

1400 cm^{-1} — (usually simultaneously with 1600 cm^{-1}) — carboxylate anions in substances where absorption typical for carboxyl groups (1730—1710 cm^{-1}) did not occur or disappeared, thus in fractions adsorbing on the column or passing through it,

1300—1030 cm^{-1} — a very numerous group of organic structures such as phenols, aromatic acids, esters, peroxides, anhydrides — mainly in whole natural humic acid and its fraction I; in model substances there is no absorption at these wavelengths.

Determination of the particle size of humates (by filtration on Sephadex G 100 and G 50 columns) revealed differences between natural and model humates. Separation of the natural substance and of its particular fractions showed two peaks: one corresponding to molecular weight of the standards 145 000—147 000, the other to size that of 13 000; the latter value being characteristic for the greater part of the solution. No significant differences were found between the particular fractions of natural humate; only in fraction I (insoluble in acetone—water mixture) particles corresponding to the second peak were somewhat larger than in other fractions, however, smaller than 15 000. Deviations from the mean molecular weight (thus size) of standards were estimated according to the sequence of the collector test-tubes collecting most of the liquid (according to colour intensity).

In contrast to natural humate the model substance and its fractions did not show the presence of particles corresponding to globulin, that is around 147 000. Their size varied between 5 000 and 15 000 (i.e. between the size corresponding to the molecular weight of insulin and cytochrome C). Moreover, part of the solution was adsorbed on Sephadex G 100, this indicating presence of particles smaller those of standard of molecular weight 5 000. Tests on Sephadex G 50 revealed a peak corresponding more or less to size of insuline molecules with molecular weight ca 5 000; only a small part of the solution contained smaller or larger particles, always, however, below 15 000 molecular weight of standards.

DISCUSSION

The present results confirm once more that model humate prepared by oxidation and polymerization of p-benzoquinone is as effective as natural humate when applied to water cultures with stagnant nutrient solution. Obtention by way of column chromatography of fractions corresponding in biological activity to those of natural humate and the finding of analogous differences in adsorption properties in respect to aluminium oxide as well as the ability of those fractions to retain iron in the solution indicate a similar mechanism of physiological action of the compared corresponding natural and model humate-derived substances.

Confrontation of the similiarities and differences in the occurence of the particular functional groups in natural and model humates and their corresponding fractions indicates that the biological activity of the particular substances is associated with the presence of free carboxyl and phenolic-hydroxyl groups and with the mutual quantitative ratio of these groups.

Chemical and spectrophotomeric investigations demonstrated in this respect a high similarity between natural and model humates. Since the fractions adsorbing on aluminium oxide did not exhibit in spectrophotometric examination light absorption typical for free carboxyl groups, this could be considered as the cause of the inability of these fractions to maintain iron in the nutrient solution and thus the inability to ensure normal development of the seedlings cultured in a stagnant medium. These fractions were strongly contaminated with ash substances so that determination of carboxyl and phenolhydroxyl groups in them was impossible. In their ash 26—39 per cent of CaO and 32—48 per cent of Al_2O_3 was found, it is, therefore, probable that blocking of the above named groups by calcium and aluminium made iron binding impossible, so these fractions could no exert a favourable influence on the plants in the stagnant nutrient solution. The ash components played some role here as indicated by the fact, in the first experiment with the use of nondialysed substances, the fractions adsorbing on aluminium oxide had a noxious effect, whereas in the second, after dialysis, they were biologically nearly inactive.

Noteworthy is also the fact that fraction II, both of natural and model humate, exhibiting a high ratio of phenol-hydroxyl to carboxyl groups, was particularly efficient in holding iron in the solution. This fraction also had a favourable influence on the growth of seedlings, in spite of being highly contaminated with ash components (less contaminated, however, that the fractions adsorbing on the column). It would seem that the ratio of these functional groups in humic compounds plays a rather important role in the performed biological test, where

iron deficiency is the growth-restricting factor (Gumiński et al. 1965; Czerwiński, 1967).

The agreement of the present results with those of Schnitzer and Poapst (1967), obtained in studies on the influence of fulvic acids on root initiation is interesting. These authors, namely, found that this effect is dependent on the presence of free phenolic hydroxyl and carboxyl groups and that iron present in the medium enhances the activity of fulvic acids. If we consider that earlier invistigations of the present authors (Gumiński and Sulej, 1967) demonstrated a positive correlation between the ability of iron binding and holding in the nutrient solution and the favourable effect of the given fraction on plant growth, the results of the present study indicate that it is the phenol-hydroxyl and carboxyl groups in similar position in model and natural humate, that may be responsible for this action of humate. Other functional groups do not seem to play any role here.

These studies do not, however, explain why certain substances of both natural and model humate adsorb on aluminium oxide, always in the form of three fractions. Thus, the possibility cannot be ruled out that the adsorbing fractions have some unknown properties which make them inert or even noxious to plant organism. The explanation that this noxious effect disappers after dialysis owing to removal of ash substances and particularly aluminium is only one of the possible suggestions.

The differences in the chemical structure of natural and model humates consisting in the presence of nitrogen and occurence of organic structure with characteristic absorption maxima in the infrared within the 2960 and 1300—1030 cm^{-1} for natural humate and their absence by the model substance did not effect the observed equipotence of the corresponding fractions of both substances in iron maintetnance in the solution and in their biological activity.

Neither was the size of the colloidal particles of the tested substances of any importance in this respect. The corresponding natural and model fractions differed widely in these values, nevertheless they showed similar effects in iron maintenance in the nutrient solution and in their biological activity.

CONCLUSIONS

1. It is possible by way of column chromatography on aluminium oxide to obtain from model substances of the character of sodium humate, derived from p-benzoquinone, fractions resembling as regards chemical structure and biological activity the corresponding fractions of natural humate.

2. The biological activity of these fractions in water cultures with a stagnant nutrient solution is associated with the maintenance of iron

in the solution and depends on the presence of free phenolic-hydroxyl and carboxyl groups in appropriate quantitative ratio.

3. The differences in the effectiveness of the particular fractions cannot be attributed to their different colloidal particle size.

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Porównanie niektórych właściwości chemicznych i fizykochemicznych naturalnego i modelowego humianu sodu oraz ich aktywności biologicznej w kulturach wodnych pomidorów

Streszczenie

W oparciu o metodykę opisaną w pracy ogłoszonej uprzednio (Gumiński i Sulejowa 1967) dokonano rozdziału humianu naturalnego otrzymanego z kompostu i modelowego uzyskanego z p-benzochinonu, stosując rozpuszczanie w mieszaninie acetonu z wodą i chromatografię kolumnową na tlenku glinu.

Zbadano wpływ tych humianów oraz ich funkcji na utrzymywanie się żelaza w roztworze pożywki oraz na rozwój siewek w kulturach wodnych w pożywkach stagnujących. Przeprowadzono badania chemiczne na zawartość grup hydroksylowych pochodzenia fenolowego i grup karboksylowych oraz spektrofotometryczne w podczerwieni na występowanie różnych grup funkcyjnych. Stosując preparaty „Sephadex" wyznaczono w przybliżeniu wielkość cząstek koloidowych poszczególnych substancji próchniczych.

Pomiędzy humianem naturalnym i modelowym znaleziono następujące różnice i podobieństwa z punktu widzenia chemicznego i biologicznego: Humian modelowy rozpuszczał się całkowicie w mieszaninie acetonu z wodą (2:1), podczas gdy naturalny rozpuszczał się tylko częściowo. Rozpuszczalne w tej mieszaninie substancje próchniczne obu humianów rozdzielały się na kolumnie chromatograficznej na cztery frakcje; trzy z nich adsorbowały się na tlenku glinu, dając odpowiadające sobie pasma, czwarta przechodziła przez kolumnę. Niezależnie od rodzaju humianu frakcje przechodzące przez kolumnę wykazywały zdolność utrzymywania żelaza w roztworze soli mineralnych pożywki. Frakcje adsorbujące się na kolumnie również niezależnie od rodzaju humianu nie wykazywały właściwości ochronnych przed wytrącaniem się osadu soli żelaza z pożywki.

W kulturach wodnych pomidorów z pożywką stagnującą, w których żelazo stanowiło czynnik ograniczający, znaleziono paralelizm pomiędzy odpowiadającymi sobie substancjami humianu naturalnego i modelowego. Korzystnie działały jedynie te, które utrzymywały żelazo w roztworze, tzn. obie substancje w całości, frakcja nierozpuszczalna w acetonie z wodą oraz frakcje przechodzące przez kolumnę uzyskane z obu humianów. Frakcje adsorbujące się na tlenku glinu nie działały korzystnie, a raczej hamowały wzrost roślin.

Poszczególne substancje były w różnej mierze zanieczyszczone składnikami popielnymi, które starano się usunąć przy pomocy dializy. W substancjach działających biologicznie korzystnie ujawniono występowanie wolnych grup fenolohydroksylowych i karboksylowych, przy czym frakcje przechodzące przez kolumnę charakteryzowały się wysokim stosunkiem grup -OH/-COOH, co było skorelowane pozytywnie ze szczególną zdolnością utrzymywania żelaza w roztworze pożywki. Spektrofotometria ujawniła duże podobieństwa w budowie chemicznej obu humianów. Humian modelowy różnił się od naturalnego głównie brakiem struktur nie aromatycznych oraz połączeń estrowych. Ponadto nie zawierał on oczywiście azotu (substancją wyjściową był p-benzochinon), podczas gdy humian naturalny zawierał, jak zawsze, azot.

Nie znaleziono korelacji pomiędzy wielkością cząstek poszczególnych substancji próchnicznych a ich aktywnością biologiczną w zastosowanym teście.

W konsekwencji upatrujemy podobieństwa w budowie chemicznej i aktywności biologicznej porównywanych substancji w występowaniu grup fenolohydroksylowych i karboksylowych na strukturach aromatycznych i chinoidowych.